

THESE DE DOCTORAT DE L'UNIVERSITE DE LA ROCHELLE
ECOLE DOCTORALE *Euclide*

Préparée au Laboratoire Centre d'Études Biologiques de Chizé - UMR 7372 -

Présentée par **Marion CHERON**

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Pour l'obtention du grade de Docteur de la Rochelle Université

Discipline : Biologie de l'environnement, des populations, écologie

**Effets sublétaux de contaminants environnementaux sur le développement
d'un amphibien (*Bufo spinosus*) : une approche expérimentale.**



Directeur de thèse : **François Brischoux**

Jury de thèse

Nathalie MONDY	Maître de conférences (Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés)	Rapportrice
Sandrine MEYLAN	Professeur (Université Sorbonne)	Rapportrice
Paco BUSTAMANTE	Professeur (La Rochelle Université)	Examineur
François BRISCHOUX	Chargé de recherche (Centre d'Etudes Biologique de Chizé)	Directeur de thèse

The cake is a lie

Préface

Remerciements

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Résumé

L'agriculture intensive homogénéise le paysage et réduit la part des habitats naturels, altérant ainsi l'écologie de la faune sauvage. Outre cette fragmentation du paysage, l'agriculture intensive est responsable du rejet de produits phytosanitaires dans les milieux non-cibles, comme les milieux aquatiques particulièrement touchés par cette contamination. Les phénomènes de ruissellement ou d'érosion des sols transfèrent ces molécules vers les milieux aquatiques. Les pesticides peuvent s'accumuler en grande quantité dans les milieux à faible courant tels les étangs ou les mares, constituant potentiellement des pièges écologiques pour les espèces y habitant. Les amphibiens sont des espèces indicatrices de leurs écosystèmes et sont particulièrement sensibles aux intrants agricoles, notamment parce qu'ils sont caractérisés par une peau particulièrement perméable. En outre, une partie de leur cycle de vie complexe (bi-phasique) a lieu dans les environnements lenticules potentiellement soumis à la contamination. Pendant ma thèse, j'ai étudié les effets des activités agricoles sur une espèce d'amphibien répandue en milieu agricole et forestier, le crapaud épineux (*Bufo spinosus*). Pour comprendre les conséquences des activités agricoles sur l'écologie et la physiologie de cette espèce, j'ai étudié les effets des caractéristiques de l'habitat (structure et contamination) sur différents stades de développement (embryons, larves, adultes).

Afin de remplir ces objectifs, j'ai utilisé deux approches méthodologiques : (i) une approche de terrain visant à mesurer les différences morphologiques et physiologiques de populations de mâles adultes selon des habitats contrastés ; puis, (ii) des expérimentations en conditions contrôlées afin de distinguer les effets de la phase d'exposition et de la concentration des différents contaminants sur les phases clés du développement de cette espèce.

Les résultats indiquent que les populations de mâles adultes montrent des relations allométriques différentes selon l'habitat (agricole, intermédiaire ou forestier) et que ces différences résultent probablement de processus liés à la plasticité phénotypique. En effet, les analyses préliminaires de génétique de population ne détectent pas de divergence génétique entre les populations étudiées ce qui suggère que les caractéristiques physiques ou chimiques de l'habitat entraînent des normes de réactions différentes chez les populations. Les relations allométriques observées pourraient être la résultante, au moins en partie, d'une plasticité développementale due à la contamination environnementale des sites de reproduction. Les études de l'exposition à deux herbicides (un métabolite de contaminant historique et un contaminant émergent) sur les phases de développement de cette espèce nous a montré que la contamination entraîne de la variabilité dans les réponses morphologiques (taille et proportion corporelle), physiologiques (stress oxydant) et comportementales qui suggèrent des mécanismes de perturbation endocrinienne opérants à très faible concentration. De plus, la contamination influence négativement des proxys de la fitness chez les individus exposés (taille et masse à la métamorphose, survie) ce qui pourrait à terme altérer le recrutement, la reproduction et la pérennité des populations de crapauds épineux.

Mot-clefs : Amphibiens, développement, contamination, herbicides, crapaud épineux, plasticité phénotypique, stress oxydant, comportement, morphologie, télomères, corticostérone, testostérone

Abstract

Intensive agriculture homogenizes landscape and reduces the proportion of natural habitats, thus altering wildlife ecology. In addition to landscape fragmentation, intensive agriculture is responsible for the release of chemicals into non-target environments, such as aquatic environments, which are particularly affected by this contamination. The transfer of these molecules to aquatic environments occurs through runoff or soil erosion. Pesticides can accumulate in large quantities in slow-flux habitats such as ponds and vernal-pools, potentially creating ecological traps for the species that inhabit them. Amphibians are sentinel species of their ecosystems and are particularly sensitive to agricultural chemicals, especially because of their particularly permeable skin. In addition, part of their complex (biphasic) life cycle takes place in lentic environments, which are likely to be highly contaminated.

During my thesis, I studied the effects of agricultural activities on an amphibian species that is widespread in agricultural and forest environments, the spined toad (*Bufo spinosus*). To understand the consequences of agricultural activities on the ecology and physiology of this species, I studied the effects of habitat characteristics (structure and contamination) on different developmental stages (embryos, larvae, adults). To achieve these objectives, I used two methodological approaches: (i) a field approach to measure morphological and physiological differences of adult male populations in contrasting habitats; and, (ii) an experimental approach under controlled conditions to distinguish the effects of exposure period and concentration of different contaminants on key developmental phases of this species. The results indicate that male adult populations show different allometric relationships depending on the habitat (agricultural, intermediate or forest) and that these differences probably result from evolutionary mechanisms related to phenotypic plasticity. Indeed, preliminary population genetic analyses do not detect genetic divergence among the populations studied, suggesting that physical or chemical habitat characteristics result in different response norms among populations. The observed allometric relationships could be the result, at least in part, of developmental plasticity due to environmental contamination within breeding sites. Studies of exposure to two herbicides (a legacy contaminant metabolite and an emerging contaminant) over ontogeny in this species showed us that contamination results in variability in morphological (size and body proportion), physiological (oxidative stress) and behavioral responses that suggest that there may be endocrine disruption mechanisms operating at very low concentrations. In addition, contamination negatively influences fitness proxies in exposed individuals (size and mass at metamorphosis, survival) which could ultimately alter recruitment, reproduction and population sustainability of spined toads.

Publications

Publications en relation avec le manuscrit de thèse

Ce manuscrit est basé sur la liste de publications suivantes. Les articles seront appelés dans le manuscrit sous la forme de « **Article I** » en gras et chiffres romains.

- I. Renoirt, M., Angelier, F., **Cheron, M.**, Bustamante, P., Cherel, Y., & Brischoux, F. (2021). Stable isotopes of a terrestrial amphibian illustrate fertilizer-related nitrogen enrichment of food webs in agricultural habitats. *Agriculture, Ecosystems & Environment*, 319, 107553. <https://doi.org/10.1016/j.agee.2021.107553>
- II. **Cheron, M.**, Angelier, F., Ribout, C., Parenteau, C & Brischoux, F. Habitat influences morphological and physiological allometry scaling in a widespread amphibian species. *In preparation for Science of Total Environment*.
- III. **Cheron, M.**, Angelier, F., Ribout, C., & Brischoux, F. (2021). Clutch quality is related to embryonic development duration, hatchling body size and telomere length in the spined toad (*Bufo spinosus*). *Biological Journal of the Linnean Society*, 133(1), 135–142. <https://doi.org/10.1093/biolinnea/blab035>
- IV. **Cheron, M.**, & Brischoux, F. (2020). Aminomethylphosphonic acid alters amphibian embryonic development at environmental concentrations. *Environmental Research*, 190, 109944. <https://doi.org/10.1016/j.envres.2020.109944>
- V. **Cheron, M.**, Costantini, D, Angelier, F., Ribout, C., & Brischoux, F. Aminomethylphosphonic acid (AMPA) alters the oxidative status during embryonic development in an amphibian species. *Chemosphere*. <https://10.1016/j.chemosphere.2021.131882>
- VI. **Cheron, M.**, Costantini, D., & Brischoux, F. (2022). Nicosulfuron, a sulfonylurea herbicide, alters embryonic development and oxidative status of hatchlings at environmental

concentrations in an amphibian species. *Ecotoxicology and Environmental Safety*, 232, 113277.

<https://10.1016/j.ecoenv.2022.113277>

- VII.** Cheron, M., Raelison, L., Kato, A., Ropert-Coudert, Y, Meyer, X, MacIntosh, JJ, A & Brischoux, F. (2021) Ontogenetic changes in activity, locomotion and behavioural complexity in tadpoles. *Biological Journal of the Linnean Society*, 133(1), 135–142.

<https://doi.org/10.1093/biolinnea/blab077>

- VIII.** Cheron, M., & Brischoux, F. (2021). Exposure to low concentrations of AMPA influences morphology and decreases survival during larval development in a widespread amphibian.

Under review in Aquatic Toxicology.

- IX.** Cheron, M., Raelison, L., Kato, A., Ropert-Coudert, Y, Meyer, X, MacIntosh, JJ, X & Brischoux, F. Nicosulfuron, a sulfonylurea herbicide, alters larval development and behaviour in an amphibian species. *In preparation for Environmental Pollution.*

Publications en collaboration dans des projet scientifiques annexes

Brischoux, F., & Cheron, M. (2019). Osmotic ‘cost’ of reproduction in breeding male toads. *Biology Letters*, 15(11), 20190689. <https://doi.org/10.1098/rsbl.2019.0689>

Brischoux, F., Cheron, M., Renoirt, M., & Lourdais, O. (2021). Getting ready for a long bath: Skin permeability decreases prior to aquatic breeding in male toads. *The Science of Nature*, 108(6), 48.

<https://doi.org/10.1007/s00114-021-01761-x>

Renoirt, M., Cheron, M., Angelier, F & Brischoux, F. (2021) Unusual lack of reproduction in toad populations from agricultural habitats. *Herpetological Journal.*

Renoirt, M., **Cheron, M.**, Angelier, F., Tartu, S & Brischoux, F. Evidence of predation by the Brown rat (*Rattus norvegicus*) on the Spined toad (*Bufo spinosus*). *Herpetological review*.

Communications orales

"L'habitat influence la morphologie, la physiologie et la phénologie de la reproduction chez les crapauds épineux mâles (*Bufo spinosus*)" **Colloque d'Ecophysiologie Animale, 4ème édition - CEPA4 - Octobre 2019**

" Quels sont les effets de la fragmentation des habitats sur les populations de Tortue d'Hermann de la plaine des Maures (Var) ?" **46ème congrès de la Société Herpétologique de France - 13 octobre 2018**

"Effets sublétaux des contaminants environnementaux sur le développement d'un amphibien : Une approche expérimentale" **Colloque de 2ème année de l'Université de la Rochelle - 23 septembre 2021**

Vulgarisation

" Effet de contaminant (AMPA) sur le développement embryonnaire et larvaire du Crapaud épineux (*Bufo spinosus*)" **11ème rencontres nationales sur la conservation des amphibiens et des reptiles - Festival de Ménigoute - Octobre 2018**

Fête de la Science CEBC " L'écologie des crapauds"

Deux journées entières de présentation avec un stand. Le vendredi 5 octobre 2018 était à destination des scolaires (500 élèves). Le samedi 6 octobre était à destination du grand public avec une journée porte ouverte.

"Chizé. Les crapauds victimes des produits phytosanitaires ?"

Publication le 23/03/2019. **Courrier de l'Ouest.**

"Deux-Sèvres. Le CNRS de Chizé révèle les effets délétères des résidus de glyphosate sur les têtards"

Publication le 07/09/2019. **Courrier de l'Ouest.**

Participation à la "**journee spéciale biodiversité**" dans l'émission "**la Terre au carré**" présentée par Mathieu Vidard sur France Inter le jeudi 8 avril 2021

<https://www.franceinter.fr/emissions/la-terre-au-carre/la-terre-au-carre-08-avril-2021>

Intervention auprès d'élèves de 4ème du collège Louis Merle (Secondigny) dans le cadre de l'opération **Sciences en Mouvement d'Elles (SEME)**

Tutorat

Encadrement stagiaire Master 2 (durée 6 mois)

Sujet : "**Influence de contaminants environnementaux sur la complexité comportementale des têtards de crapauds épineux au cours de l'ontogénie : une approche expérimentale** "

Encadrants : **Marion Cheron**, François Brischoux, Akiko Kato, Yan Ropert-Coudert

Encadrement 4 stagiaires master 1 (durée 1 semaine)

Analyse d'un jeu de données et présentation orale.

Sujet : "**Influence du Tébuconazole sur la complexité comportementale des têtards de crapauds épineux au cours de l'ontogénie : une approche expérimentale** "

Encadrement stage d'observation de 3ème (2019 et 2020)

Formation

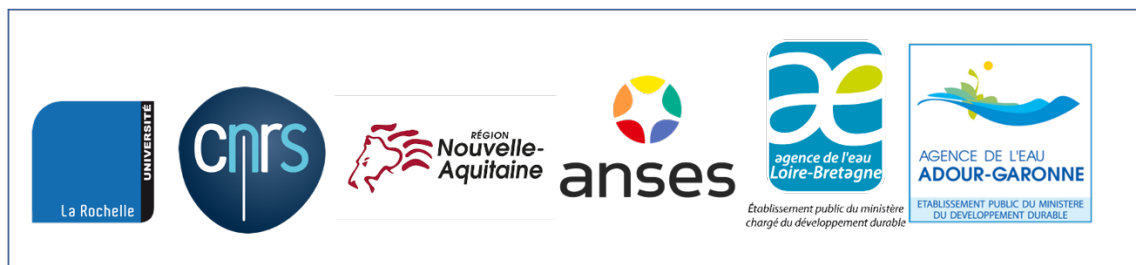
Formation expérimentation faune sauvage : Formation à l'utilisation d'animaux de la faune sauvage non-hébergée à fins scientifiques. Dispensée par le *Museum National d'Histoire Naturelle*. Formation théorique au MNHN, formation pratique au SETE de Moulis sur amphibiens.

Formation intégrité scientifique dans les métiers de la recherche. Université de Bordeaux MOOC.

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Préambule

Cette thèse est structurée de façon à synthétiser les **9 articles** (4 publiés, 2 soumis, 3 prêts pour soumission) écrits dans le cadre des 3 ans de thèse.

Elle se compose d'une courte « **Introduction générale** » synthétisant le contexte général des études réalisées, une partie « **Méthodes générales** » expliquant les approches terrain et expérimentales. Trois **chapitres** découpés selon le cycle de vie du modèle d'étude (crapaud épineux, *Bufo spinosus*) et qui présentent chacun une introduction et une discussion spécifique.

- **Chapitre I** : Effets de l'habitat sur la morphologie et la physiologie des mâles adultes
- **Chapitre II** : Effets de la contamination environnementale pendant l'embryogénèse
- **Chapitre III** : Effets de la contamination environnementale pendant le développement larvaire

Enfin, une dernière partie « **Perspectives** » propose des axes de recherche connexes.

Les 9 articles sont disponibles à la fin du manuscrit pour plus d'informations sur les méthodes, le contexte associé à chaque étude et des résultats complémentaires.

Terminologie

En écotoxicologie, une différenciation est faite entre les termes « dose » et « concentration ». La « dose » sous-entend une quantité d'une substance qui doit être administrée, généralement exprimée par le poids. On mesure la dose dans le corps ou une matrice. La « concentration » d'une substance est la quantité de soluté présent dans une quantité donnée de solution. Elle peut être exprimée en mol/L (concentration molaire) ou par g/L (concentration massique). Le terme concentration est utilisée tout au long du manuscrit. Enfin, le terme « xénobiotique » désigne une substance étrangère dans un organisme ce qui est le cas des pesticides.

Les notions de « contaminants » et « polluants » ont des définitions similaires mais c'est l'échelle considérée qui diffère. Contaminants et polluants sont tous deux des produits chimiques (Walker et al., 2012). Cependant, un polluant est un contaminant présentant des effets clairs nocifs à des concentrations environnementales. Le terme contaminant est utilisé tout au long du manuscrit par mesure de simplicité.

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Introduction générale



“I am glad of all details ... whether they seem to you to be relevant or not.” — **Sir Arthur Conan Doyle** (The Adventure of the Copper Beeches, collected in Adventures of Sherlock Holmes (1892), 294)

D) Contamination environnementale

Les activités humaines ont causé des changements à large échelle menant à un déclin de la biodiversité (Brooks et al., 2002; Myers & Knoll, 2001). Ce déclin est lié à de multiples stressseurs tels que les modifications d'habitats, l'introduction d'espèces exotiques et invasives, les maladies, le changement climatique ou une combinaison de ces différents facteurs (Brooks et al., 2002; Vitousek et al., 1997). Ce déclin, lié aux activités humaines, semble être à l'origine de l'extinction de certains groupes taxonomiques (Brooks et al., 2002).

La révolution industrielle de la fin du 19^{ème} siècle a profondément marqué l'empreinte des activités humaines sur la Terre. A tel point que des théoriciens ont introduits un nouveau terme marquant une nouvelle ère géologique l'« Anthropocène ». Ce terme indique que les activités humaines sont devenues l'une des principales forces de changements sur Terre modelant les écosystèmes, la biodiversité et/ou leurs interactions. La signature stratigraphique mondial (« PSM » ou « clou d'or »), outil marquant un changement d'époque géologique, inclut les extinctions de masse, l'agriculture intensive, les particules aériennes, les retombées radioactives et les relargages de produits synthétiques dans l'environnement (Meineke et al., 2019) qui ont pris naissance durant « la grande accélération » peu après la seconde guerre mondiale.

Avec l'avènement des activités humaines modernes est arrivée l'industrialisation massive et la prolifération des produits chimiques synthétiques. En 2015, l'American Chemical Society dans sa division « *Chemical Abstracts Service* » servant à recenser le nombre de produits organiques et inorganiques répertorie 100 millions de substances dévoilées dans la littérature depuis début 1800 (CAS, 2015). En 2021, ce registre recensait 183 millions de substances (CAS, 2021). Ces produits chimiques sont utilisés dans notre vie de tous les jours, en agriculture, en cosmétiques, dans les industries pharmaceutiques, textiles, les nouvelles technologies. Ces produits sont potentiellement des contaminants environnementaux dont les effets sur la biodiversité restent à déterminer pour la plupart. En effet, les activités humaines sont responsables de la production et du relargage de ces nouvelles molécules dans l'environnement naturel, même dans des milieux éloignés des sources directes de pollution (Cessna et al., 2015).

La contamination environnementale peut amener à des effets ponctuels ou chroniques sur les organismes (Crump et al., 2002; Giaquinto et al., 2017; Tsui & Chu, 2003). La prise de conscience générale de l'impact des contaminants sur la biodiversité et la santé humaine a été rendue possible grâce aux travaux pionniers de nombreux scientifiques tels que Clair C. Patterson sur le danger du plomb (Patterson, 1965), Rachel Carson et son livre « *Silent spring* » (Carson et al., 2000) ou encore Tyrone Hayes montrant les effets tératogènes de l'atrazine sur les amphibiens (Hayes et al., 2006). De plus, la convention de Stockholm (2001, en vigueur en 2004) a permis de réguler l'utilisation des

polluants dit « polluants organiques persistants » (POPs) dont les particularités résident dans leur toxicité, leur persistance dans l'environnement et leur bioaccumulation dans les tissus vivants et le long des réseaux trophiques. Parmi les POPs dont la convention préconise l'élimination ou la restriction, 18/35 sont des pesticides issus de l'agriculture.

II) Agriculture et intrants chimiques

30 à 50% de la surface des terres dans le monde ont été transformées sous les effets de l'action humaines (Vitousek et al., 1997).

La France se situe au premier rang européen en termes de surface agricole utile (SAU) avec près de 30 millions d'hectares de surface agricole cultivée, dont 18 millions d'hectares de terres arables, le reste se composant de cultures pérennes et surface de prairies. Les milieux agricoles sont particulièrement exposés à la contamination environnementale, car ce sont les premiers lieux de mise en place de nouvelles techniques d'agriculture qui se basent sur l'utilisation de l'agrochimie (pesticides, fertilisants). Certaines de ces substances et molécules sont créées dans le but d'altérer des composants de l'écosystème (plantes, insectes, champignons) qui induisent des effets négatifs sur les récoltes/rendements. Par exemple, les insecticides se doivent d'être toxiques pour les insectes ravageurs de cultures mais non toxiques pour les insectes qui bénéficient à la plante comme les pollinisateurs. Les herbicides, ont pour rôle de détruire les adventices qui sont en compétition avec les plantes pour les ressources mais ne doivent pas endommager les cultures. Enfin, les fongicides éradiquent les champignons qui induisent des maladies aux plantes sans interférer avec certains processus de fermentation comme dans l'industrie du vin. Ces mécanismes sélectifs des produits phytosanitaires sont souvent étudiés pour interagir spécifiquement sur un organisme d'un point de vue physiologique. La contamination environnementale par des substances non naturelles est observée intensément dans les zones tempérées ou tropicales où les activités agricoles sont importantes (Brinson et al, 2002). A cause de la modernisation des pratiques agricoles et l'augmentation de l'utilisation des intrants agricoles, il est nécessaire d'étudier l'impact de ces produits sur les organismes non-cibles (de Brito Rodrigues et al., 2019; Hasenbein et al., 2017; Ware, 1980).

A) Intrants chimiques : la fertilisation

Les sources d'azote utilisées dans l'agriculture sont principalement synthétiques (engrais chimiques) et appliquées sur les cultures à des quantités plus importantes que l'azote fixée naturellement. La vente d'engrais est en constante augmentation depuis les années 60 dans le monde et en France après une croissance exponentielle entre les années 60 et 80, le nombre de tonnes d'engrais azotés reste stable mais élevé chaque année (Figure 1). L'application excessive d'engrais riches en azote et phosphore a mené à l'eutrophisation des eaux de surface et les nappes phréatiques autour du monde (Ansari & Gill,

2014; Khan & Mohammad, 2014). En effet, en arrivant dans les milieux non-cibles comme les milieux aquatiques, les engrais azotés accélèrent la croissance des algues et des plantes aquatiques altérant les assemblages des communautés d'espèces au sein de ces milieux. L'eutrophisation est l'une des principales causes de dégradation de la qualité de l'eau. L'augmentation des cyanobactéries dans les eaux de surfaces liée à cette supplémentation excessive en azote entraîne des zones anoxiques lorsque ces bactéries se décomposent dans le sédiment. Ces zones anoxiques peuvent être responsables de la mort massive d'espèces sauvages (Dodds et al., 2009).

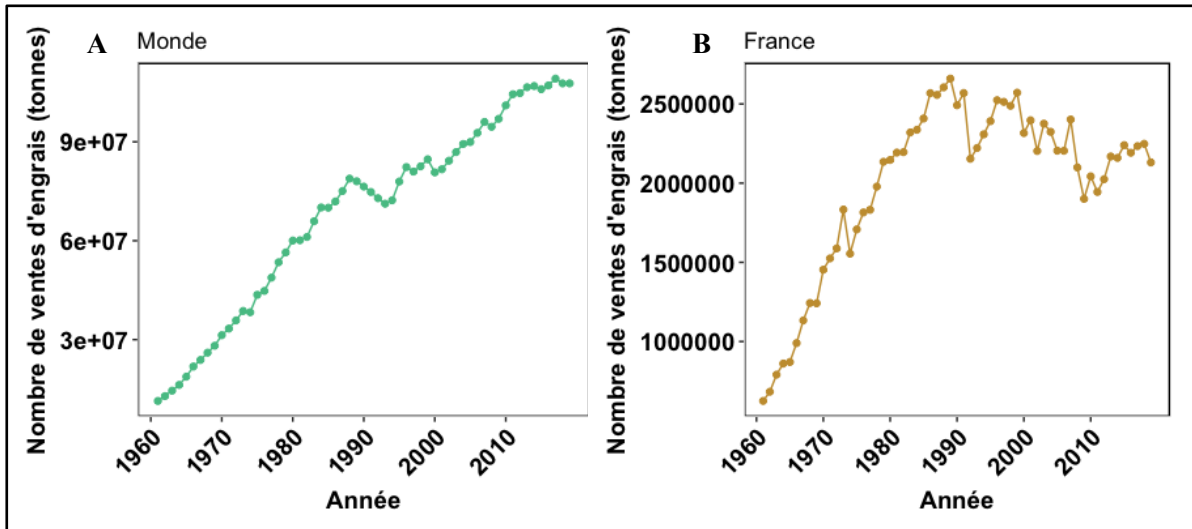


Figure 1 : Nombre de tonnes d'engrais azotés vendus dans le monde (A) et en France (B) depuis les années 1960. Données issues du site de la Food and Agriculture Organization (FAOSTATS).

B) Intrants chimiques : les pesticides

Les pesticides sont des substances utilisées pour contrôler ou éradiquer les nuisibles des cultures (Casida, 2009). Les années 1930 et 1940 ont représenté les années les plus prolifiques en termes de création de nouvelles substances, d'utilisation des intrants et les études de toxicologie avaient pour principal but de mesurer l'efficacité (toxicité) de ces produits (Casida, 2009). Un pesticide se doit d'être efficace, sélectif et peu coûteux. La structure chimique d'un pesticide définit son site d'action et son mode d'action dans ce site (Casida, 2009) et la perturbation est physiologique pour les trois catégories de pesticides (p ex., insecticides, fongicides et herbicides). En effet, les pesticides sont destinés à perturber une cible primaire chez le ravageur de sorte qu'il ne soit plus nuisible. La cible désigne le site de liaison spécifique ou la lésion biochimique impliquée dans la perturbation physiologique (Casida, 2009; Krieger, 2001; Tomlin, 2003; Ware, 1980). Le pesticide en tant que tel ou sous sa forme bioactive se lie à la cible ou interagit avec elle, déclenchant une série d'événements qui sont délétères ou létaux pour l'organisme (Casida, 2009; Krieger, 2001; Tomlin, 2003; Ware, 1980). Cela signifie qu'il peut y avoir une ou plusieurs cibles, sites de liaison ou modes de liaison

uniques dans une enzyme, un récepteur, un canal, une protéine ou une membrane donnée (Casida, 2009; Krieger, 2001; Tomlin, 2003; Ware, 1980).

a) Mode d'action des pesticides

Les $\frac{3}{4}$ des ventes d'insecticides et d'herbicides dans le monde ont les mêmes 4 à 6 cibles principales. La plupart des insecticides perturbent rapidement la neurotransmission pour modifier le comportement ou la survie des insectes (Ishaaya & Degheele, 1998). Une action rapide est normalement requise car les insectes peuvent causer des dommages économiquement importants en quelques heures ou quelques jours (Casida & Quistad, 1998).

- (i) Les insecticides peuvent être efficaces sur une gamme biologique limitée comme les pucerons ou les chenilles. Les insecticides actuels agissent principalement sur quatre cibles nerveuses, à savoir l'acétylcholinestérase, les canaux chlorés, le récepteur de l'acétylcholine et le récepteur de l'acide γ -aminobutyrique, systèmes présents chez les animaux mais pas chez les plantes (Ishaaya & Degheele, 1998; Yu, 2015).
- (ii) Les herbicides agissent principalement sur des voies spécifiques aux plantes en bloquant la photosynthèse, la synthèse des caroténoïdes ou la synthèse des acides aminés aromatiques et à chaîne ramifiée, essentiels chez les plantes mais pas chez autres organismes pour détruire les adventices en quelques jours (Monaco et al., 2002).
- (iii) Les fongicides agissent sur de nombreuses fonctions cellulaires importantes pour la croissance des extrémités des hyphes. De nombreux fongicides bloquent l'ergostérol (un stérol fongique), la biosynthèse de la tubuline ou la cytochrome c réductase, tandis que d'autres perturbent les fonctions cellulaires de base (C. Yang et al., 2011).

Une très faible partie de la dose de pesticide appliquée atteint la cible primaire. La plus grande partie est détournée vers des cibles secondaires ou est dégradée par le métabolisme. Les premières interactions avec les cibles des pesticides agissent à des concentrations picomolaires ou nanomolaires, alors que le niveau est plus important sur les interactions secondaires (Casida, 2009; Krieger, 2001; Tomlin, 2003; Ware, 1980).

Plusieurs classes de pesticides de structures chimiques très diverses agissent parfois sur la même cible. La découverte des herbicides très efficaces à base de sulfonylurée et d'imidazolidinone ont mené à la prise de conscience que ces deux familles avaient la même cible primaire malgré leurs structures très différentes (Duggleby et al., 2008). Certaines cibles primaires sont très spécifiques quant aux ligands qui s'y fixent (pesticides sélectifs à mode d'action spécifique), tandis que d'autres ont un champ de reconnaissance moléculaire plus large (pesticides à large spectre) (Martin, 1987).

b) Utilisation des pesticides

Le nombre d'études d'impact sur l'environnement et la santé humaine sous influence des pesticides a augmenté de 58% ces 10 dernières années (Web of Science). Parmi les trois classes de pesticides, les insecticides sont la classe la plus étudiée (65%), puis les herbicides (33%) et enfin les fongicides (2%). Pourtant les herbicides représentent les pesticides les plus utilisés dans le monde (52%). De même que les engrais azotés, le nombre de tonnes vendues de pesticides et d'herbicides est en constante augmentation depuis 1990 dans le monde. En France, après une baisse d'utilisation des herbicides au début des années 2000 expliquée principalement par la baisse d'utilisation du cuivre et du soufre utilisés comme fongicides entre 2001 et 2011 mais également par le remplacement de molécules anciennes par des nouvelles molécules s'utilisant à des doses plus faibles par hectare, la vente de pesticides et d'herbicides a repris son augmentation depuis 2012 (Figure 2).

L'interdiction de nombreux produits tels que les insecticides atrazine (2003 en Union Européenne), le Carbaryl, Carbofuran, Carbosulfan, Endosulfan (2008) ou l'herbicide Paraquat (2003) a été décidée suite à de nombreuses études sur la toxicité humaine des produits phytosanitaires (Giri et al., 2002; Gupta, 1994; Lu et al., 2000; Proudfoot, 1979).

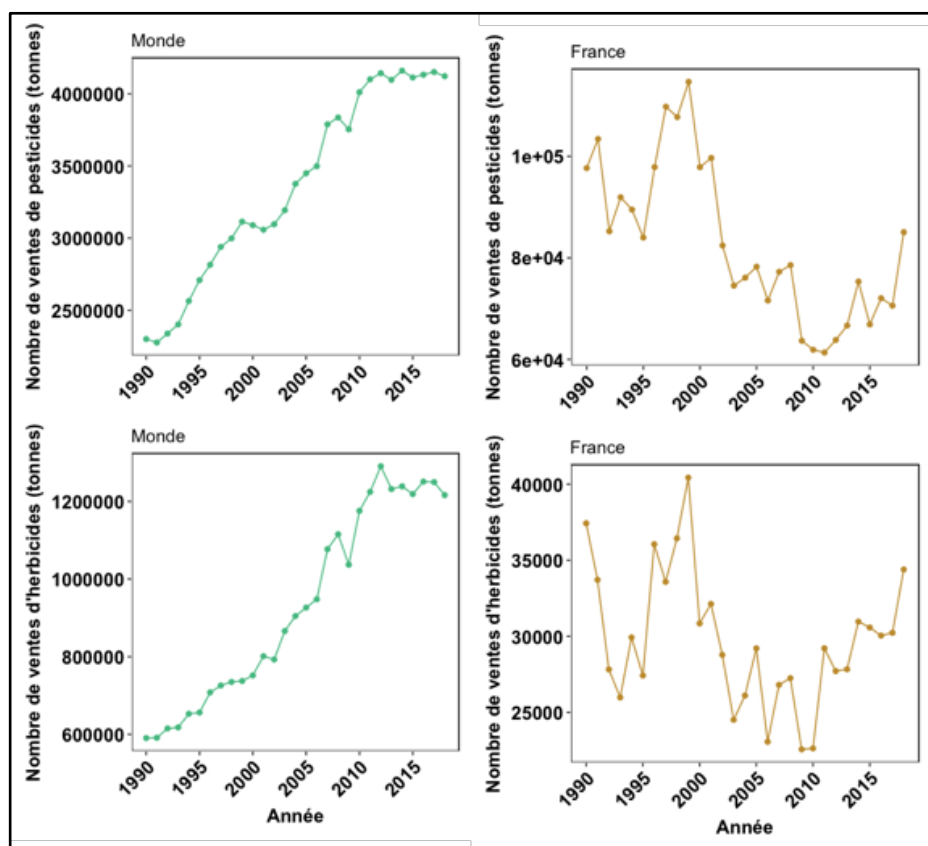


Figure 2 : Nombre de tonnes de pesticides vendues dans le monde (à haut à gauche) et en France (à haut à droite) et de tonnes d'herbicides vendus dans le monde (à bas à gauche) et en France (à bas à droite) depuis les années 1990. Données de issus du site de la Food and Agriculture Organization (FAOSTATS).

Dans le cadre de cette thèse, nous nous sommes intéressés à la catégorie de pesticide la plus vendue dans le monde et en France, les herbicides.

III) Comment explorer les effets de la contamination ?

“*The dose makes the poison*” (Paracelsus, 16^{ième} siècle), un des principes les plus reconnus de toxicologie ne s’applique pas dans tous les cas. Ce principe repose sur le fait qu’une concentration importante d’une molécule pourrait produire des effets néfastes à la santé humaine ou environnementale. C’est sur ce principe que repose la plupart des tests de toxicité actuels visant à déterminer la toxicité d’une dose minimale détectable dans les matrices (eaux, sédiment, nourriture). Ce principe suggère qu’à des doses faibles, aucune réponse perceptible n’est susceptible d’apparaître, contrairement à des doses plus importantes. L’écotoxicologie offre la possibilité de tester ces relations doses-spécifiques et d’étudier la sensibilité de différentes espèces aux contaminants environnementaux.

A) Tests de toxicité classique : DL₅₀

La majorité des études se concentrent sur les tests de toxicité à court terme (dit « toxicité aigüe ») conduites sous des conditions standards régies par des instances gouvernementales comme l’OCDE conçue pour estimer la dose médiane létale (DL₅₀) (Døving, 1991; Hodson, 1985). Ces tests exposent des organismes modèles à une série de concentrations plus ou moins diluées du produit à tester dans le but d’estimer la concentration qui cause 50% de mortalité sur les organismes testés. Les tests sont conduits sur des périodes de 96 h ou moins. Ce sont ces valeurs qui sont utilisées par les instances gouvernementales pour déterminer des seuils maximums autorisés dans l’environnement (Kramer et al., 2009).

D’autre part, il existe des tests de toxicité chronique, plus représentatifs des potentielles expositions des organismes dans le milieu naturel. Les individus sont mis en contact avec le produit pendant une période plus importante en fonction à l’espérance de vie de l’espèce et de la demi-vie des molécules testées.

Cependant, dans la plupart des cas, ces doses sont fortes et ne sont pas représentatives des concentrations retrouvées dans l’environnement (Annett et al., 2014). En conséquence, les effets de toxicité à faibles concentrations sont peu représentés dans la littérature (Beiras, 2018; Hasenbein et al., 2017).

B) Les limites des organismes modèles dans les études de toxicité

Les études de toxicité sont communément effectuées sur des organismes modèles vertébrés, la souris (*Mus musculus*) pour les mammifères, le diamant mandarin (*Taeniopygia guttata*) (Griffith et al., 2021) pour les oiseaux, le danio (*Danio rerio*) pour les poissons ou encore le xénope lisse (*Xenopus laevis*) pour les amphibiens (Segner & Baumann, 2016). Une espèce devient un modèle en science lorsqu'elle combine des propriétés pratiques qui facilitent son maintien en captivité, son élevage et sa manipulation avec des caractéristiques qui la rendent apte à répondre à des questions de recherche particulières (Dietrich et al., 2020; Matthews & Vosshall, 2020). Les organismes modèles ont des temps de génération rapides qui facilitent la sélection de lignées, et peuvent être facilement manipulées à l'aide d'outils génétiques expérimentaux de plus en plus complexes et puissants pour visualiser et manipuler des cellules spécifiques du développement. Les organismes modèles sont irremplaçables pour l'étude des aspects fondamentaux de la biologie, mais leur capacité à aborder les spécificités biologiques de leur espèce sous l'effet de leur environnement naturel complexe est insuffisante. Par exemple, en prenant l'exemple du diamant mandarin très largement utilisé comme organisme modèle, les effets combinés des périodes de reproduction non dépendantes de la saison, du caractère particulier écologique et climatique de la zone aride australienne dont il est originaire rend cette espèce particulière parmi les oiseaux. Les stratégies évolutives associées à un large niveau de tolérance aux variations environnementales peuvent avoir déterminé leur succès en tant que modèles de laboratoire, mais peuvent également faire du diamant mandarin une espèce à part parmi les oiseaux. On peut observer que les réponses des diamants mandarins dans le cadre d'études portant sur leur physiologie, leur comportement et leur histoire de vie (typique en écologie) peuvent différer de la réponse de la plupart des autres espèces aviaires (Griffith et al., 2021).

De plus, la sélection dans l'environnement simplifié des conditions de captivité au laboratoire peut canaliser l'expression de phénotypes qui sont normalement sélectionnés dans des environnements complexes. En effet, une fois qu'une espèce est sélectionnée comme modèle, sa biologie change (Bolker, 2019). Les traits développés par une espèce dans la nature ne seront pas les mêmes que ceux des organismes modèles qui ont subi une forte sélection dans l'environnement d'un laboratoire de recherche. Finlay (2019) résume cette « laboratorisation » (concept de Robinson, 1965) comme suit : « selection on little but breeding and surviving in a sterile and changeless environment ». En prenant l'organisme modèle de référence, la souris de laboratoire, la divergence évolutive rapide s'expliquent par des tailles de populations faibles, consanguines et soumises à une sélection stricte. Les souris de laboratoire montrent des modifications de comportement (Blanchard et al., 2013; Chalfin et al., 2014), de génétiques de populations (H. Yang et al., 2011), de la fonction immunitaire (Beura et al., 2016; Reese et al., 2016), du métabolisme, de la physiologie (Martin, 1987; Zolfaghari et al., 2013). Ces observations s'appliquent à la plupart des autres espèces modèles.

C) La contamination à des concentrations pertinentes pour l'environnement

L'utilisation d'espèces indicatrices et sentinelles de leur environnement sur le terrain peut être largement plus représentative des effets des stressés environnementaux (contamination) rencontrés par les espèces dans leur milieu (Hopkins, 2007; Segner & Baumann, 2016).

Les effets sublétaux sur les organismes non-cibles peuvent entraîner des altérations morphologiques et physiologiques qui peuvent avoir des impacts sur la persistance des populations naturelles. En effet, les effets sublétaux provoquent des changements comportementaux, physiologiques et développementaux (Bernabò et al., 2008; Brunelli et al., 2009; Park et al., 2020). L'exposition à un seul produit à une concentration ponctuelle trouvée dans l'environnement peut produire des effets différents de ceux induits par une concentration chronique à des concentrations trouvées dans l'environnement à plusieurs échelles temporelles (Hayes et al., 2006; Relyea, 2009; Relyea & Diecks, 2008). Chez toutes les espèces, chaque produit a le potentiel d'être bénéfique ou délétère à la structure et la physiologie d'une cellule, d'un tissu, d'un organe ou au niveau de l'organisme dans son ensemble. Les substances chimiques peuvent augmenter, réduire ou interférer avec les fonctions endocrines sous-jacentes aux processus de digestion, de reproduction, de croissance, les fonctions du système nerveux, le métabolisme. Même une substance naturelle biologiquement active comme une hormone ou médiateur chimique essentiels au maintien de l'homéostasie, peut s'avérer délétère pour l'organisme lorsqu'elle est sécrétée de façon chronique à forte dose.

C'est le cas de la testostérone (Wingfield et al., 2001), une hormone stéroïdienne sécrétée, à l'approche de la période de reproduction, via l'axe hypothalamo-hypophysaire. Ses effets sur l'organisme incluent des modifications morphologiques (développement de caractères sexuels secondaires), la régulation de la spermatogénèse, la voie rétroactive d'hormones signaux de la reproduction (gonadotropine, GnRH), et des effets sur le comportement d'agression ou de parade nuptiale. Cependant, la sécrétion de la testostérone en trop grande quantité en dehors des périodes de reproduction implique des effets importants comme l'augmentation de la consommation d'énergie, le risque de blessure, la réduction du stockage de réserves lipidiques. Le compromis entre les avantages évidents de la testostérone sur le système reproducteur des mâles pendant la période de reproduction et les coûts lorsque les taux plasmatiques de testostérone restent élevés pendant trop longtemps a pu conduire à une forte sélection de mécanismes permettant d'éviter ces coûts chez les espèces (Wingfield et al., 2001).

Cependant, les xénobiotiques sont nouveaux à l'échelle évolutive et les espèces ne sont probablement pas adaptées pour répondre à ces perturbations. Malgré les a priori des études en toxicologie, certains produits chimiques ne montrent pas la relation dose-réponse linéaire classique et des doses faibles peuvent mimer l'action de certaines molécules ou se comporter comme des antagonistes modifiant les fonctions physiologiques, comme c'est le cas des perturbateurs endocriniens (EDC) (Vandenberg et al., 2012).

D) Les différentes réponses à des concentrations environnementales

a) Relation concentration-effet

L'effet d'un pesticide sur la survie ou sur une fonction d'un organisme est usuellement décrit via une relation concentration-effet. Ces relations suivent souvent une courbe sigmoïdale (Ekelund et al., 1994). De très faibles concentrations de pesticides sont supposées n'avoir aucun effet nocif, suivi d'une gamme de concentrations pour lesquelles la survie ou la fonction de l'organisme diminue. Les augmentations de la concentration de pesticide au-delà d'une certaine concentration n'entraînent pas de diminution supplémentaire de la survie ou de la fonction (**Encadré n°1**). Les concentrations étudiées ne sont souvent pas pertinentes avec les concentrations rencontrées par les individus dans l'environnement. Dans le cadre du modèle concentration-effet linéaire, les tests à forte concentration sont utilisés pour extrapoler à des concentrations plus faibles qui sont supposées être " sûres " pour les expositions humaines. Cependant, des faibles concentrations peuvent provoquer des effets biologiques importants pour la survie de l'organisme.

Contrairement aux tests toxicologiques classiques où " la dose fait le poison " (Paracelsus, 16^{ème} siècle), l'évaluation d'une potentielle perturbation endocrinienne pose d'autres défis (Fuhrman et al., 2015) :

- (i) L'existence de réponses non monotones aux expositions remet en question la détermination de doses seuils " sûres " gouvernementales (Vandenberg et al., 2012).
- (ii) Le système endocrinien intégratif et complexe peut entraîner une diaphonie entre différentes voies hormonales (Couderq et al., 2020).
- (iii) Les fenêtres critiques du développement sont particulièrement vulnérables à l'exposition aux perturbateurs endocriens (EDC) (Greulich & Pflugmacher, 2003).
- (iv) Les effets de l'exposition peuvent survenir plus tard dans la vie, voire s'étendre aux générations futures (Kadokami et al., 2004).

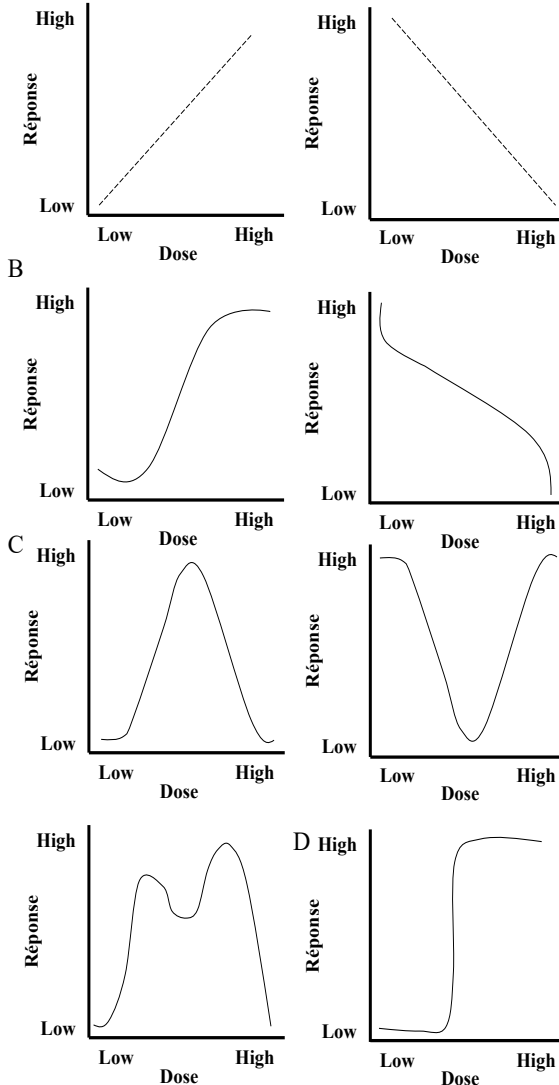
b) Non-monotonie

Les courbes concentration-réponse non monotones (NMDRC, **Encadré n°1**) sont définies mathématiquement comme un changement de signe (positif/négatif) de la pente d'une relation concentration-réponse sur la gamme de concentrations testées. De nombreuses études ont reconnu la présence de NMDRC dans les réponses des organismes aux nutriments, aux vitamines, aux composés pharmacologiques et autres médiateurs chimiques qui interagissent avec les récepteurs, y compris les hormones. Au fur et à mesure que l'étude des perturbateurs endocriens (« endocrine disrupting chemicals » EDC) s'est développée au cours des deux dernières décennies, de nombreux exemples de NMDRC ont été identifiés dans la littérature scientifique et comprennent des études menées sur des cellules en culture, des animaux de laboratoire et des populations humaines. L'existence de NMDRC dans la gamme des niveaux d'exposition humaine semblerait donc falsifier une hypothèse

fondamentale, à savoir que les dangers à forte concentration peuvent être utilisés pour prédire la sécurité à faible concentration. Les exemples qui correspondent aux cas 2 et 3 indiquent que la non-monotonie se produit à des concentrations qui sont négligées par la toxicologie réglementaire telle qu'elle est couramment pratiquée aujourd'hui (Vandenberg et al., 2012) (**Encadré n°1**).

Il convient de noter que la faible concentration et la non-monotonie se distinguent du concept d'hormèse, qui est défini comme un type spécifique de réponse dans lequel "*les différents points de la courbe peuvent être interprétés comme bénéfiques ou néfastes, selon le contexte biologique ou écologique dans lequel ils se produisent*" (Costantini, 2014; Shi et al., 2016). Les estimations des effets bénéfiques ou néfastes ne peuvent pas être déterminées à partir de la direction de la pente d'une courbe concentration-réponse. Enfin, il convient de noter que tout effet biologique, qu'il soit observé pour suivre des relations linéaires avec la concentration administrée ou non, constitue une preuve concluante qu'un EDC a une activité biologique. Ainsi, d'autres effets biologiques sont susceptibles d'être présents mais peuvent rester non détectés ou non examinés. De nombreux EDC, y compris ceux utilisés comme pesticides, ont été conçus pour avoir des effets biologiques (par exemple, les insecticides conçus pour imiter l'hormone de la mue). Ainsi, la question de savoir si ces produits chimiques ont des effets biologiques et quels autres effets sont induits par ces agents biologiquement actifs, et non s'ils existent.

Encadré n°1



Exemples de courbes dose-réponse à un contaminant. A, Les réponses linéaires, qu'il y ait des associations positives ou inverses entre la dose et l'effet, permettent des extrapolations d'une dose à une autre. Par conséquent, la connaissance des effets d'une dose élevée permet de prédire avec précision les effets à faible dose. B, Exemples de réponses monotones et non linéaires. Dans ces exemples, la pente de la courbe ne change jamais de signe, mais elle change de valeur. Ces types de réponses ont souvent une composante linéaire, et les prédictions peuvent être faites dans la plage linéaire, comme pour les autres réponses linéaires. C, trois types différents de NMDRC sont affichés, notamment une courbe en forme de U inversé, une courbe en forme de U et une courbe multiphasique. Toutes ces courbes sont considérées comme des NMDRC car la pente de la courbe **change de signe** une ou plusieurs fois. Ces courbes montrent clairement que la connaissance de l'effet d'une dose, ou de plusieurs doses, ne permet pas de faire des hypothèses sur les effets d'autres doses. D, Une réponse binaire est représentée, où une gamme de doses n'a aucun effet, puis un seuil est atteint, et toutes les doses supérieures ont le même effet. Adapté de Vandenberg et al., 2012).

Dans le cadre de cette thèse, on se demande donc si les molécules retrouvées dans l'environnement peuvent représenter des contraintes sur les espèces non-ciblées sauvages.

IV) Les milieux non-cibles : exemple des milieux humides

Les milieux d'eaux douces représentent 0,01% de l'eau de la Terre avec 2,3% de surface en eau (Lehner & Döll, 2004) et sont peu considérés par les instances gouvernementales ou par le grand public (Cooke et al., 2016). Pourtant, le taux d'extinction annuel des espèces de milieux d'eau douce est de 3,9% ce qui est presque 4 fois supérieur au taux d'extinction des espèces terrestres (1.1%) (WWF). Parmi les menaces anthropiques pesant sur les milieux d'eaux douces, la contamination des eaux de surfaces est particulièrement importante car les sources de pollutions sont multiples (de Souza et al., 2020). Les sources de contamination proviennent des activités industrielles, agricoles, aquacoles, et des activités domestiques. Chaque source peut impacter la biodiversité aquatique indirectement en influençant la qualité de l'habitat ou par toxicité directe.

Cependant, depuis les années 2000, des substances persistantes et qui s'accumule facilement dans les matrices environnementales comme l'insecticide dichlorodiphényltrichloroethane (DDT) connues pour être toxiques pour de nombreux taxons sont peu à peu remplacées par une pléthore de produits à demi-vie moins importantes et supposés moins toxiques. De nos jours, les études de toxicités sur les contaminants se concentrent sur des études à long-terme de toxicité chronique des contaminants émergents ou des POPs historiques. En effet, un champ important de la littérature se concentre le rôle de perturbateurs endocriniens (EDC) de ces contaminants (Hayes et al., 2006; Leemans et al., 2019; Muñoz et al., 2021).

A) Transport et accumulation

De plus, les contaminants peuvent être transportés dans les milieux non-cibles (Relyea, 2009). En effet, à cause de l'érosion, du drainage des sols et des phénomènes météorologiques, les intrants agricoles sont transférés dans des milieux aquatiques où ils ont tendance à s'accumuler (Solomon & Thompson, 2003). Certains contaminants se transfèrent dans les milieux aquatiques, et leur propriétés chimiques (haute solubilité dans l'eau, polarité ...) favorisent la persistance dans les milieux aquatiques (**Figure 3**) (Giesy et al., 2000; Mackay & Boethling, 2000). Les pesticides ont le potentiel d'entrer dans les habitats aquatiques via l'application directe, le ruissellement, l'érosion des sols ou le transport lié au vent. De nombreux pesticides trouvés dans les milieux aquatiques sont des contaminants depuis longtemps interdits ou non destinés à l'utilisation en milieux aquatiques (Solomon & Thompson, 2003).

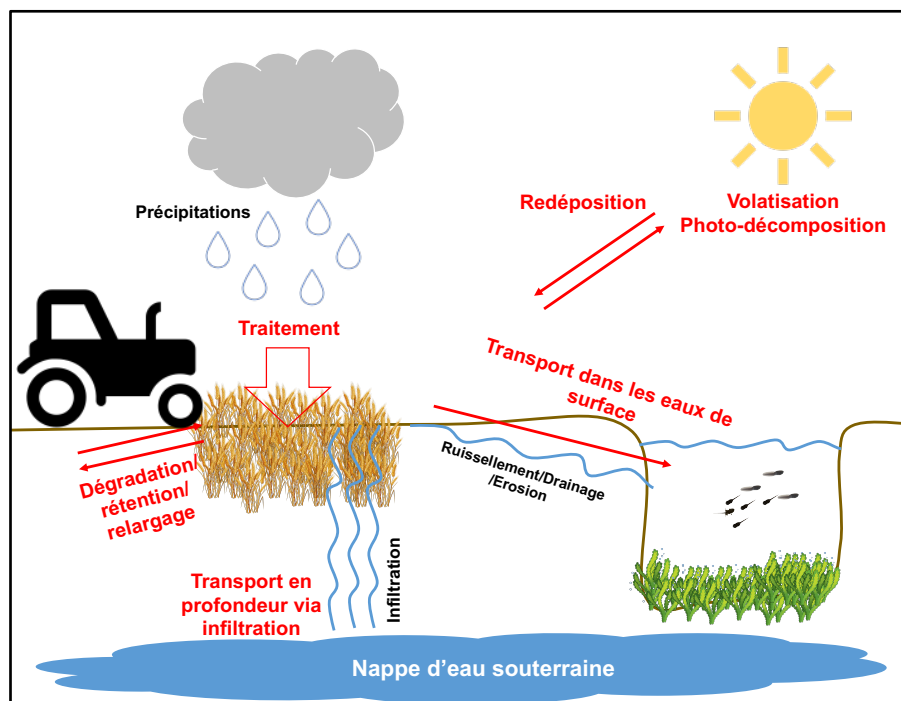


Figure 3 : Dispersion des polluants dans l'environnement. Les flèches rouges représentent le transfert et /ou la dégradation des produits. Les traits bleus représentent la circulation de l'eau.

Les concentrations trouvées dans l'environnement sont bien plus faibles que les concentrations utilisées dans les tests de toxicité expérimentaux à cause de la dégradation du produit dans le sol, l'eau et sous l'effet de la photo-dégradation ou la dégradation microbienne.

Étant donné la facilité des pesticides à se retrouver en milieux lenticules ou lotiques, on se demande comment ces substances affectent les espèces non-cibles dans ces systèmes.

B) Effets des contaminants environnementaux sur les espèces non-cibles en milieux aquatiques

De nombreux pesticides sont des perturbateurs endocriniens qui modifient les processus physiologiques (Mnif et al., 2011; Muñoz et al., 2021). A échelle globale, la contamination environnementale par les pesticides affecte les interactions interspécifiques et altère les fonctions des écosystèmes (Köhler & Triebskorn, 2013; Schäfer et al., 2007). La présence de pesticides dans les cours d'eau induit un stress associé à une diminution de l'abondance relative et du nombre d'espèces sensibles dans les communautés (Beketov et al., 2013) avec des taux pouvant aller jusqu'à 42% dans certaines régions (Schäfer et al., 2007). Des changements structuraux dans les cours d'eau contaminés résultent des effets fonctionnels des pesticides avec une réduction de 2,5 fois du taux de

décomposition de la litière de feuilles. Des concentrations environnementales ont induit ces changements dans la structure de la communauté. Si ce déclin se situe au niveau d'espèces à des niveaux trophiques bas, toute le réseau trophique peut être impactée. Par exemple, un déclin d'invertébrés change la ressource en nourriture d'un milieu ce qui se répercute sur les prédateurs naturels (Köhler & Triebkorn, 2013).

Des méta-analyses récentes résument la toxicodynamique des pesticides sur divers taxons habitant les milieux d'eaux douces comme les invertébrés, les poissons, les amphibiens, allant d'effets neurotoxiques, une altération du comportement, du métabolisme, de la toxicité développement et de l'immunotoxicité (Baker et al., 2013; Egea-Serrano et al., 2012; Relyea, 2009). Ces effets induisent une surcharge en parasites et/ou des mortalités massives ce qui altère les capacité de reproduction et peut mener à un déclin des populations (Beketov et al., 2013; Leemans et al., 2019; Slaninova et al., 2009). De plus, les facteurs abiotiques tels que les variations de température influencent la toxicité de certains pesticides et la réponse des espèces soumises à cette exposition et ces variations (Baier et al., 2016; Gomes & Juneau, 2017).

Ci-dessous sont résumées les principales fonctions impactées par les pesticides chez les espèces de milieux d'eaux douces en concentrations environnementales.

(i) Biochimique - De nombreuses études (Deb & Das, 2013; Gibbons et al., 2015; Slaninova et al., 2009) ont résumé les effets sublétaux des pesticides sur les poissons incluant une inhibition de l'acétylcholinestérase (AChE), une perturbation de la production d'hormones thyroïdiennes, une altération de la morphologie des branchies ou du foie et une augmentation du stress oxydant.

(ii) Dommages moléculaires - L'exposition aux pesticides chez les espèces aquatiques peuvent mener à des altérations sur l'ADN notamment via des mutations, des aberrations chromosomiques ou des effets métaboliques. Ces dommages entraînent des malformations morphologiques ou la létalité. Ces dommages sont souvent entraînés par un stress oxydant important résultant en une augmentation des espèces réactives de l'oxygène (ROS) (Pašková et al., 2011; Polard et al., 2011). De plus, des effets épigénétiques Trans générationnels peuvent être induits sous l'effet de la contamination environnementale par des mécanismes épigénétiques (méthylation de l'ADN, modifications des histones, ARN non codant) et la modification du phénotype d'un organisme sans changer la séquence d'ADN (Perez & Lehner, 2019; Sharma et al., 2019).

(iii) Développement - Chez les amphibiens, l'impact des contaminants environnementaux sur les traits de développement comme la durée de métamorphose, le succès d'éclosion ou le taux de déformation est soit absent (Egea-Serrano et al., 2012) soit montre des effets notables (Jones et al., 2010, 2011; Karlsson et al., 2021). Baier et al. (2016) a étudié les effets interactifs d'herbicides à base de glyphosate et de paramètres environnementaux (température et algues) et a montré qu'une accélération du développement chez le crapaud commun, *Bufo bufo*. L'exposition à des concentrations environnementales de pesticides réduit la survie pendant le développement dans la plupart des études comprenant les herbicides organochlorés, les insecticides carbamates ou chlorporidinyll (Baker et al., 2013). Les effets sur les amphibiens comprennent une mortalité accrue, une croissance réduite, des anomalies de développement et une sensibilité accrue aux maladies (Daszak et al., 2003; Relyea, 2005). L'effet de ces produits chimiques peut varier selon les classes chimiques et les espèces. Par exemple, la survie de la grenouille verte (*Rana clamitans*) a diminué lorsqu'elle a été exposée à un pesticide organophosphoré, alors qu'un chloropyridinyle, n'a pas entraîné de diminution de la survie de la même espèce (Sparling et al., 2001; Wojtaszek et al., 2005) De plus, le carbaryl, un carbamate, réduit la survie de la salamandre tachetée (*Ambystoma maculatum*) mais ne réduit pas celle de la grenouille léopard (*Rana sphenoccephala*) (Boone et al., 2004, 2007).

(iv) Phénotype - Les changements morphologiques intègrent les multiples effets d'un ou de plusieurs facteurs de stress sur le développement des organismes exposés. Une étude sur le goujon (*Gobio gobio*) a montré que des variations en traits morphologiques étaient corrélés à la toxicité des pesticides (Shinn et al., 2015). Après élimination des effets confondants comme la variabilité génétique, 3 des 17 traits morphologiques étudiés étaient significativement corrélés à la toxicité des pesticides, ce qui suggère une réponse de ces traits au stress induit par les pesticides (Shinn et al., 2015). Le diamètre des yeux du goujon, la position des yeux et la hauteur du corps sont les trois traits morphologiques qui ont présentés une réponse aux niveaux de toxicité des pesticides : des différences croissantes (entre les sites d'échantillonnage) dans ces traits correspondent à des différences croissantes dans la toxicité.

(v) Reproduction - Les effets sublétaux altèrent la morphologie des gonades et la concentration d'hormones sexuelles (Karlsson et al., 2021; Slaby et al., 2019). Les contaminants environnementaux connus comme étant des perturbateurs endocriniens, peuvent agir comme anti-androgènes et auraient un effet négatif sur la gamétogenèse et la fertilité après une exposition au stade larvaire chez les amphibiens (Gyllenhammar et al., 2009; Hayes et al., 2010).

(vii) Comportement - L'exposition aux pesticides à des concentrations sublétales peut entraîner des modifications comportementales. On peut citer la baisse de vitesse de nage chez les poissons sous l'influence des pyréthroïdes, carbamates, et organophosphonates. Des impacts sur l'activité, la réponse aux prédateurs ou la personnalité (Brunelli et al., 2009; Ford et al., 2021; Giaquinto et al., 2017; Shuman-Goodier & Propper, 2016). Chez le Pacu (*Piaractus mesopotamicus*), des concentrations de glyphosate diminuent la prise alimentaire inhibant la croissance à long terme (Giaquinto et al., 2017). Les amphibiens à différentes concentrations d'endosulfan ont présenté des schémas de nages altérés, tels que l'hyperactivité, la nage erratique (*Rana sylvatica*, *Rana clamitans* et *Bufo americanus*) (Shuman-Goodier & Propper, 2016).

(viii) Populationnel – L'impact des niveaux résiduels d'insecticides dans le milieu aquatique a été étudié dans une synthèse centrée sur les insecticides néonicotinoïdes (Sánchez-Bayo et al., 2016). Les néonicotinoïdes modifient les assemblages et la structure des communautés aquatiques avec une déplétion importante des populations d'invertébrés. En conséquence, les espèces dépendantes des insectes comme ressource sont en déclin incluant les amphibiens et les oiseaux. Un autre groupe peu représenté dans les études d'impacts des contaminations sont les champignons aquatiques (Ittner et al., 2018). Pourtant, nécessaires dans les dynamiques de réseau trophique étant une source d'alimentation des petits invertébrés aquatiques, ils sont impactés par la contamination environnementale. Des études sur les effets des fongicides sur d'autres fonctions écologiques importantes, comme le contrôle des proliférations d'algues dans les eaux de surface lenticues par des champignons chytrides parasites sont des voies d'investigations prometteuses.

V) Les amphibiens comme sentinelles de leur environnement

Les amphibiens sont un des taxons les plus touchés par cette crise de la biodiversité du dernier siècle (Blaustein et al., 2011) avec 41% des espèces d'amphibiens connues menacées d'extinction (IUCN, 2020; Monastersky, 2014). Les causes du déclin des amphibiens sont complexes et multiples (Figure 4), elles diffèrent entre espèces, mode de vie, populations et stades de développement (Blaustein et al., 2011; Brühl et al., 2011; Greulich & Pflugmacher, 2003). Elles peuvent se trouver à l'échelle globale (Stuart et al., 2004) comme l'exploitation et le trafic d'animaux sauvages, les espèces invasives, le changement climatique (Todd et al., 2011) ou locales avec les changements climatiques au sein de micro-habitats, la dégradation de l'habitat (Alford, 2010; Blaustein et al., 2003; Cooke, 1981), les maladies (Daszak et al., 2003) ou la pollution (Blaustein et al., 2003; Hopkins, 2007; Sparling et al., 2001). L'exposition aux contaminants environnementaux est l'une des principales menaces auxquels

font face les amphibiens avec au moins 1/5 des espèces affectées (Brühl et al., 2013; Hayes et al., 2006).

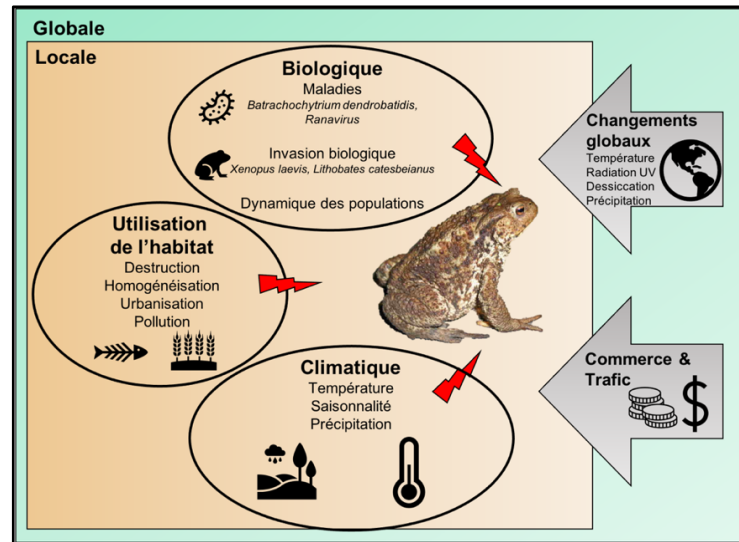


Figure 4 : Facteurs affectant les populations d'amphibiens à l'échelle globale et locale.

L'utilisation à large échelle des pesticides est intimement liée aux pratiques agricoles qui est un facteur majeur de déclin des populations d'amphibiens. Le lien entre contamination environnementale et le déclin des populations d'amphibiens est montré par une proximité aux milieux agricoles et urbanisés (Berger et al., 2018; Bishop et al., 2010; Lenhardt et al., 2015; Taylor et al., 2005). Les milieux utilisés par les amphibiens sont des petits plans d'eau à faibles courants tels que les mares ou les étangs. Ces sites sont souvent nichés au sein d'une mosaïque d'habitats agricoles et sont donc les premiers habitats touchés par la contamination environnementale depuis les zones agricoles adjacentes. L'absence de courant peut aussi influencer la dynamique temporelle des contaminants, ce qui peut augmenter les concentrations de ces molécules et faciliter leur bioaccumulation ou leur métabolisation.

A) Susceptibilité à la contamination environnementale

Malgré cela, de nombreuses espèces d'amphibiens sont capables de survivre et persister dans les milieux modifiés, notamment grâce aux mares temporaires et bassines créés pour certaines activités agricoles (abreuvement des troupeaux, irrigation) et servant de mares de reproduction (da Silva et al., 2012; Knutson et al., 2004; Smalling et al., 2015). De plus, l'écologie des amphibiens les rend pertinents pour étudier l'impact des changements de qualité de l'eau pour plusieurs raisons.

(i) La peau de ces espèces est très perméable à l'eau, aux ions et échanges gazeux ce qui facilite l'entrée de substances tels que les xénobiotiques (Quaranta et al., 2009).

(ii) Chez de nombreuses espèces, les têtards filtrent l'eau, broutent le biofilm, les algues ou le sédiment où les pesticides sont connus pour s'accumuler pendant des durées importantes même lorsque les concentrations sont faibles (Greulich & Pflugmacher, 2003).

(iii) Les développements embryonnaire et larvaire des amphibiens se passent dans le milieu aquatique alors que le stade adulte a souvent lieu dans le milieu terrestre, ils sont donc potentiellement soumis à la contamination dans les deux milieux. Chez les amphibiens terrestres, le transfert de contaminant par exposition percutanée est une voie d'exposition peu étudiée (Brühl et al., 2013). Pourtant, les amphibiens, pour boire, absorbent l'eau à travers leur peau (zone pelvienne en position ventrale) en contact direct sur les substrats humides (McClanahan & Baldwin, 1969). Ces patches cutanés sont très vascularisés et leur structure augmente la susceptibilité aux xénobiotiques (Brühl et al., 2011, 2013; Quaranta et al., 2009). Pendant les périodes sèches, les amphibiens tendent à préférer les milieux agricoles qui restent humides à cause de l'irrigation des cultures (Fryday & Thompson, 2012). Cette coïncidence spatiale avec les activités agricoles peut faciliter le contact de ces animaux avec les pesticides et fertilisants (Fryday & Thompson, 2012; Mann et al., 2009). Ces pesticides peuvent s'accumuler dans le derme des amphibiens et entraîner des charges de contamination corporelles mesurables (Van Meter et al., 2014), des altérations physiologiques et la mortalité (Brühl et al., 2013; Henson-Ramsey et al., 2008; Storrs Méndez et al., 2009). Malgré l'absence de prise en compte explicite de la voie de contact dermique chez les amphibiens dans les évaluations des risques écologiques, cette voie est potentiellement une route d'exposition importante aux pesticides (Van Meter et al., 2014, 2015).

(iv) Enfin, le développement des amphibiens est soumis à un contrôle hormonal, notamment pendant la métamorphose. Les embryons et têtards sont sensibles aux xénobiotiques connus comme étant des perturbateurs endocriens qui peuvent altérer les voies hormonales (Chai et al., 2017; Crump et al., 2002; Kashiwagi et al., 2009). Notamment, les hormones thyroïdiennes (TH) qui jouent un rôle important dans le métabolisme, la croissance et le développement. Chez les amphibiens, les hormones thyroïdiennes ont un rôle prédominant dans la métamorphose. La métamorphose des amphibiens est déclenchée et contrôlée par les TH, et il semble donc très probable que la synthèse, le transport et le métabolisme des TH soient tous des domaines cibles potentiels pour les effets perturbateurs des produits chimiques environnementaux. La perturbation des hormones thyroïdiennes est causée par divers mécanismes, notamment l'augmentation du métabolisme de la thyroxine (T₄) par les uridine diphosphate glucuronyl transférase (UDPGT), le blocage de la signalisation des TH par les récepteurs des hormones thyroïdiennes (TR).

B) Espèces sauvages sentinelles

Les méthodes pour explorer les effets de la contamination sur les population d'amphibiens incluent des expérimentations en milieux contrôlés utilisant des espèces modèles (Babalola et al., 2021; Coady et al., 2005). Une approche courante de la recherche toxicologique sur les amphibiens consiste à exposer une seule espèce d'anoures ou de salamandres aquatiques embryonnaires et/ou larvaires à différentes concentrations d'un seul contaminant (par exemple, aucun et 5 concentrations selon les normes OCDE) dans des aquariums en laboratoire. Deux espèces modèles d'amphibiens sont couramment utilisées de cette manière : *Xenopus laevis* et *Xenopus tropicalis* (Matthews & Vosshall, 2020; Segner & Baumann, 2016). Il s'agit de deux des nombreuses espèces entièrement aquatiques de grenouilles africaines. *X. laevis* et *X. tropicalis* sont des modèles à partir desquels on extrapole des effets pour les autres espèces d'amphibiens, mais aussi pour les vertébrés en général, y compris les humains (Blum & Ott, 2018). Ils sont utilisés depuis des décennies dans la recherche fondamentale sur les mécanismes du développement et sur les produits chimiques toxiques qui peuvent interférer avec le développement (Babalola et al., 2021; Crump et al., 2002; Gyllenhammar et al., 2009). L'avantage de ces deux espèces, *X. laevis* et, de plus en plus, *X. tropicalis*, sont leur disponibilité, leur coût d'entretien faible, et ces espèces sont relativement grandes et tolérantes aux conditions de laboratoire (Blum & Ott, 2018). Il est important de noter que les nombreuses études précédentes sur xénopes fournissent une base de littérature importante. En revanche, le génome, la morphologie et le cycle de vie des xénopes diffèrent considérablement de ceux de la plupart des autres amphibiens. Il reste donc nécessaire d'incorporer d'autres espèces plus représentatives des espèces d'amphibiens sauvages dans les études de stress environnementaux (Bolker, 2019; Hopkins, 2007).

Outre les approches purement expérimentales, l'échantillonnage en milieu naturel sur des sites contaminés ou non permet d'observer l'état d'une population soumise à plusieurs stress environnementaux. Les paramètres étudiés sont la mortalité, la survie, la durée de développement, le taux de malformation, les effets sur la différenciation sexuelle, la fécondité, les fonctions immunitaires et hormonales, le stockage d'énergie ou la génotoxicité (Bishop et al., 2010; Bókony et al., 2019; Cooke, 1981; Fleeger et al., 2003; Fryday & Thompson, 2012; Gibbons et al., 2015; D. A. Morrison, 2017; M. L. Morrison, 2001). L'exposition aux pesticides peut varier dans le temps et impacter de manière différentielle les stades de développement des amphibiens.

C'est dans ce cadre que ma thèse s'inscrit et j'ai cherché à savoir comment les herbicides altèrent les phases clés du développement d'un amphibien présent dans les milieux agricoles et forestiers.

VI) Objectifs de la thèse

Les stressseurs environnementaux peuvent influencer différentes échelles d'intégration biologique, des molécules aux réponses populationnelles (**Figure 5**). Étant donné la structure chimique et la capacité de certains pesticides à mimer les médiateurs chimiques, le stress induit par la contamination pourrait avoir des conséquences importantes sur l'intégrité moléculaires et cellulaires entraînant des modifications de comportement, de stratégie de développement menant à terme à de la variabilité au sein des populations.

En conséquence, je me suis intéressée aux effets de la contamination environnementale sur l'écologie et le développement d'un amphibien répandu de l'Ouest de la France, le crapaud épineux (*Bufo spinosus*). L'intérêt de cette espèce réside dans son écologie. Sa faible capacité de dispersion et son mode de vie bi-phasique propre aux amphibiens l'obligent à faire face à une contamination terrestre et aquatique à tous les stades de vie (voir ci-dessus). On le retrouve aussi bien en milieu forestier qu'en milieu agricole (Guillot et al., 2016; Janin et al., 2011) avec des abondances importantes en période de reproduction.

Tout d'abord, nous avons cherché à savoir si des individus adultes issus d'habitats contrastés, qui diffèrent structurellement et en termes de contamination environnementale, présentaient des différences morphologiques, physiologiques ou comportementales pouvant résulter de réponses aux stressseurs des milieux agricoles (**Articles I, II**). Pour cela, nous avons collecté des mâles de crapauds épineux adultes pendant la période de reproduction sur 8 sites contrastés et mesuré leur morphologie. Les contaminants environnementaux étant connus comme étant des perturbateurs endocriniens, nous avons également dosé les hormones critiques pour l'homéostasie en période de reproduction, à savoir la testostérone et la corticostérone. Nous avons également mesuré la longueur de leur télomères, proxy de sénescence cellulaire et donc d'espérance de vie.

Dans un second temps, nous avons cherché à isoler les potentiels effets de contaminants environnementaux sur l'écologie du crapaud épineux. Pour cela, nous avons mené des expérimentations en milieux contrôlés sur le développement de *Bufo spinosus*. Pendant le développement, deux périodes sont critiques pour le futur individu, le développement embryonnaire (**Articles III, IV, V, VI**) et le développement larvaire (**Articles VII, VIII, IX**). Le développement embryonnaire va déterminer la qualité du têtard pouvant évoluer librement et continuer son développement dans son environnement aquatique et le développement larvaire jusqu'à la métamorphose va déterminer la qualité de l'individu à l'âge adulte (Brühl et al., 2011; DiGiacopo & Hua, 2020; Greulich & Pflugmacher, 2003). Nous avons donc soumis les deux périodes clés du développement à deux herbicides, un métabolite d'un contaminant d'héritage (AMPA, voir

méthodes, **Articles IV, V, VIII**) et un herbicide émergent (Nicosulfuron, voir méthodes, **Articles VI, IX**) à des concentrations sublétales croissantes dans le but de déterminer si ces deux phases de développement (embryonnaire ou larvaire) diffèrent dans leur sensibilité à ces herbicides. Nous avons étudié la survie, la morphologie, la durée de développement, les télomères, les mécanismes de défenses antioxydants, le comportement et la viabilité des individus post-métamorphose.

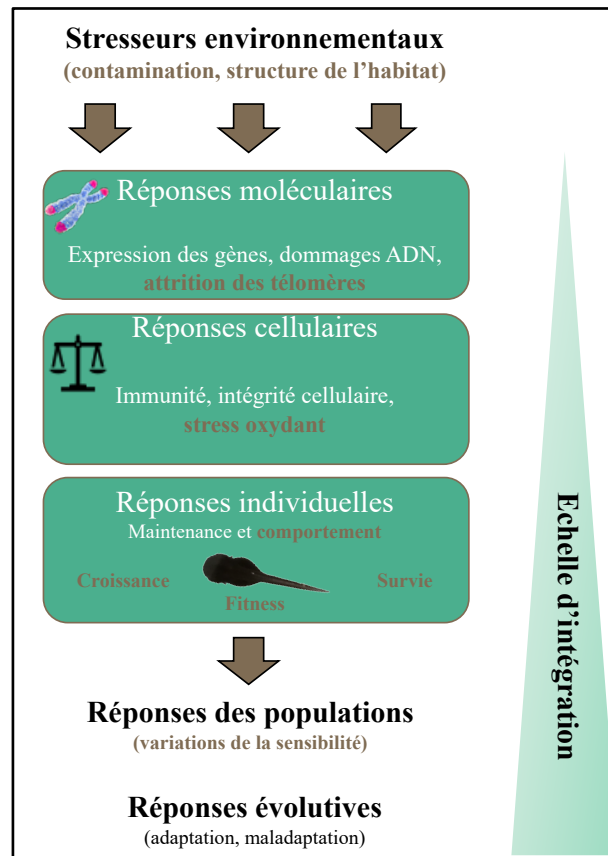


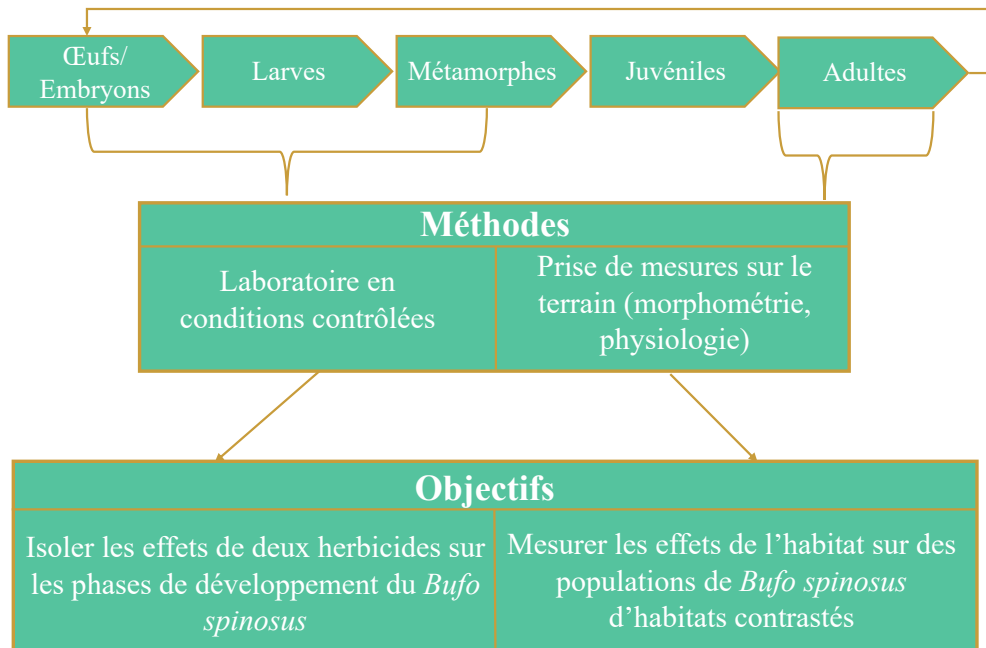
Figure 5 : Schéma conceptuel de la réponse aux stress simples ou multiples (ici contamination et altération de la structure de l'habitat) de l'échelle moléculaire à l'échelle populationnelle. En marron sont représentés les réponses étudiées dans cette thèse. Le **chapitre I** se concentre sur les réponses des populations de mâles crapauds épineux (variabilité en morphologie et physiologie). Le **chapitre II** sur les réponses moléculaires, cellulaires et individuelles des embryons éclos de crapaud épineux (Télomères, stress oxydant, survie et croissance). Le **chapitre III** se concentre sur les réponses individuelles de têtards et métamorphes de crapaud épineux (Comportement, croissance, survie et fitness).

Méthodes générales



“Data! Data! Data! ... I can’t make bricks without clay!” — **Sir Arthur Conan Doyle** (The Adventure of the Copper Beeches, dans *Adventures of Sherlock Holmes* (1892), 289)

Cette section aborde les méthodes propres à chaque article inclue dans la thèse. Des méthodes plus spécifiques seront retrouvées au sein des articles correspondants en fin de manuscrit.



I) Modèle d'étude : Le crapaud épineux

Le crapaud épineux *Bufo spinosus* appartient à l'ordre des anoures. Autrefois faisant partie de l'espèce *Bufo bufo* (crapaud commun), il a été élevé au rang d'espèce en 2012 sur la base de critères morphologiques et génétiques (Recuero et al., 2012, Arntzen et al., 2013). De plus, les aires de répartition diffèrent entre le crapaud épineux et le crapaud commun (*Bufo bufo*) auquel il était autrefois rattaché.

Le crapaud épineux est l'une des espèces d'amphibiens les plus communes d'Europe de l'Ouest. C'est une espèce généraliste qui est retrouvée dans une grande diversité d'habitat, incluant les milieux modifiés soumis à l'agriculture intensive (Guillot et al., 2016). Ses périodes d'activités se situent de Janvier à Octobre avec une période d'hivernation en début d'hiver (Figure 6). Comme la plupart des amphibiens semi-terrestres, la phase adulte est principalement terrestre exceptée pendant les périodes de reproduction où les femelles et les mâles se retrouvent, parfois à des effectifs importantes, dans les sites (mares) de reproduction. Cette espèce est qualifiée d'« explosive breeder » et les individus montrent également une grande fidélité à leur mare de naissance (Reading et al., 1991).

Les phases de développement embryonnaire et larvaire sont entièrement aquatiques de la ponte jusqu'à la métamorphose.

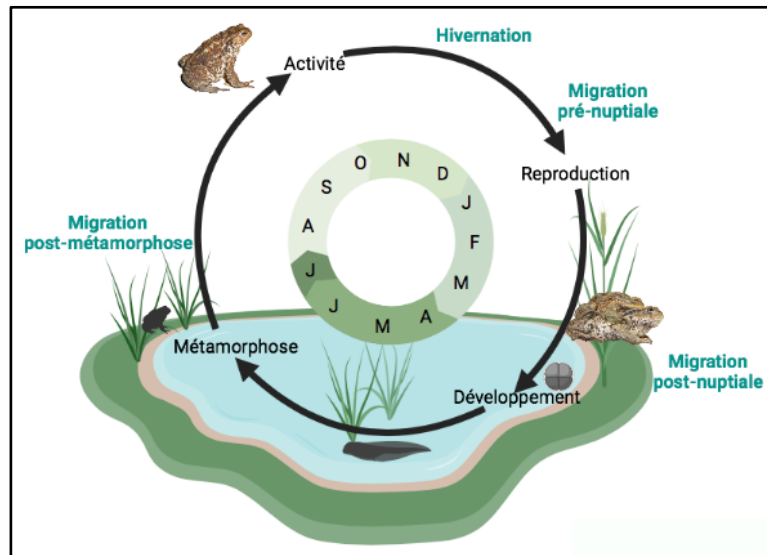


Figure 6 : Cycle de vie du crapaud épineux (*Bufo spinosus*)

Plusieurs phases de migrations sont observées chez le crapaud épineux, une migration pré et post nuptiale et une migration post-métamorphose (Hartel & Demeter, 2015; Kovar et al., 2009). Les distances de migrations sont faibles allant de 170 à 1835 mètres en moyenne (Kovar et al., 2009). A la fin de l'hiver, les crapauds émergent d'hivernation et migrent vers les sites de reproduction. Les mâles arrivent les premiers sur ces sites dans lesquels ils restent toute la durée de la période de reproduction (en moyenne 36 jours de reproduction (Kovar & Brabec, 2007)). Les femelles arrivent plus tardivement, y restent de manière très transitoire et repartent dans leur micro-habitat terrestre juste après la ponte. Les mâles surpassent donc en nombre les femelles et c'est pour cette raison que nos études de terrain se concentrent sur les mâles exclusivement (Reading & Clarke, 1983).

II) Approche terrain

A) Sites d'échantillonnage

Dans le but de mesurer les différences phénotypiques, physiologiques, et comportementales entre les individus issus d'habitats contrastés, nous avons mis en place un plan d'échantillonnage réparti sur 8 sites différents situés dans le sud Deux Sèvres et le nord de la Charente-Maritime.

Autour de chaque mare, nous avons caractérisé l'habitat dans un diamètre de 1000m sachant les distances de migration des crapaud épineux (Kovar et al., 2009; Miaud & Sanuy, 2005). Les sites ont été monitorés toutes les nuits du 7 février 2019 au 11 mars 2019. Nous nous sommes concentrés sur les premiers mâles de chaque site pour limiter les différences phénotypiques et physiologiques liées à la phénologie de la reproduction (Phillimore et al., 2010; Urban et al., 2014). Cependant, un site en particulier (Prissée la Charrière) a été monitoré pendant 5 semaines consécutives dans le cadre d'un

projet annexe à ce projet de thèse (Brischoux & Cheron, 2019) (**Annexe I**). Au total, 229 mâles *Bufo spinosus* ont été capturés dans le cadre de cet échantillonnage (**Tableau 1**).

Site	Nombres de sessions	Première date de session	Dernière date de session	Nombre de mâles capturés
Aulnay	2	19/02/2019	22/02/2019	19
LeLac	2	17/02/2019	19/02/2019	20
Javarzay	1	18/02/2019	18/02/2019	20
Labo	2	18/02/2019	26/02/2019	22
Préraut	2	19/02/2019	25/02/2019	10
Prioulet	1	09/02/2019	09/02/2019	20
Prissée la Charrière	5	07/02/2019	11/03/2019	100
Zoo	1	08/02/2019	08/02/2019	18

Tableau 1 : Site, date d'échantillonnage et nombre d'individus capturés dans le cadre de l'étude sur les mâles adultes d'habitats contrastés.

La caractérisation de l'habitat a été effectuée en utilisant le logiciel de cartographie QGIS. Autour de chaque site d'étude, des zones tampons, d'un diamètre de 1000m correspondant à la distance maximale parcourue par un individu pour rejoindre une mare de reproduction, ont été dessinées (Kovar et al., 2009). L'habitat était caractérisé selon l'aire de forêt, l'aire de zones agricoles, l'aire de bâti et l'aire de prairies au sein du diamètre de 1000m (**Figure 7**).

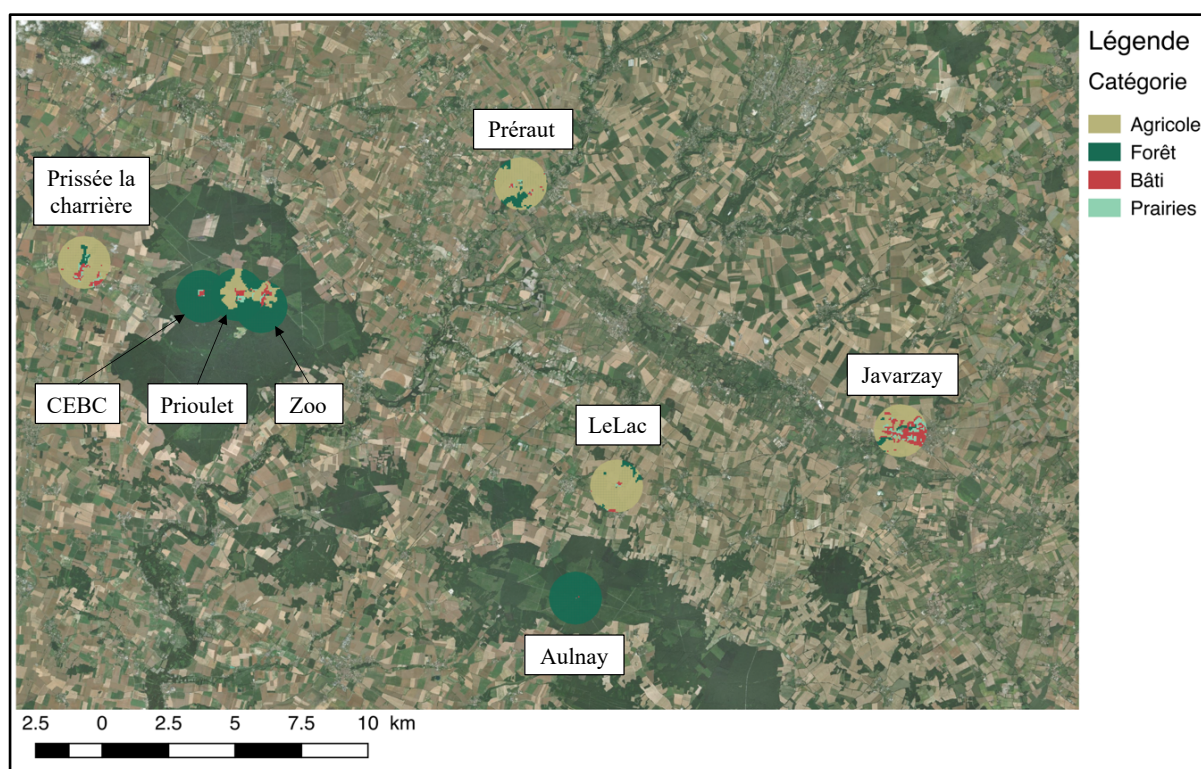


Figure 7 : Sites de prélèvements de *Bufo spinosus* et caractérisation de l'habitat (QGIS V2.18). L'aire agricole (jaune), l'aire forestière (vert), l'aire de bâti (rouge) et l'aire de prairies (bleu clair).

Les surfaces de couvert forestier et de champs agricoles ont été retenues comme étant représentatives de la qualité d'un habitat pour cette espèce. Les deux types de surface étaient corrélées négativement ($r=-0.96$ [-0.99: -0.80), $t(6)=-8.60$, $p<0.001$). L'aire de couvert forestier nous a permis de créer des catégories d'habitats : les sites >90% d'aire de forêt ont été classifiés : « **Forestier** », les sites <15% d'aire de forêt ont été classifiés « **Agricole** » et les sites restants (entre 60 et 70%) comme « **Intermédiaire** » (Tableau 2).

Site	Catégorie	N	Couvert forestier (%)
Prissé	Agricole	21	4.45
Javarzay	Agricole	20	5.22
LeLac	Agricole	20	5.61
Préaut	Agricole	7	13.14
Prioulet	Intermédiaire	20	61.01
Zoo	Intermédiaire	18	73.06
Labo	Forestier	22	93.19
Aulnay	Forestier	19	99.84

Tableau 2 : Classement par catégories d'habitats selon le couvert forestier des sites d'études.

B) Dosages de contaminants dans les mares

Dans le but de déterminer la composition en pesticides des sites d'étude, nous avons collaboré avec un laboratoire d'analyse indépendant « QUALYSE ». Deux sites forestiers et deux sites potentiellement contaminés ont été analysés : Aulnay, CEBC, Ensigné et Bernegoue, un site non compris dans cette thèse du mois de Février à Juin avec un prélèvement par mois. Les molécules analysées comprennent Phtalates, Pesticides (Triazole, Organophosphonate, multi-famille), Eaux usées, Chrome VI, Tritium et Algues. Le but de ces analyses était de déterminer la charge en pesticides des mares de reproduction de crapaud épineux et d'observer l'évolution temporelle en fonction de l'épandage provenant de l'agriculture. A terme, nous pouvions déterminer un indice de toxicité (Toxic Unit, TU, De Castro-Català et al., 2016, Kuzmanovic et al., 2016) pour classer ces mares en fonction de leur toxicité et comparer la potentielle exposition des adultes et des individus en développement. Ces dosages pour le site « CEBC » a pu nous assurer que les pontes échantillonnées pour l'approche expérimentale étaient des pontes naïves à la contamination.

C) Mesures

Trois types de mesures ont été effectués soit directement sur le terrain soit en laboratoire. Dès qu'un individu était repéré à l'aide d'une lampe frontale, il était capturé par l'observateur et ramené sur un site de prélèvement adjacent au site de capture. Nous avons effectué des prélèvements de sang intracardiaque (100µl) en utilisant des seringues d'1ml héparinées. Le but étant de mesurer les hormones

(i.e, corticostérone et testostérone), mesurer la longueur des télomères, doser les isotopes mais également effectuer des analyses de génétiques des populations. Nous avons également prélevé à l'aide d'écouvillons le microbiote cutané pour possiblement mesurer la prévalence de chytridiomycose dans les populations. Les prises de sang ont été effectuées en moins de 5 minutes (moyenne±se, 2,58±1,08) pour mesurer les taux basaux d'hormones. Les crapauds étaient pesés et leur température corporelle a été mesurée à l'aide d'un thermomètre. Les individus étaient ensuite placés dans des boîtes individuelles et ramenés au laboratoire.

a) Morphométrie

Au laboratoire, les crapauds étaient mesurés en utilisant un pied à coulisse ($\pm 0,01\text{mm}$). Les mesures effectuées sont résumées dans la **figure 8** (Orton et al., 2020; Watters et al., 2016).

Un indice de condition corporelle a été quantifié à partir des scores résiduels de la régression linéaire entre la taille corporelle et la masse corporelle.

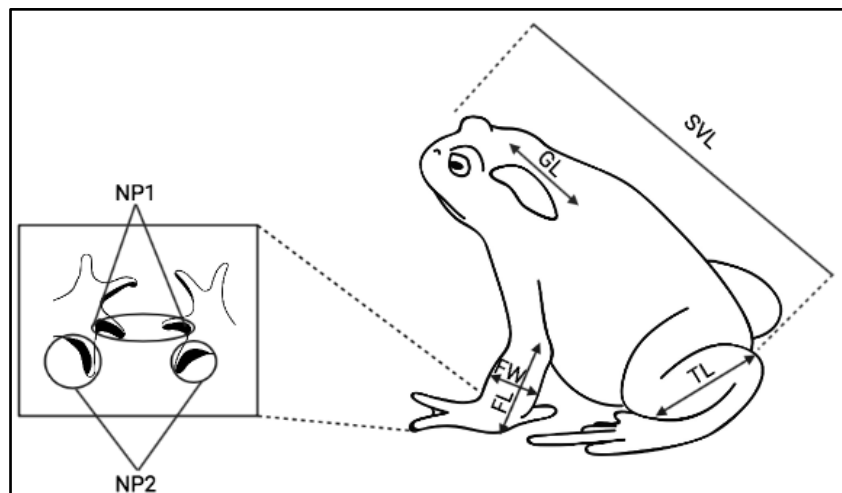


Figure 8 : Mesures morphométriques prises sur les mâles adultes de crapaud épineux (*Bufo spinosus*). *SVL*: Longueur museau-cloaque, *TL*: Longueur du tibia, *GL*: Longueur des glandes parotoïdes, *FL*: Longueur de l'avant-bras, *FW*: Largeur de l'avant-bras, *NP1*: Aire de la callosité nuptiale du deuxième doigt, *NP2* : Aire de la callosité nuptiale du premier doigt.

Parmi ces variables morphométriques, nous nous sommes intéressés à des caractères sexuels secondaires connus pour être liés au succès reproducteur chez les amphibiens, les callosités nuptiales (Orton et al., 2020). Les androgènes (comme la testostérone) contrôlent le développement des callosités nuptiales (Epstein & Blackburn, 1997) avec des niveaux importants de récepteurs androgènes dans les pattes avant des mâles comparés aux femelles (Emerson et al., 1999). De plus, la castration d'adultes, de même que l'exposition à des estrogènes, résultent en une baisse de taille des callosités nuptiales réversible à l'aide d'implants hormonaux (Taisen, 2011). Les callosités nuptiales servent à agripper la femelle pendant l'amplexus. De même que la reproduction, la couleur et la taille

de ces callosités sont régulés par des androgènes (Norris & Lopez, 2011; Rastogi et al., 2011). De plus, nous avons mesuré la largeur des avant-bras comme caractères sexuels secondaires et proxy de la musculature chez les mâles crapauds épineux. La largeur des bras chez les mâles est plus importante que chez les femelles ce qui suggère que ce trait est sous sélection sexuelle (Greene & Funk, 2009), une largeur plus importante augmentant le succès d'amplexus (Bowcock et al., 2009).

b) Allométrie statique

Des différences entre traits et tailles corporelles peuvent être vues d'un point de vue ontogénétique ou au niveau des relations allométriques. L'allométrie statique est l'étude des relations allométriques au niveau intraspécifique et à un stade ponctuel. La régression log-log d'un trait en fonction de la taille corporelle permet de renseigner sur la croissance d'un stade de développement en particulier et est souvent mesurée chez les adultes (Sanger et al., 2013). Les relations allométriques entre traits peuvent différer en pente, en ordonnées à l'origine (intercept) mais également en décalage (**Figure 9**) ou les trois simultanément. Chaque scénario peut mener à des conclusions différentes. Des différences en pentes peuvent indiquer qu'un facteur (ici l'habitat) induit un changement inégal de trait avec l'augmentation de la taille corporelle. Des différences en ordonnées à l'origine sous une pente commune signifie que l'habitat influence les proportions de structures morphologiques de manière uniforme entre les tailles corporelles. Une inégalité entre les intercepts peut indiquer que les taux de croissance pendant l'ontogénie sont différents selon les facteurs étudiés (Sanger et al., 2013; Taskinen & Warton, 2013; Warton et al., 2006).

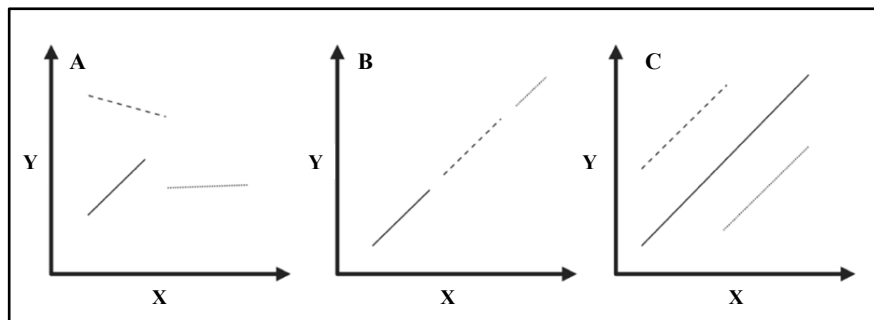


Figure 9 : Types de différences entre relations allométriques (adaptée de Warton et al., 2006). **(A)** Différence entre pente, par ex., la relation entre x et y diffère entre les catégories d'habitat **(B)** Différence de décalage, par ex., les lignes allométriques sont décalées le long de leur pente commune, par ex., x et y varient de la même façon à travers les catégories. **(C)** Différence dans les ordonnées à l'origine, les relations allométriques diffèrent en élévation, elles sont décalées en parallèle les unes par rapport aux autres, par ex., les valeurs de x diffèrent entre les groupes pour une même valeur de y .

c) Physiologie

Analyse isotopique

Conjointement aux analyses hormonales, nous avons réalisé des analyses isotopiques dans le sang des crapauds provenant de différents habitats. Les analyses isotopiques ont été effectuées au LIENSS sur du culot de sang de *Bufo spinosus*. Les analyses ont été réalisées par Matthias Renoirt dans le cadre de sa thèse avec l'aide de Paco Bustamante. Les analyses isotopiques ont été réalisées sur du sang lyophilisé au laboratoire LIENSS (La Rochelle, France). Les aliquotes de ~0,3 mg de masse sèche ont été analysées à l'aide d'un spectrophotomètre de masse à flux continu (Thermo Scientific Delta V Advantage) couplé à un analyseur élémentaire organique (Thermo Scientific Flash EA 1112). Les résultats sont calibrés en notation δ conformément à la norme Vienna PeeDee Belemnite (VPDB) et le taux de N₂ atmosphérique pour $\delta^{13}\text{C}$ et $\delta^{15}\text{N}$, respectivement. Les standards (acétanilide) ont été utilisés pour contrôler les résultats. Les erreurs de mesures sont <0.15‰ pour les valeurs de $\delta^{13}\text{C}$ et $\delta^{15}\text{N}$.

Analyses hormonales

Les analyses de corticostérone (CORT) et de testostérone (T) ont été réalisées au CEBC sur du plasma de sang de *Bufo spinosus*. Le sang a été centrifugé, le plasma et les érythrocytes séparés et gardés dans un congélateur à -20°C jusqu'à ce que les analyses soient effectuées. Les concentrations de T et CORT ont été déterminées par radio-immuno-essai (Brischoux et al., 2018) en utilisant 50µL de plasma. Les niveaux de détection minimum étaient de 0,83 et 0,28 ng ml⁻¹, respectivement, pour T et CORT, et les coefficients de variation intra et inter dosages étaient 13,16% et 17,56% et 10,66% et 11,77%, respectivement, pour T et CORT (qui ont été exécutés deux fois au sein et entre les dosages). Les échantillons ont été analysés en dupliquât, en 5 dosages.

Mesure de longueur des télomères

Les analyses de télomères ont été effectuées au CEBC par Frédéric Angelier, Cécile Ribout et moi-même (extraction d'ADN). L'extraction ADN a été effectuée sur environ 4µL de culot de sang en utilisant un kit « Blood MN » (Macharey-Nagel). La longueur des télomères a été déterminé par PCR quantitative (qPCR; BioRad CFX 96; Bio-Rad, USA) en utilisant un protocole établi (McLennan et al., 2019), adapté pour le crapaud épineux. Toutes les qPCRs ont été effectués en trois plaques pour chaque gène (RAG1 et télomère) en utilisant 7.5ng d'ADN par réaction. La longueur relative des télomères (exprimée en T/S ratio) a été calculée comme le nombre de copies de télomères (T) relatif aux nombres de gènes à copie unique (S ; RAG1) selon Cawthon, 2002.

d) Comportement

Le comportement et la personnalité a des implications importantes pour la conservation (Merrick & Koprowski, 2017). Certains traits de personnalité ou une combinaison de traits peut avoir le potentiel d'influencer la migration, le succès de reproduction ou encore la survie. Ici nous avons choisi d'étudier le comportement selon les stades de développement (Bredeweg et al., 2019; Bremner-Harrison et al., 2004; Sarrazin & Legendre, 2000). Le comportement change au cours de l'ontogénie car il influence des traits d'histoires de vie. Étudier le comportement chez les amphibiens est particulièrement pertinent chez des espèces particulièrement susceptibles aux facteurs responsable de l'érosion de la biodiversité (Stuart et al., 2004). Cependant, la métamorphose est une caractéristique des amphibiens et les changements comportementaux associés à ce cycle de vie bi phasique à de grandes implications dans la persistance des populations (Brown & Cai, 2007; Earl & Whiteman, 2015). Cette étape constitue une transition dans l'habitat et la niche écologique impliquant des modifications de la physiologie, morphologie et l'écologie. Kelleher et al ont synthétisé les différentes études sur le comportement chez les amphibiens, du têtard à l'adulte. Ces expériences varient en fonction du milieu de vie de l'espèce, notamment entre espèces totalement aquatiques ou terrestres. *Bufo spinosus* étant une espèce semi-terrestre, nous avons adapté les méthodologies pour répondre aux caractéristiques écologiques de l'espèce.

Réale et al. (2007) a développé un cadre pour étudier la personnalité animale en 5 axes définissant les traits de personnalité majeurs (1) témérité/timidité, (2) exploration/évitement, (3) activité, (4) sociabilité, and (5) agressivité. Chez les amphibiens, la sociabilité est limitée à quelques espèces et les tests d'agressivité ne sont pas adaptés à la plupart des espèces. Les études de comportement se concentrent donc sur (1) témérité / timidité, (2) exploration/évitement, (3) activité. Dans notre étude, nous avons donc cherché à étudier si le comportement était différent entre mâles en reproduction provenant de différents habitats.

Suite à la capture, les mâles étaient ramenés en laboratoire pour participer à des expériences de comportement. Les crapauds étaient placés seuls dans une arène (73L x 111 x 17H cm) en conditions contrôlées. Deux abris étaient placés à chaque extrémité de l'arène. Le crapaud était placé sous un des abris et les expérimentateurs sont sortis de la salle pendant les 15 minutes d'acclimatation. A la suite de cette acclimatation, l'abri sous lequel se trouvait le crapaud a été enlevé et nous avons enregistré son comportement pendant 30 minutes en utilisant une caméra (GoPro hero, GoPro, Inc., San Mateo, CA, USA) placée au-dessus de l'arène enregistrant à 60 images.s⁻¹. Cette expérience a permis d'étudier 4 variables représentant des proxys d'exploration, d'activité et témérité/timidité. Tout d'abord, nous avons mesuré si le crapaud est sorti de l'abri pendant la phase d'acclimatation qui peut être un proxy de témérité en test de nouvel environnement (González-Bernal et al., 2014; Gruber et al., 2017). Puis, si, pendant l'enregistrement, le crapaud est allé dans le second abri ou pas également un proxy de la témérité (Videlier et al., 2014). Nous avons également discriminé les types de positions adoptées par

les crapauds lorsque qu'on enlevait l'abri comme proxy de défense ("Crouching-down" or "Body-raising" position, Toledo et al., 2011). La position de type « body-raising » est une posture typique utilisée par les espèces toxiques lors d'une attaque par un prédateur (Toledo et al., 2011). Et enfin, nous avons mesuré si les individus ont exploré ou non et le temps d'activité des individus pendant les 30 minutes.

III) Approche expérimentale

A) Site de prélèvement

Parmi les sites d'étude de l'approche terrain, deux sites en particulier ont été utilisés pour récolter les pontes de crapaud épineux nécessaire à l'approche expérimentale (Prissée la charrière, Labo, Figure 7). Ces sites présentent des configurations particulières qui facilitent la collecte d'individu sans impacter la population. De plus, sa proximité avec le laboratoire d'étude a permis de monitorer le site matin et soir pour éviter aux pontes de rester dans leur environnement d'origine assez longtemps pour induire des effets développementaux.

B) Type de contaminant

Dans le cadre de cette thèse, nous nous sommes intéressés à deux herbicides largement retrouvés dans les cours d'eaux, l'acide aminométhylphosphonique (AMPA) et le nicosulfuron. Les agences de l'eau Loire-Bretagne et Adour-Garonne ayant collaboré à cette thèse, nous avons eu accès aux occurrences et concentrations de ces herbicides dans les eaux de surface de la région. Ce sont grâce à ces données que nous avons fixé les concentrations utilisées dans le cadre des expérimentations.

a) Acide aminométhylphosphonique (AMPA)

Les herbicides contenant du glyphosate sont utilisés partout dans le monde sur plus de 100 cultures différentes (Monsanto, 2009). Le glyphosate a d'abord été enregistré sous la forme du Roundup au États-Unis en 1974 et a été largement utilisé. En 2007 aux États-Unis ainsi qu'au Canada, le glyphosate représentait 40% des parts d'herbicides utilisés. Le glyphosate est souvent appliqué en post-émergence sur les cultures et peut être appliqué plus d'une fois pendant la saison. Cet herbicide est populaire auprès du public et des agriculteurs de par son efficacité mais également parce qu'il est considéré comme étant peu toxique et peu persistant dans l'environnement (Giesy et al., 2000). Cependant, des études montrent sa toxicité et particulièrement la toxicité des formulations à base de glyphosate. Ces formulations contiennent des surfactants et adjuvants (POEA) pour accroître l'efficacité qui s'avèrent être plus toxiques que le glyphosate pur (Giesy et al., 2000; Vincent & Davidson, 2015). Des effets sur le développement et la survie chez les amphibiens ont été observés à

différents niveaux d'exposition au glyphosate et au POEA (Cauble & Wagner, 2005; Howe et al., 2004; Lajmanovich et al., 2003; Moore et al., 2012; Relyea, 2005; Williams & Semlitsch, 2010). Le glyphosate est un composé amphotère polaire qui se lie fortement aux sols, mais qui est également très soluble dans l'eau (plus de 10 000 mg/l à 25 °C). Dans le sol, sa demi-vie se situe entre 2 et 215 jours et dans le milieu aquatique de 2 à 91 jours (Battaglin et al., 2014; Giesy et al., 2000). Il se dégrade rapidement dans l'environnement en acide aminométhylphosphonique (AMPA), par dégradation microbienne en premier lieu. La demi vie de l'AMPA dans le sol est de 60 à 240 jours et dans le milieu aquatique comparable au glyphosate (Battaglin et al., 2014; Giesy et al., 2000). L'AMPA peut également être formée par la dégradation des acides phosphoniques présents dans certains détergents et produits de nettoyage ménagers et industriels (Skark et al., 1998), ce qui fait des usines de traitement des eaux usées et des fosses septiques une source potentielle d'AMPA dans certaines régions (Figure 10). De plus, les acides phosphoniques sont fortement adsorbés dans les sédiments et particules en suspension, et récalcitrants à la dégradation biologique ou non biologique (HERA, 2004).

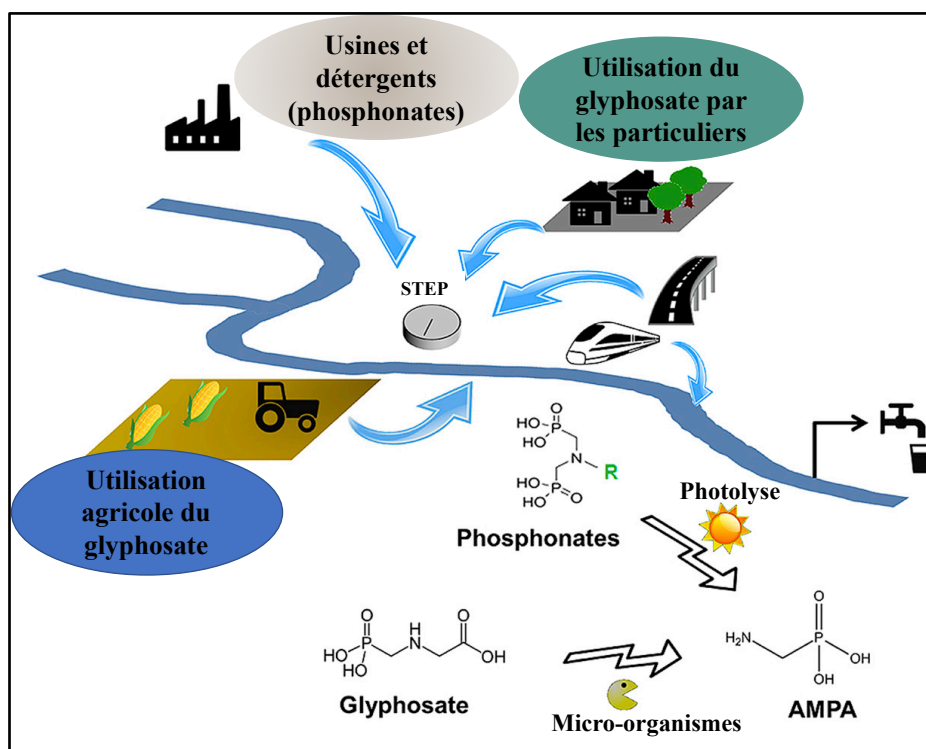


Figure 10 : Sources potentielles pour l'acide aminométhylphosphonique (AMPA). (STEP) représente les stations de traitement des eaux usées domestiques.

Les concentrations dans l'environnement en milieu aquatique en France varient de 0,1µg.l⁻¹ à 6,6µg.l⁻¹ (données « Agence de l'eau Loire Bretagne » et « Agence de l'eau Adour-Garonne ») et sont semblables aux concentrations observées dans les milieux aquatiques en Amérique du Nord et en Europe (Bonansea et al., 2017; Coupe et al., 2012; Grandcoin et al., 2017). Notre objectif étant de mimer la gamme des concentrations trouvées dans la nature, nous avons produit trois traitements allant

de concentrations faibles à relativement élevées (**Tableau 5**). Des solutions mères (0,1 g/L) d'AMPA ont été obtenues en dissolvant de la poudre cristalline commerciale (acide aminométhylphosphonique, pureté de 99 %, ACROS ORGANICS™) dans de l'eau du robinet déchlorée. Ces solutions mères ont été diluées avec de l'eau du robinet déchlorée pour atteindre chacun des traitements (**Tableau 5**). Nous avons produit 3 échantillons supplémentaires pour chaque traitement afin d'effectuer des vérifications analytiques des concentrations. Ces vérifications analytiques des concentrations réelles ont été effectuées par un laboratoire d'analyse indépendant accrédité (QUALYSE, Champdeniers-Saint-Denis, France). Dans l'ensemble, ces vérifications ont révélé que les concentrations réelles étaient semblables à celles observées dans les milieux aquatiques (voir ci-dessus) et que les différences entre les traitements « Faible », « Moyenne » et « Forte » étaient significatives (**Tableau 5**). Pour plus de clarté, nous désignerons les traitements comme Faible, Moyenne et Forte ci-après.

Traitement	Concentration d'AMPA
Témoin	0
Faible	0.07±0.01
Moyenne	0.32 ±0.052
Forte	3.57 ±0.153

Tableau 5 : Concentrations mesurées dans les aquariums ($\mu\text{g.l}^{-1}$) (Mean± SD)

b) Nicosulfuron

Depuis quelques années, de nombreux articles font état de nouveaux composés appelés « contaminants émergents » (Deblonde et al., 2011). Ces contaminants sont de nouvelles substances chimiques qui n'ont pas de statut réglementaire ou d'études sur leurs effets à moyen et à long terme sur la faune et la santé humaine (Deblonde et al., 2011). Cette vaste gamme de contaminants est de plus en plus introduite dans l'environnement par l'application directe, le ruissellement provenant d'applications agricoles et forestières ou de l'exploitation minière, les eaux usées urbaines et industrielles et les dépôts atmosphériques (Relyea & Hoverman, 2006; Solomon & Thompson, 2003). Parmi les herbicides, il existe plusieurs groupes d'inhibiteurs d'acides aminés, dont la famille des sulfonylurées. Ces herbicides sont appliqués dans une grande variété de cultures, y compris le blé, l'avoine, le soja ou le maïs. Habituellement, les applications d'herbicides à base de sulfonylurées se font au printemps après la levée et donc sont particulièrement susceptibles d'être en contact avec les amphibiens pendant leur reproduction (adultes) ou leur développement (embryons, têtards) (Berger et al., 2018). Un des herbicides sulfonylurées le plus couramment utilisé est le nicosulfuron. Il est fréquemment détecté à de très faibles concentrations (moins de $1 \mu\text{g.l}^{-1}$) dans les eaux de surface des rivières et des mares aux États-Unis ou au Canada (Battaglin et al., 2000, 2009; de Lafontaine et al., 2014), dans les sédiments des milieux humides (Degenhardt et al., 2009) et dans les eaux de surface en France (Agences de

l'Eau). Les milieux humides sont particulièrement sensibles à la contamination par ces herbicides en raison de leur emplacement dans les zones agricoles (étangs artificiels, rivières, étangs éphémères...). Dans les étangs, le nicosulfuron présente une demi-vie de dissipation de 75 jours dans la colonne d'eau (Cessna et al., 2015). Bien que les étangs soient soumis à la photolyse et à l'hydrolyse, la photodégradation dans l'eau est lente (Fenoll et al., 2012). Cette stabilité dans la colonne d'eau indique également que la dégradation abiotique et microbienne est faible dans les étangs éphémères (Cessna et al., 2015). Cette persistance du nicosulfuron dans les milieux aquatiques suggère que les concentrations environnementales peuvent avoir une incidence sur les organismes non-cibles des milieux humides. En raison de sa phytotoxicité élevée et sa facilité à se retrouver dans l'environnement via le ruissellement, on peut se s'interroger sur ses effets potentiels sur les amphibiens. Les concentrations de nicosulfuron dans les eaux de surface en France varient de 0,005 $\mu\text{g l}^{-1}$ à 10,702 $\mu\text{g l}^{-1}$ avec une moyenne de 0,054 $\mu\text{g l}^{-1}$ (données de l'Agence de l'eau Loire Bretagne et de l'Agence de l'eau Adour-Garonne, **Tableau 6**).

Molécules	Nombre de dosage positif	Min ($\mu\text{g/l}$)	Max ($\mu\text{g/l}$)	Moyenne ($\mu\text{g/l}$)
Amidosulfuron	27	0.01	0.36	0.059
Chlorsulfuron	1	0.005	0.005	0.005
Flazasulfuron	139	0.003	0.25	0.018
Flupyrsulfuron methyle	127	0.006	10.72	0.096
Iodosulfuron	1	0.021	0.021	0.021
Mesosulfuron methyle	9	0.006	0.054	0.023
Metsulfuron méthyle	47	0.005	0.714	0.0351
Myclobutanil	42	0.007	0.553	0.041
Nicosulfuron	2018	0.005	10.702	0.054
Prosulfuron	28	0.006	10.36	0.442
Sulfosufuron	4	0.02	0.417	0.134
Thifensulfuron methyl	6	0.005	0.03	0.017
Tribenuron-Methyle	27	0.043	20.099	1.820
Triflusaluron-methyl	8	0.009	0.093	0.027

Tableau 6 : Concentrations en herbicides sulfonylurés trouvées dans les eaux de surface en Deux-Sèvres.
Données de l'Agence de l'Eau Adour-Garonne.

Par conséquent, nous avons utilisé trois concentrations pour imiter la gamme des concentrations trouvées dans la nature. Traitement témoin ($0\mu\text{g.l}^{-1}$), faible ($0,15\mu\text{g.l}^{-1}$) et concentration élevée ($0,83\mu\text{g.l}^{-1}$, **Tableau 7**). De même que pour l'AMPA, les solutions mères ont été diluées avec de l'eau du robinet déchlorée pour atteindre la concentration requise pour chaque traitement (**Tableau 7**). Nous avons obtenu des solutions mères ($0,1\text{g.l}^{-1}$) de Nicosulfuron en dissolvant la poudre cristalline commerciale (PESTANAL, Sigma-Aldrich) dans l'eau du robinet déchlorée. Les vérifications analytiques de la concentration ont été effectuées en triplicat par une analyse indépendante accréditée

(QUALYSE, Champdeniers-Saint-Denis, France, LQ=0.01 $\mu\text{g l}^{-1}$). Dans l'ensemble, ces vérifications ont révélé que les concentrations réelles étaient semblables à celles observées dans les milieux aquatiques et que les différences entre les traitements « Faible » et « Forte » étaient significatives (**Tableau 7**). Pour plus de clarté, nous appellerons les traitements Faible et Forte ci-après.

Treatment	Concentration en nicosulfuron
Témoin	0.00 \pm 0.00
Faible	0.15 \pm 0.05
Forte	0.83 \pm 0.04

Tableau 7: Concentrations mesurées dans les aquariums ($\mu\text{g.l}^{-1}$) (Mean \pm SD)

C) Design expérimental

Le design expérimental de cette thèse a été fait pour mesurer les effets de la contamination environnementale sur les phases de développement du crapaud épineux (**Figure 11**). Les œufs et les têtards ont été soumis à différentes concentrations d'herbicides et à un témoin pendant tout le développement embryonnaire jusqu'à l'éclosion. Afin de maintenir des niveaux d'exposition relativement constants tout au long de l'expérience, mais d'éviter des perturbations mécaniques excessives aux œufs en développement, l'eau a été changée une fois par semaine. La gangue des œufs a été maintenue tout au long du développement embryonnaire. Des cordons d'œufs de crapaud épineux (*B. spinosus*) (N = 22 pour l'AMPA, N= 20 pour le nicosulfuron) ont été recueillis dans un étang près du Centre d'étude biologique de Chizé et ramenés au laboratoire et conservés dans des conditions contrôlées. Les œufs ont été recueillis immédiatement après la ponte (observations directes).

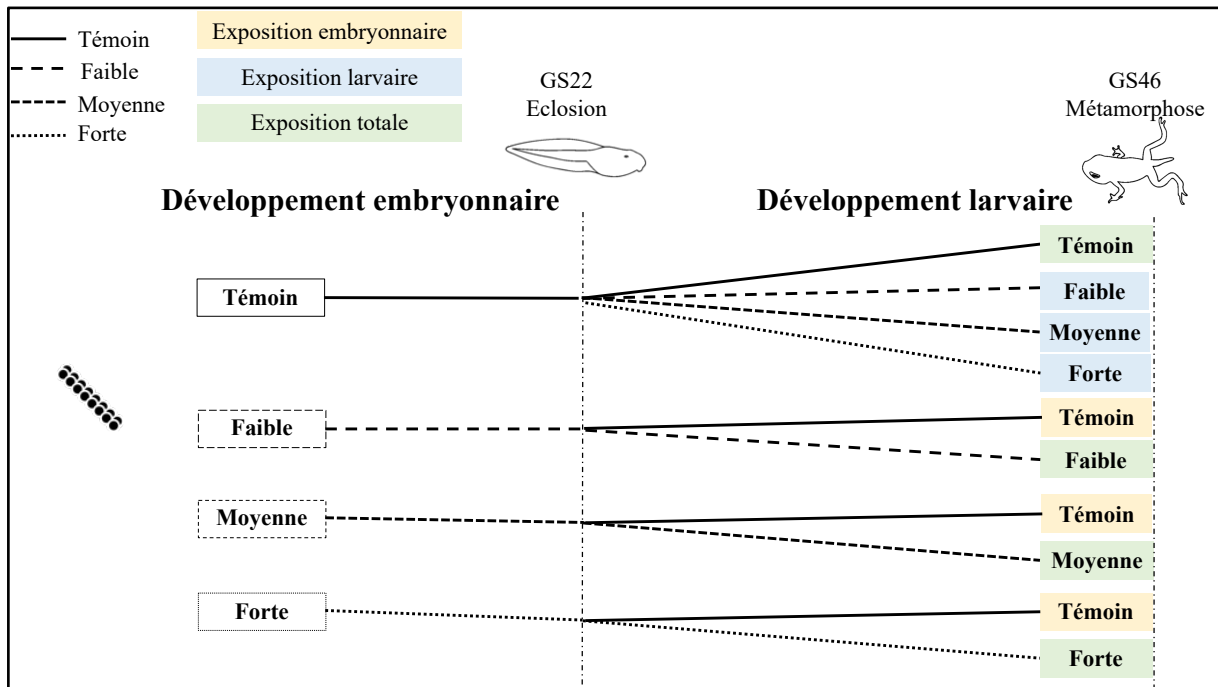


Figure 11 : Design expérimental destiné à étudier l'influence d'herbicides sur les phases de développement du crapaud épineux à différentes concentrations (« Témoin », « Faible », « Moyenne », « Forte » pour AMPA ; (« Témoin », « Faible », « Forte » pour nicosulfuron).

Chaque ponte a été séparée en segment de 30 œufs placés dans des aquariums en verre moulés de 2 L. Chaque ponte était donc représentée dans un traitement. Pour l'expérience AMPA, on avait donc 22 (pontes) * 4 (traitements) = 88 aquariums * 30 œufs = 2 640 embryons. Pour l'expérience nicosulfuron 20 (pontes) * 3 (traitements) = 60 aquariums * 30 œufs = 1 800 embryons. Les œufs étaient surveillés jusqu'à l'éclosion (**phase embryonnaire**). Les œufs restants ont été relâchés sur le site de capture.

AMPA - A l'éclosion, un têtard parmi chacune des 15 pontes et par traitement a été sélectionné au hasard et conservé pour l'expérience (tous les autres têtards ont été libérés dans l'étang de capture). L'expérience AMPA comprenait donc 150 têtards (Un têtard par ponte (15 ponte) * 10 traitements = 150, **Figure 11**).

Nicosulfuron - A l'éclosion, parmi 10 pontes, deux têtard par ponte et par traitement ont été sélectionnés au hasard et conservés pour l'expérience (tous les autres têtards ont été libérés dans l'étang de capture). L'expérience nicosulfuron comprenait donc 140 têtards (Deux têtards par ponte (10 ponte) * 7 traitements = 140, **Figure 11**).

Les têtards sélectionnés ont été placés individuellement dans des aquariums individuels de 2L, et leur développement a été surveillé jusqu'à la fin de la métamorphose. Ces méthodes d'élevage permettent de monitorer individuellement le développement de chaque têtard. Le maintien des têtards

individuellement dans les aquariums ne devrait pas générer de conditions stressantes et représente au mieux le milieu naturel (Bókony et al., 2021; Melvin & Houlihan, 2014). Les têtards ont été nourris *ad libitum* avec des épinards congelés issus de l'agriculture biologique. A l'approche de la métamorphose (GS42), les métamorphes étaient transférés dans des boîtes en plastique avec un peu d'eau de l'aquarium dans laquelle se situait une rampe entourée de papier absorbant mouillé pour leur permettre de sortir de l'eau. Dès que les métamorphes étaient observés sur leur rampe, l'eau était enlevée et remplacée par un papier absorbant humide. Les métamorphes étaient nourris avec *ad libitum* avec *Acyrtosiphon pisum* et *collembola sp* ou relâché après 5 jours dans le cadre de l'expérience sur le nicosulfuron.

D) Mesures

a) Développement

Parmi les mesures effectuées, nous nous sommes intéressés aux traits de développement. Pour déterminer un stade de développement, la table de Gosner était utilisée (Gosner, 1960). Le choix des stades de développement étaient déterminés en fonction de la pertinence biologique et la facilité de reconnaissance à l'œil nu par l'expérimentateur. La **figure 12** résume les stades de développement clefs biologiquement pertinents pour l'espèce. Les stades compris entre 21 et 24 sont les stades d'éclosion qui marquent la transition entre des embryons immobiles et des têtards actifs et capables de se nourrir. Les stades compris entre 25 et 37 correspondent au stade de croissance somatique, qui est une phase de développement des membres postérieurs. Enfin, les stades 42 à 46 sont des stades de transition entre la vie aquatique et la vie terrestre, des stades de préparation à la métamorphose avec au stade 46 une métamorphose complète.

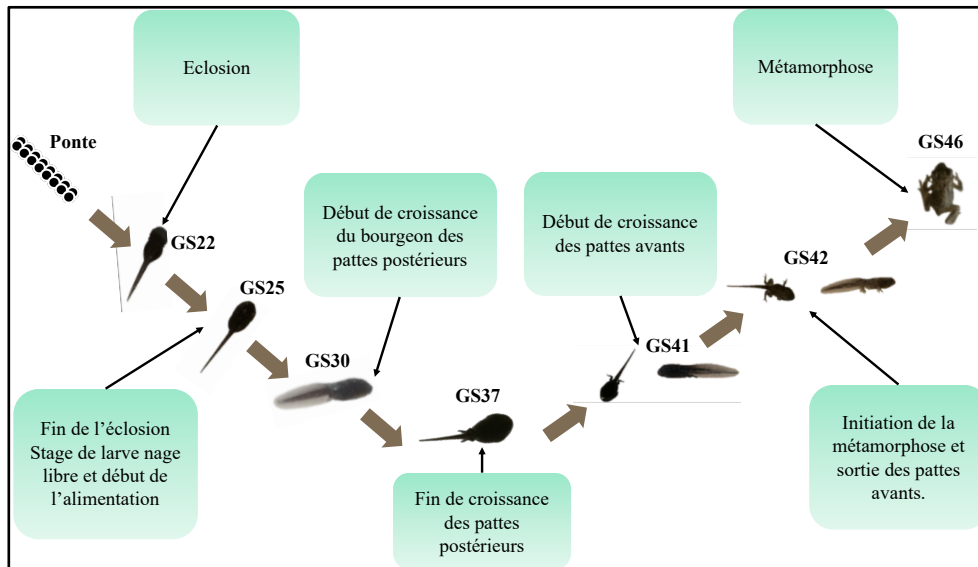


Figure 12 : Stades de Gosner étudiés pendant la thèse (Gosner, 1960)

A l'écllosion, la proportion d'œufs ayant écloso était comptée dans chaque segment ce qui nous donnait une valeur de **succès d'écllosion**. Le **taux de déformation** était déterminée en dénombrant le nombre de têtards déformés au sein des segments parmi les déformations connues chez les têtards : malformation axiale, œdème, scoliose de la queue, (Cooke, 1981; Wagner et al., 2014). Le **temps de développement** était déterminé entre phase de développement et stade pour déterminer si le contaminant étudié modifiait la durée de développement. Nous n'avons pas observé de différences dans les temps de développement intra-segment.

b) Morphologie

Les mesures morphométriques ont été effectuées en utilisant le logiciel ImageJ (Schneider et al, 2012). Les différentes mesures effectuées sont résumées dans le **figure 13**. A l'écllosion (GS22), les mesures effectuées étaient la longueur totale (LT), la longueur du corps (LC) et la longueur de la queue (LQ). Au total pour l'expérience AMPA, 2043 embryons ont été mesurés, pour l'expérience nicosulfuron, 1675 embryons. Les mesures de la **figure 13** sont les mesures effectuées sur les têtards en phase larvaire. Pour la prise de mesure, les têtards étaient placés dans une boîte de pétri et photographiés du dessus et de côté. Les traits morphométriques pris en compte sont connus pour montrer la plasticité chez les amphibiens (Watters et al., 2016). Au total pour l'expérience AMPA, les 150 têtards ont été mesurés à 4 stades différents (600*6 types de mesures = 3 600 mesures), pour l'expérience nicosulfuron, 140 têtards ont été mesurés à 5 stades différents (700*8 types de mesures = 5 600 mesures). Les mesures ont été effectuées par la même personne pour toutes les expériences de cette thèse.

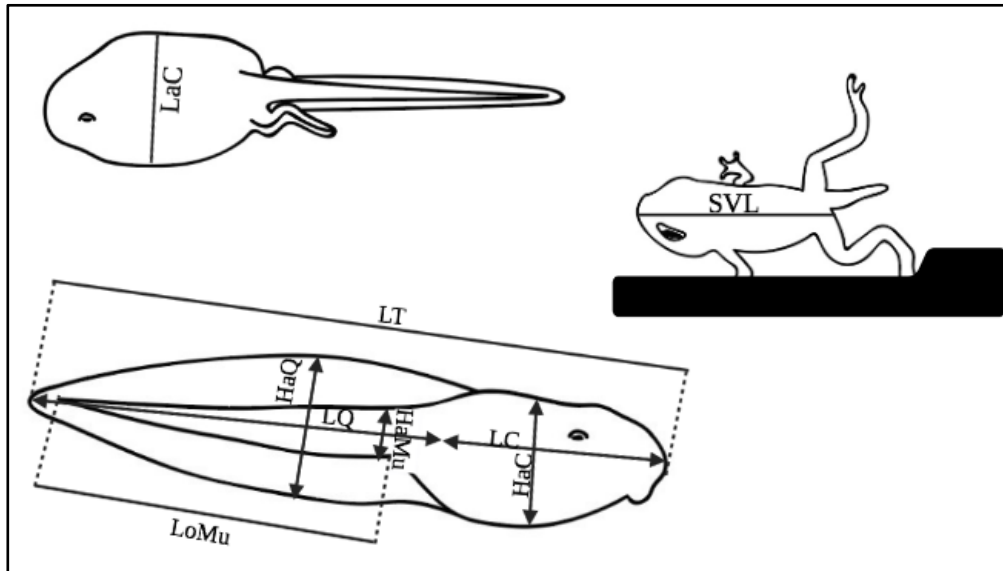


Figure 13 : Mesures morphométriques prises sur les têtards de crapaud épineux (*Bufo spinosus*). **LT** : Longueur totale, **LC** : Longueur du corps, **LQ** : Longueur de la queue, **LoMu** : Longueur du muscle de la queue, **HaC** : Hauteur du corps, **HaQ** : Hauteur de la queue, **HaMu** : Hauteur du muscle de la queue, **LaC** : Largeur du corps. Au moment de la métamorphose, les crapelets étaient pesés J1 et J5 et la **SVL** (longueur museau-cloaque) était mesuré. **LoMu** et **HaMu** n'étaient pas pris en compte dans l'expérience AMPA.

A la métamorphose, les crapelets étaient pesés tous les jours et pris en photo tous les cinq jours. Pour l'expérience AMPA, les crapelets ont été gardés pendant 130 jours après la métamorphose, pour l'expérience nicosulfuron, les crapelets ont été gardés 5 jours après la métamorphose.

c) Physiologie

Deux mesures de physiologie ont été effectuées sur les têtards soumis à la contamination. Le développement du dosage des hormones thyroïdiennes (**T4** (thyroxine) et **T3** (triiodothyronine)) est en cours.

Longueur des télomères

Les analyses de télomères ont été effectuées au CEBC par Frédéric Angelier, Cécile Ribout et moi-même (extraction d'ADN). Les méthodes précises peuvent être retrouvées dans **l'Article III**. Au moment de l'éclosion, deux embryons éclos ont été pris de manière aléatoire parmi les embryons éclos dans chaque ponte pour les analyses de télomères. Les embryons éclos ont été euthanasiés et l'individu entier a été utilisé pour l'analyse. La longueur des télomères a été déterminée par PCR quantitative (qPCR; BioRad CFX 96; Bio-Rad, USA) en utilisant un protocole établi (McLennan et al., 2019), adapté pour le crapaud épineux. Toutes les qPCRs ont été effectuées en trois plaques pour chaque gène (RAG1 et télomère) en utilisant 7.5ng d'ADN par réaction. La longueur relative des télomère (exprimée en T/S ratio) a été calculée comme le nombre de copies de télomères (T) relatif aux nombres de gènes à copie unique (S ; RAG1) selon Cawthon, 2002.

Marqueurs de stress oxydant

Les analyses de marqueurs de balance oxydative ont été effectuées par David Costantini à l'UMR 7221 (CNRS/MNHN). 20 têtards par groupes expérimentaux ont été sélectionnés (2 têtards par ponte) pour les analyses de stress oxydant. Les détails peuvent être retrouvés dans [l'Article V](#) et [l'Article VI](#). Les têtards entiers ont été utilisés et les deux têtards par ponte ont été poolés ensemble pour palier la faible taille des individus à l'éclosion. Des kits ont été utilisés pour mesurer : la superoxide dismutase (**SOD**), la glutathione peroxydase (**GPx**), l'activité de la catalase (**CAT**) et les niveaux de **thiols**. Toutes les analyses ont été faites en dupliquât et les coefficients de variation étaient <10%. Les valeurs de marqueurs ont été standardisées selon la quantité de protéines et quantifiées selon les méthodes de Bradford avec l'albumine comme standard de référence (Sigma-Aldrich, France).

d) Comportement

Les analyses de comportement ont été effectuées sur les têtards soumis au nicosulfuron et plus de détails peuvent être trouvés dans [l'Article VII](#) et [l'Article IX](#). Cette étude comportementale était en collaboration avec deux collaborateurs de l'équipe Prédateurs Marins du CEBC - Yan Ropert-Coudert et Akiko Kato - en collaboration avec Xavier Meyer (European Science Foundation) et Andrew J.J MacIntosh (Kyoto University Primate Research Institute). Les prises de mesures de comportement ont été effectuées à l'aide d'une stagiaire de Master 2 (Léa Raoélison) et moi-même. Les mesures de comportement étaient effectuées à chaque stade de développement montré dans la [figure 13](#). A chaque stade de développement, les têtards ont été retirés de leur aquarium et placés dans une arène individuelle (boîte de Pétri, diamètre 13,5 cm, niveau d'eau 1,5 cm) placée au-dessus d'un fond blanc. Après 15 minutes d'acclimatation ([Dayton et al., 2005](#)), les têtards étaient filmés pendant 30 minutes à l'aide d'un caméra type GoPro (GoPro HERO (2018); GoPro, San Mateo, CA, USA) placée au-dessus de l'arène. Les données collectées ont été analysées à l'aide du logiciel *ToxTrac* ([Rodriguez et al., 2018](#)). Le nombre de vidéos analysées est de 140 (têtards) * 5 (stades) = 700 vidéos ce qui correspond à 350 heures de vidéos. Grâce à ce logiciel et des scripts améliorés par Akiko Kato et Xavier Meyer nous avons pu déterminer les variables suivantes : **nombre de fenêtres d'activité, durée totale d'activité, distance totale parcourue, vitesse maximale observée, vitesse moyenne**. Pour minimiser le biais des observateurs, des méthodes à l'aveugle ont été utilisées lorsque toutes les données comportementales ont été enregistrées et analysées.

De plus, une mesure de complexité comportementale a été effectuée grâce à la collaboration avec Yan Ropert-Coudert, Akiko Kato, Xavier Meyer et Andrew J.J MacIntosh. Une analyse de séries chronologiques a été effectuée pour mesurer la dépendance à long terme comme indice de complexité temporelle dans les séquences d'activité des têtards ([Peng et al., 1995](#); [Peng et al., 1994](#)).

L'application de cet outil analytique, dérivé de la théorie de la complexité, repose sur les propriétés fractales fondamentales (c'est-à-dire l'échelle dans la variance) qui caractérisent le comportement

animal dans son organisation temporelle et spatiale. Les structures ou processus fractals présentent des motifs qui se répètent à travers une série d'échelles de mesure différentes. Elles sont omniprésentes dans la nature, non seulement dans le monde physique sous la forme de nuages, d'arbres, de montagnes et de lignes côtières (Mandelbrot, 1967, 1983), mais aussi dans divers systèmes biochimiques et physiologiques, des fluctuations du rythme cardiaque aux taux respiratoires et aux réseaux neuronaux (Goldberger, Rigney, & West, 1990 ; Ivanov et al., 1999 ; Peng, Havlin, Stanley, & Goldberger, 1995) (Figure 14).

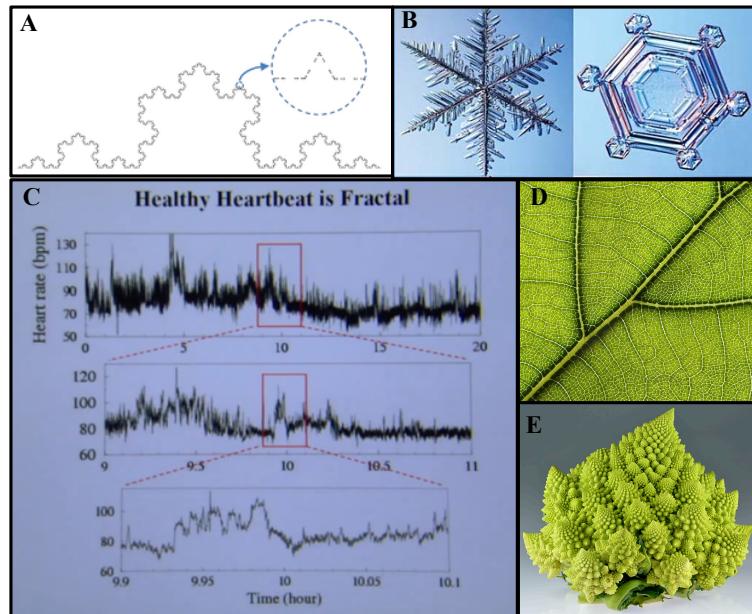


Figure 14 : Structures fractales retrouvées dans la nature. A : Courbe de Koch, B, Flocons de neige, C : Battements cardiaque, D : Feuille, E : Chou romanesco.

Ces modèles invariants à une échelle peuvent être observés dans la chronologie de comportements spécifiques (Shimada et al., 1995 ; Cole, 1995 ; Alados et Weber, 1999 ; MacIntosh et al., 2011), ainsi que dans les trajectoires de déplacement des animaux (Bartumeus 2007 ; Sims et al., 2008 ; Viswanathan et al., 2011). Les avantages pour la santé et la flexibilité comportementale ont tous deux été associés à des modèles de type fractal, car on pense qu'ils permettent aux organismes de mieux faire face aux perturbations internes ou externes imprévisibles (MacIntosh, 2014, 2015 ; West, 1990). Par exemple, le caractère fractal dans l'architecture bronchique des mammifères est plus tolérant aux fluctuations de l'environnement physiologique pendant la morphogenèse (West, 1990). En outre, dans de nouvelles conditions environnementales, les mouches des fruits, *Drosophila melanogaster* (Shimada et al., 1995), et les cailles japonaises, *Coturnix coturnix* (Kembro et al., 2009), produisent des modèles fractals dans les séquences de comportement de recherche de nourriture pour mieux explorer et exploiter les ressources. La complexité de l'organisation structurelle d'un système est définie par son échelle fractale, c'est-à-dire par le degré auquel les fluctuations du comportement d'un système changent avec l'échelle de mesure. L'analyse fractale est un outil de diagnostic non invasif

émergent qui permet aux expérimentateurs de mesurer la complexité spatiale ou temporelle d'un système, c'est-à-dire son degré de variabilité ou d'irrégularité dans l'espace ou dans le temps.

De nombreuses études ont montré une perte de complexité (c'est-à-dire une moindre irrégularité) dans les systèmes biologiques dans des conditions de stress ou pathologiques. En effet, une réduction de la variabilité dans l'espace ou dans le temps par le vieillissement et la maladie a été démontrée dans de nombreux systèmes physiologiques, cognitifs et moteurs humains (Lipsitz et Goldberger, 1992 ; Ribeiro et al., 2002 ; Vaillancourt et Newell, 2002 ; Benoit et al., 2011). Chez les animaux domestiques, une complexité moindre (c'est-à-dire une plus grande périodicité ou stéréotypie) dans la dynamique temporelle a été signalée dans la fréquence cardiaque de moutons ressentant de la douleur (Stubsj en et al., 2010) et de porcs pendant un choc h morragique (Batchinsky et al., 2007). En ce qui concerne le comportement animal, les analyses fractales, en particulier, ont r v l  une diminution de la complexit  des comportements d'individus stress s ou en difficult  physiologique, chez de nombreuses esp ces (voir les m ta analyse de Seuront, 2010, 2015 ; MacIntosh, 2014 ; Seuront et Cribb, 2017).

Les analyses de l'organisation comportementale sont particuli rement prometteuses, car elles peuvent r v ler des informations suppl mentaires par rapport aux approches plus traditionnelles bas es sur des mesures de dur es de comportements sp cifiques (Alados et al., 1996 ; Kembro et al., 2009 ; Rutherford et al., 2003).

  ce jour, les analyses fractales ont permis de d tecter efficacement les alt rations de l'organisation comportementale en fonction des conditions dans une vari t  de contextes, qu'il s'agisse d'exp riences toxicologiques (Alados & Weber, 1999 ; Kembro et al, 2009 ; Motohashi, Miyazaki, & Takano, 1993), des r glages de production (Maria, Escos, & Alados, 2004 ; Rutherford, Haskell, Glasbey, & Lawrence, 2006) et pour le suivi de la faune sauvage (Alados et al., 1996 ; Cottin et al., 2014 ; Cribb & Seuront, 2016 ; MacIntosh, Alados, & Huffman, 2011 ; Seuront & Cribb, 2011).

Ce ph nom ne, dans lequel l'organisation structurelle du comportement change en r ponse   un ou plusieurs facteurs de stress, est d sormais connu sous le nom de " perte de complexit  ". Il repose sur l'hypoth se qu'il existe une configuration optimale dans l'organisation comportementale (MacIntosh, 2014).

IV) Données récoltées pendant la thèse

Le tableau suivant résume les types de données récoltées pour les articles incluent dans cette thèse (en bas de manuscrit).

	Article I	Article II	Article III	Article IV	Article V	Article VI	Article VII	Article VIII	Article IX
Stade	Adultes	Adultes	Embryogénèse	Embryogénèse	Embryogénèse	Embryogénèse	Larvaire	Larvaire	Larvaire
Paramètres physiologiques									
<i>Testostérone</i>									
<i>Corticostérone</i>									
<i>Marqueurs de stress oxydatif</i>									
<i>Isotopes</i>									
<i>Longueur des télomères</i>									
Traits de développement									
<i>Durée de développement</i>									
<i>Déformation</i>									
<i>Succès d'éclosion</i>									
<i>Survie</i>									
Traits morphologiques/phénotypiques									
<i>Masse corporelle</i>									
<i>Condition corporelle</i>									
<i>Taille/proportion</i>									
Comportement									
<i>Activité</i>									
<i>Exploration</i>									
<i>Complexité comportementale</i>									

Le tableau suivant résume les données récoltées pour des projets annexes à la thèse. Deux articles ont été rédigés dans la catégorie « coûts osmotiques de la reproduction » ([Annexe I](#) et [Annexe II](#)). La catégorie « Exposition chronique » est expliquée dans le chapitre « Perspective » et concerne des expérimentations toujours en cours au moment du rendu de cette thèse.

	Coûts osmotiques de la reproduction	Exposition chronique
Stade	Adultes	Adultes
Paramètres physiologiques		
<i>Testostérone</i>		
<i>Corticostérone</i>		
<i>Marqueurs de stress oxydatif</i>		
<i>Osmolalité</i>		
<i>Perméabilité cutanée (TEWL)</i>		
<i>Immunologie</i>		
<i>Taux métabolique basal</i>		
<i>Télomères</i>		
<i>Microbiote intestinal</i>		
Traits de développement		
<i>Survie</i>		
Traits morphologiques/phénotypiques		
<i>Masse corporelle</i>		
<i>Condition corporelle</i>		
<i>Taille/proportion</i>		
<i>Réflectance UV</i>		

Chapitre I

Effets de l'habitat sur la morphologie et la physiologie du crapaud

épineux



Crédit photo : Auxence Foreau

Ce ne sont pas les circonstances de la naissance qui importent, mais ce que l'on fait du don de la vie qui détermine qui nous sommes – Mewtwo - Le pouvoir est en toi

Ce chapitre résume les **articles I, II** et les résultats préliminaires de génétique des populations portant sur l'étude des variations allométriques entre populations d'habitats contrastés de *Bufo spinosus*. Pour plus d'informations, voir les articles en fin de manuscrit.

I) Contexte

A) Habitats homogènes

Depuis le début du XXIème siècle, l'agriculture moderne s'est intensifiée, menant à des changements dans la structure de l'habitat à une grande échelle (Benton et al., 2003). Dans le monde, la proportion d'environnement naturel a régressé en faveur de milieux modifiés par l'Homme, avec 38% de zones agricoles en exploitation (Foley et al., 2011). En France en 2018, les milieux agricoles occupaient 45% du territoire sous la forme de prairies et zones cultivées. L'homogénéisation du paysage est l'une des premières conséquences de l'agriculture intensive (Endenburg et al., 2019; Stoate et al., 2009; Sumasgutner et al., 2019; Tschardt et al., 2005).

Ces changements dans l'exploitation du milieu mènent à une perte de la biodiversité et des changements de la structure taxonomique des habitats (Chopin et al., 2019; Endenburg et al., 2019, 2019; Put et al., 2019). Les monocultures alignées remplacent les prairies et haies connues comme étant des zones de refuges pour la biodiversité (Graham et al., 2018; Lecq et al., 2017; Litza & Diekmann, 2020). L'homogénéisation de l'habitat peut se traduire à travers de nombreux effets sur la biodiversité :

(i) L'altération de l'habitat peut limiter la disponibilité des micro-habitats et refuges menant à une altération des traits d'histoire de vie affectant à terme la fitness (Haberkmann et al., 2020; Kim et al., 2019; Lecq et al., 2017; Zamora-Camacho, 2020). C'est notamment le cas des haies qui fournissent les ressources en nourriture, refuges, sites de nidification ou de reproduction et agissent comme corridor écologique pour la dispersion (Batáry et al., 2010). L'homogénéisation du paysage a mené à une perte de ces haies menant à une perte massive de biodiversité (Burel et al., 2013).

(ii) De plus, la pression de prédation peut également être différente ce qui peut mener à des conséquences sur la dynamique de population (Warkentin, 1995). Une pression de prédation accrue en bordure d'habitat est souvent associée à une activité accrue des prédateurs. Des études montrent que les pressions de prédation sont plus importantes en bordure de haies (Červinka et al., 2013; Šálek et al., 2010). On observe donc une forte abondance de carnivores associée à une augmentation du nombre de petits mammifères. L'abondance des prédateurs et des proies a augmenté de façon constante en bordure d'habitat à cause de la pression imposée par l'homogénéisation de l'habitat (Šálek et al., 2010).

(iii) Les ressources alimentaires peuvent varier affectant les traits d'histoire de vie (Hoset et al., 2017; Paterson & Blouin-Demers, 2018). Par exemple, chez le Plectrophane à ventre noir (*Calcarius ornatus*), une baisse de la disponibilité en nourriture dans les habitats fragmentés entraîne une baisse du succès reproducteur (Lloyd & Martin, 2005). Chez la bernache nonnette (*Branta leucopsis*), la limitation de la ressource en nourriture dans les habitats agricoles impacte la survie pendant la migration hivernale (Black et al., 1992).

B) Comment se traduit ce changement d'habitat sur les organismes ?

Les espèces qui exploitent ces habitats ont donc besoin de faire face à ces modifications, ce qui peut mener à des modifications du phénotype (via des processus de plasticité, d'adaptation locale ou des deux) et refléter un stress chronique chez la faune sauvage (Conith et al., 2020; Kelley et al., 2018; Moran et al., 2017; Van Buskirk, 2002). Par exemple, la condition corporelle peut varier en réponse à des stressseurs intrinsèques ou extrinsèques (par ex., migration, investissement reproductif). Les effets indirects de ces stressseurs sont principalement médiés par la dépendance croissante aux intrants chimiques qui visent à améliorer la productivité des cultures (Köhler & Triebkorn, 2013). L'utilisation de grandes quantités d'engrais peut éventuellement conduire à des perturbations du fonctionnement des écosystèmes (Huang et al., 2017). En outre, les effets toxiques des pesticides sur les composants non cibles ont suscité un intérêt considérable (Köhler & Triebkorn, 2013).

Il est clair que les effets indirects devraient affecter les espèces habitant les paysages agricoles à cause des modifications d'habitat sur l'écologie des espèces animales (par exemple, en l'absence des arbres ou arbustes de nidification pour les oiseaux, Mohring et al., 2021). Pourtant, dans la plupart des cas, les paysages agricoles seront entrecoupés de vestiges d'autres types d'habitats (petits bois, haies) qui devraient permettre la persistance des populations.

a) Plasticité phénotypique

La plasticité phénotypique peut permettre aux organismes de faire face aux variations environnementales. Elle se définit comme l'expression de différents phénotypes indépendamment de la variation du génotype, souvent en réponse à la variabilité de l'environnement (Bradshaw, 1965, Fusco & Minelli, 2010). La gamme de ces phénotypes qui s'expriment est définie par **la norme de réaction**.

La plasticité phénotypique se divise en deux grandes catégories : celle induite par l'empreinte gamétique parentale et l'environnement embryonnaire de la descendance (*plasticité développementale*), et la *flexibilité phénotypique* qui se produit au sein des organismes matures et qui se caractérise par une réversibilité. Les organismes peuvent montrer des réponses adaptatives aux changements environnementaux et les phénotypes associés sont supposés conférer une meilleure

fitness que les phénotypes mal adaptés à leur environnement (Price et al., 2003). Par exemple, un décalage entre l'environnement vécu au cours du développement précoce peut induire un phénotype particulier et ce phénotype ne sera pas adapté à l'environnement (maladaptation) vécu plus tard dans la vie si celui-ci change (*Phenotype-environment mismatch*) (Figure 15).

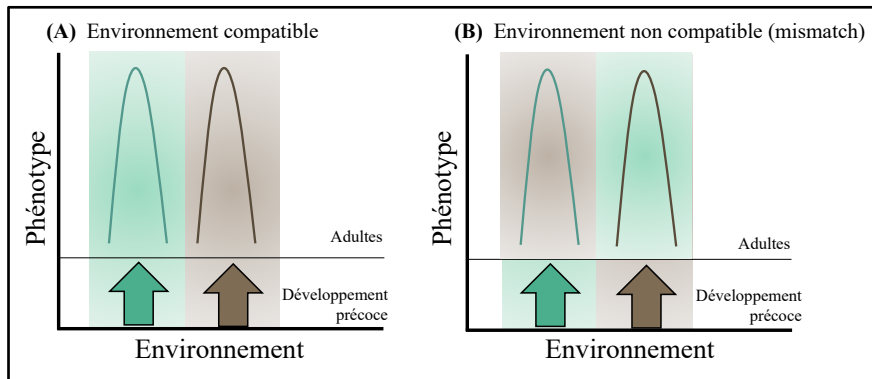


Figure 15 : Représentation schématique des réponses plastiques à des environnements variables comme l'homogénéisation de l'habitat. Les conditions environnementales rencontrées au cours du développement (indiquées par des couleurs différentes sous la ligne horizontale dans chaque panneau) peuvent déterminer la trajectoire phénotypique (flèches vertes et marrons) plus tard dans la vie pour aboutir à **des normes de réaction** (courbes) différentes dans les organismes adultes. (A) La plasticité développementale est bénéfique si les conditions vécues au cours du développement permettent de prévoir les conditions de l'adulte et d'adapter les phénotypes à ces conditions. (B) Cependant, la plasticité développementale devient inadaptée si les phénotypes adultes correspondent aux conditions du développement, mais que l'environnement adulte change ensuite de sorte qu'il y a un décalage entre le phénotype et l'environnement (**mismatch**) (adaptée de Beaman et al., 2016)

La plasticité phénotypique est favorisée dans les populations qui habitent des environnements variant et peut être qualifiée d'adaptative quand le phénotype sélectionné confère une fitness relative plus importante dans cet environnement (Agrawal, 2001; Ghalambor et al., 2007; Pigliucci, 2005). Des différences environnementales entre les milieux naturels et les milieux modifiés peuvent entraîner des variations de phénotypes et permet la persistance de la population et l'utilisation de la ressource (niveau trophique, performance locomotrice, compétition, prédation) menant à terme à de la divergence morphologique (Alberti et al., 2017; Bókony et al., 2019; Geng et al., 2007; Van Buskirk & Arioli, 2005a).

La variation des traits phénotypiques, incluant ceux affectant la fitness, peut émerger comme étant un résultat de la réponse individuelle aux variabilités environnementales. Par exemple, les milieux urbains sont connus comme étant un fort facteur de variation chez de nombreuses espèces. Le paysage adaptatif en milieux anthropisés peut avoir le potentiel de mener à une divergence phénotypique rapide (Alberti et al., 2017; Seress & Liker, 2015). Par exemple, les lézards d'habitats urbains diffèrent de leur compères en milieux forestiers par des adaptations uniques liées à la performance (nombre de

lamelles, doigts plus longs, Winchell et al., 2016). En milieux agricoles, un cocktail de fragmentation de l'habitat, destruction de l'habitat et présence d'intrants chimiques pourrait influencer des phénotypes plastiques. Chez les anoues, les défenses chimiques sont plus importantes en milieux agricoles et urbains qu'en milieux forestiers (Bókony et al., 2019). Chez les têtards de *Rana temporaria*, le phénotype est corrélé avec les caractéristiques de l'habitat comme la localisation de la mare (Van Buskirk & Arioli, 2005a).

b) L'agriculture comme stresser physiologique

Les pratiques agricoles peuvent être perçues comme des perturbations endocrines et se refléter sur un des médiateurs de l'allostase, les glucocorticoïdes (Romero et al., 2009). Les glucocorticoïdes comme la corticostérone chez les amphibiens, reptiles et oiseaux jouent un rôle dans la mobilisation des réserves énergétique (lipides: Peckett et al., 2011). Cette mobilisation peut affecter les fonctions essentielles de l'organisme comme la reproduction, le comportement et la croissance (Denver, 2009). Une augmentation des niveaux de corticostérone peut être vue comme un proxy des contraintes énergétiques d'un organisme (Bonier et al., 2009; Romero et al., 2009). La sécrétion chronique ou la perturbation de corticostérone peut perturber des fonctions métaboliques et avoir des conséquences sur la fitness (Bonier et al., 2009; Lattin & Romero, 2015).

Par exemple, la variation des niveaux de corticostérone peut influencer les niveaux d'espèces réactives de l'oxygène (ROS) et perturber la balance oxydative ce qui peut mener à terme à des dommages ADN (Costantini et al., 2011). Les activités anthropiques induisent une perturbation dans la sécrétion de corticostérone en réponse au stress chez les ectothermes (Baxter-Gilbert et al., 2014; Polich, 2016; Polich et al., 2018). Chez le rollet d'Europe (*Coracias garrulus*), les oisillons élevés dans les zones de broussailles présentaient des niveaux élevés de corticostérone induits par le stress, peut-être en raison du risque élevé de prédation dans cet habitat. En outre, le petit duc (*Otus scops*) montre des relations opposées avec l'activité agricole et les perturbations humaines. C'est dans les zones où l'activité agricole est intense les rollets ont présenté les niveaux de corticostérone les plus élevés dans les plumes (Expósito-Granados et al., 2020). Cela suggère que les espèces ajustent leur réponse au stress en réponse aux conditions environnementales.

Dans les zones agricoles, d'autres facteurs en plus de la présence d'intrants chimiques peuvent modifier les niveaux de corticostérone. Dans une étude sur l'impact du type de substrat, les crapauds communs adultes (*Bufo bufo*) présentaient des concentrations élevées de corticostérone lorsqu'ils étaient exposés à des sols labourés par rapport aux sols recouverts de litière de feuilles ou d'herbes, et les individus évitaient activement les sols labourés dans une expérience de choix (Janin et al., 2012). Les crapauds juvéniles avaient tendance à préférer les sols nus et il n'y avait aucune réaction de stress associée à ce choix de substrat. Les juvéniles risquent davantage d'être exposés à des produits agrochimiques sur des sols nus. Dans le contexte des déplacements des amphibiens dans les paysages agricoles, le stress

associé aux sols labourés peut être aggravé par le stress déclenché par l'exposition aux produits chimiques qui coïncident avec la sortie de l'eau chez ces espèces.

La sécrétion de glucocorticoïdes se produit en réponse :

- (i) Aux contraintes environnementales (par exemple, la disponibilité de la nourriture : Lewanzik et al., 2012 ; Bourbonnais et al., 2013, le climat : de Bruijn et Romero, 2011 ; Houser et al., 2011)
- (ii) Aux contraintes intrinsèques (par exemple, le statut reproducteur : Bacci et al., 2014 ; Viblac et al., 2016)
- (iii) Aux contraintes comportementales (par exemple, le stress social : Corlatti et al., 2014 ; Stocker et al., 2016)

En outre, lorsque les individus sont confrontés à des contraintes énergétiques, le métabolisme de base diminue et les niveaux de glucocorticoïdes augmentent pour favoriser la mobilisation des réserves énergétiques stockées (Sapolsky et al., 2000).

De plus, la reproduction peut être altérée et se refléter sur les caractères sexuels secondaires et les gonades (McCoy et al., 2008) mais également au niveau de la perturbation endocrine (par ex., niveaux de testostérone). Ces perturbations peuvent réduire le succès de reproduction (Orton & Tyler, 2015). Il est intéressant de noter que peu de publications montrent un effet de l'habitat et plus particulièrement de l'agriculture sur les niveaux de testostérone chez les espèces.

C) Les amphibiens en milieux agricoles

Malgré les preuves des effets négatifs de l'agriculture, certaines espèces sont positivement associées aux milieux agricoles (Koumaris & Fahrig, 2016). Les milieux agricoles sont régulièrement utilisés par les amphibiens (Bishop et al., 2010; Christin et al., 2013; Collins & Fahrig, 2017; Guillot et al., 2016; Knutson et al., 2004; Koumaris & Fahrig, 2016) et certains endroits sont considérés comme critiques pour la persistance de la population (Bishop et al., 2010; Knutson et al., 2004). Les plans d'eau, mares temporaires ou zones de drainage de l'agriculture et de l'élevage sont utilisés comme zones de reproduction avec des populations importantes (Gagné & Fahrig, 2007; Knutson et al., 2004; Koumaris & Fahrig, 2016). Bien qu'il soit clair que les zones naturelles comme les parcelles forestières et les milieux humides dans les paysages agricoles sont importantes pour les amphibiens habitant dans les milieux agricoles (da Silva et al., 2012; Knutson et al., 2004), on ne sait pas si la superficie de zone cultivée dans ces paysages peut être structurée de manière à ce qu'elle soit avantageuse pour ces espèces.

L'abondance et la richesse spécifique d'anoures sont positivement liées au couvert forestier dans les paysages agricole (Collins & Fahrig, 2017). Ce qui montre que le couvert forestier est un indicateur de l'occurrence et de la diversité d'anoures en milieu majoritairement agricole.

Les terres agricoles comportant de petites zones cultivées devraient faciliter le mouvement des anoures et fournir des refuges accessibles (Collins & Fahrig, 2017). La plupart des amphibiens pratiquent des migrations saisonnières entre différents types d'habitats (Miaud & Sanuy, 2005). Les déplacements sont facilités lorsque le substrat est humique (Trochet et al., 2019), car les anoures sont sujet à la dessiccation dans des zones plus ouvertes (Youngquist & Boone, 2014). L'abondance en proies est également plus élevée dans les paysages agricoles avec des champs de taille moyenne, car les lisières des champs de cultures sont plus abondantes en arthropodes que les cultures (Lecq et al., 2017).

II) Résultats et discussion

A) Les marqueurs isotopiques pour connaître l'utilisation des milieux

Dans les milieux modifiés à forte composante agricole, l'ajout d'intrants chimique tels que les engrais ou les fertilisants sont connus pour avoir des impacts sur la biodiversité (Huang et al., 2017). Cependant, connaître l'exposome des espèces aux intrants chimiques est relativement compliqué à déterminer car il dépend de facteurs intrinsèques tels que les capacités de dispersion ou les capacités de bioaccumulation. On sait que le crapaud épineux a des faibles capacités de dispersion.

On a cherché à savoir si l'habitat dans lequel on le retrouve en période de reproduction était représentatif de l'habitat qu'il utilise le reste du temps. Si c'est le cas, on peut s'attendre à ce que l'exposition à des stressseurs, tels que les pesticides, soient maximale dans les zones agricoles aussi bien en milieu terrestre que pendant les phases de développement en milieu aquatique. L'analyse d'isotopes stables nous a permis de montrer que les sites d'études sont représentatifs de la zone d'utilisation de l'habitat du crapaud épineux et qu'il intègre les changements liés aux pratiques agricoles dans son écologie (Article I). Le concept de niche isotopique est basé sur le principe que la composition chimique d'un animal est influencée par ce qu'il consomme (Fry, 2006). L'azote 15 (^{15}N) est un proxy de la position trophique ou de l'habitat utilisé pour l'alimentation (Kelly, 2000). Nos sites études étant discriminés par le fait qu'ils soient soumis ou non à l'agriculture, on s'attend à ce que des intrants type engrais chimiques soient utilisés. Les engrais azotés montrent des taux de $\delta^{15}\text{N}$ important (e.g., fumier et compost [$\delta^{15}\text{N}$ jusqu'à 16,2‰], sulfate d'ammonium [$\delta^{15}\text{N}$ jusqu'à 6,6‰] and nitrate d'ammonium [$\delta^{15}\text{N}$ jusqu'à 2,2‰], Bateman & Kelly, 2007). Les chaînes trophiques influencées par la fertilisation devraient montrer des $\delta^{15}\text{N}$ importants (Bateman & Kelly, 2007). La migration est limitée dans l'espace et son spectre de proie étant limité ; le crapaud épineux intègre donc la modification de son habitat environnant (Zamora-Camacho & Comas, 2017). Enfin, la durée de vie des érythrocytes chez les amphibiens est remarquablement longue (Altland & Brace, 1962) ce qui

indique que le dosage d'isotope stables dans les globules rouges reflète l'utilisation de l'habitat (Cloyed et al., 2015).

L'analyse des isotopes stables chez ces crapauds a montré une différence importante entre habitat pour le $\delta^{15}\text{N}$ avec des taux 4,72 fois plus importants chez les crapauds issus de zones agricoles comparés aux crapauds de forêt (Figure 16).

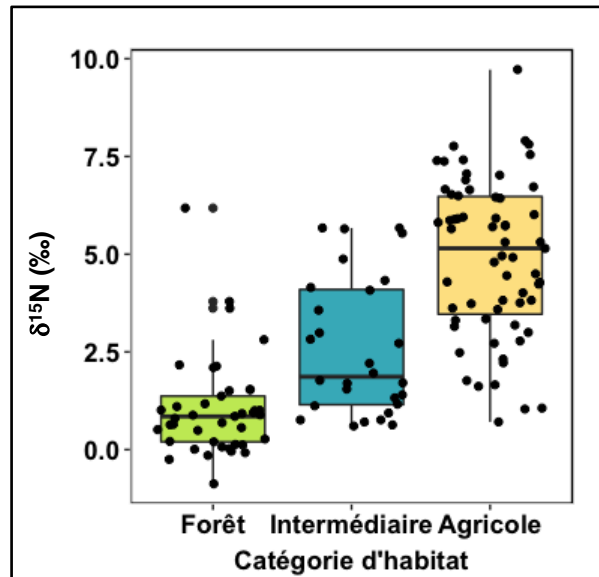


Figure 16 : Valeur de $\delta^{15}\text{N}$ (‰) sanguin chez les mâles de crapaud épineux issus de milieux forestiers (vert), intermédiaires (jaune) et agricoles (marron). Les données représentées représentent le premier et le troisième quartile, la médiane et les valeurs minimales et maximales. Les points représentent les points de données individuelles.

Ces différences pourraient s'expliquer par l'alimentation. Les crapauds issus de zones agricoles peuvent se nourrir de proies à des niveaux trophiques plus hauts que de ceux de zones forestières. Le régime alimentaire des crapauds ne diffère pas entre habitats (Zamora-Camacho and Comas, 2017) et donc que ces différences observées ne sont pas dues à un régime alimentaire différent. La fertilisation semble expliquer les différences observées en $\delta^{15}\text{N}$. Des valeurs $\delta^{15}\text{N}$ relativement élevées de ces engrais augmentent niveaux basaux $\delta^{15}\text{N}$ qui se propagent à travers les réseaux trophiques dans les paysages agricoles (Anderson et Cabana, 2005). Ce processus a déjà été démontré chez des têtards se développant dans des eaux à hautes concentrations en nitrate (Trakimas et al., 2011). Il est important de souligner les grandes variations des valeurs $\delta^{15}\text{N}$ entre les individus d'un même type d'habitat ou d'un même site d'étude. Un tel résultat semble suggérer différentes stratégies individuelles d'utilisation du micro-habitat pour s'alimenter dans un paysage similaire (Miaud et Sanuy, 2005 ; Indermaur et al., 2009). Cela indique que l'influence de la fertilisation azotée sur les valeurs $\delta^{15}\text{N}$ des crapauds est limitée dans l'espace. Un tel résultat suggère que, au moins dans notre étude, les valeurs $\delta^{15}\text{N}$ peuvent aider à comprendre l'utilisation de micro-habitats, les mouvements et/ou la dispersion et les stratégies

individuelles dans les paysages agricoles (Rickers et al., 2006 ; Dammhahn et Goodman, 2014). D'autres menaces telles que la bioaccumulation d'intrants chimiques peuvent impacter les populations d'amphibiens. De nombreuses études ont montré que des pesticides pouvaient se bioaccumuler et remonter dans les réseaux trophiques chez les invertébrés et les amphibiens (Crayton et al., 2020; Katagi & Tanaka, 2016; Mansuripur, 2011). Certains pesticides possèdent les caractéristiques chimiques possibles pour l'accumulation dans les tissus à long terme et le transfert peut s'appliquer à notre espèce. **L'Article I** nous montre donc que l'utilisation de marqueurs isotopiques pouvait discriminer les habitats soumis plus ou moins aux activités anthropiques. Il est intéressant de noter que les valeurs $\delta^{15}\text{N}$, en tant qu'indice de l'utilisation de l'habitat agricole, peuvent également aider à révéler **la vulnérabilité individuelle aux perturbations liées aux pratiques agricoles modernes (p. ex., les pesticides)**. Les différences allométriques observés sur les mâles adultes dans **l'Article II** pourraient donc être une résultante des conditions de développement et de croissance dans ces habitats.

B) Effets de l'habitat sur la morphologie et la physiologie des crapauds épineux

Notre étude (**Article II**) a permis de mettre en évidence les variations morphologiques et physiologiques liées aux types d'habitats (forêt, terres agricoles ou habitats mixtes) chez le crapaud épineux. Notre modèle d'étude a permis d'accéder à une large gamme de tailles et de conditions corporelles chez des mâles sexuellement matures (se reproduisant) provenant de types d'habitats contrastés et un tel contexte fut une opportunité intéressante pour étudier les variations de l'architecture corporelle et de l'échelle physiologique à travers de tels types d'habitats.

a) Différences phénotypiques entre habitat

Nos résultats ont mis en évidence un fort contraste entre les comparaisons simples des traits entre les types d'habitats (avec presque aucune différence entre les habitats) et les forts effets médiés par l'habitat sur les relations allométriques de ces mêmes traits. En termes de relation allométriques, nos résultats indiquent des réponses différentes des pentes (indiquant des changements inégaux avec l'augmentation de la taille corporelle) et des ordonnées à l'origine (ou intercept, indiquant des variations dans les proportions des traits) entre les habitats (**Figure 17**).

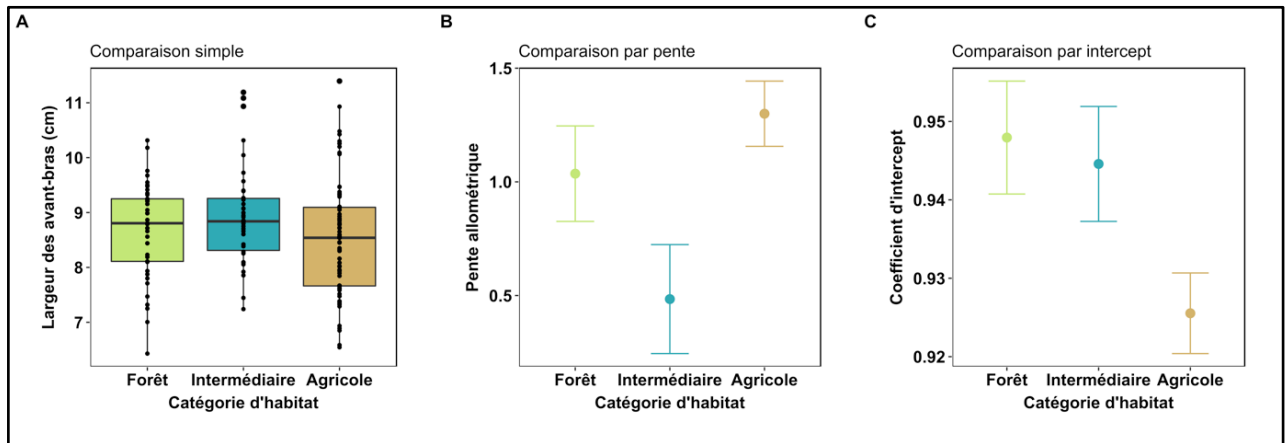


Figure 17 : Comparaison simple (A), Pentas allométriques (B) et coefficient d'intercept (C) de la largeur de l'avant-bras (mm, SVL). Toutes les mesures pour les relations allométriques sont exprimées en \log_{10} . Les mesures ont été effectuées sur des mâles adultes reproducteurs. Les couleurs représentent des habitats différents (vert pour "Forêt", bleu pour milieu "Intermédiaire", jaune pour milieu "agricole").

Ce ne sont donc pas les tailles corporelles *per se* qui diffèrent mais les proportions corporelles potentiellement à cause de différence de taux de croissance pendant le développement.

De plus, les différences de pentes de relations allométriques de divers traits en fonction de la taille corporelle des individus ont mis en évidence une réponse spécifique des individus provenant de types d'habitats mixtes par rapport aux individus provenant de sites forestiers ou agricoles. Chez ces individus, les relations allométriques étaient caractérisées par des pentes plus faibles, un résultat qui était convergent pour la plupart des traits morphologiques étudiés (masse corporelle, longueur et largeur des avant-bras, longueur des membres postérieurs, longueur des glandes parotoïdes, **Figure 18**).

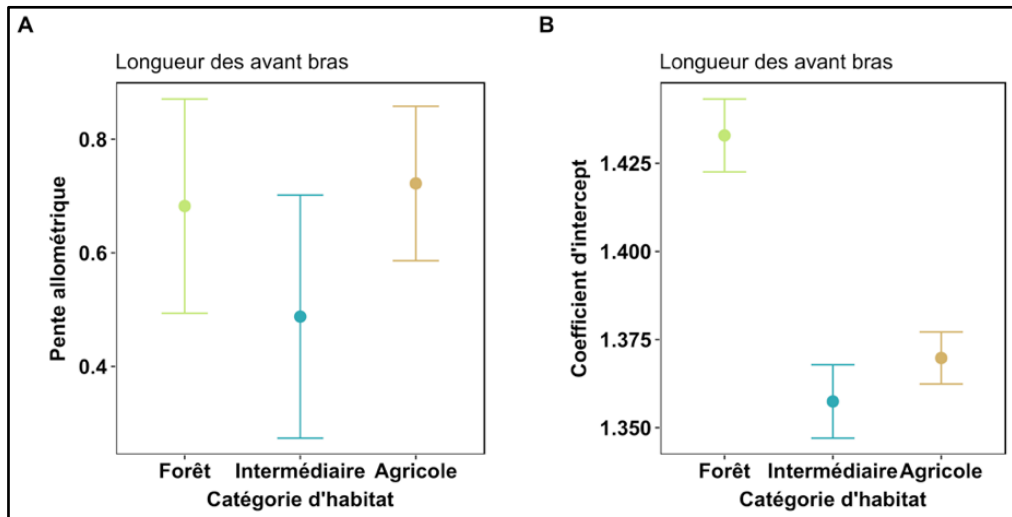


Figure 18 : *Pentes allométriques (A) et coefficient d'intercept (B) de la longueur de l'avant-bras (mm, SVL). Toutes les mesures pour les relations allométriques sont exprimées en log10. Les mesures ont été effectuées sur des mâles adultes reproducteurs. Les couleurs représentent des habitats différents (vert pour "Forêt", bleu pour milieu "Intermédiaire", jaune pour milieu "agricole").*

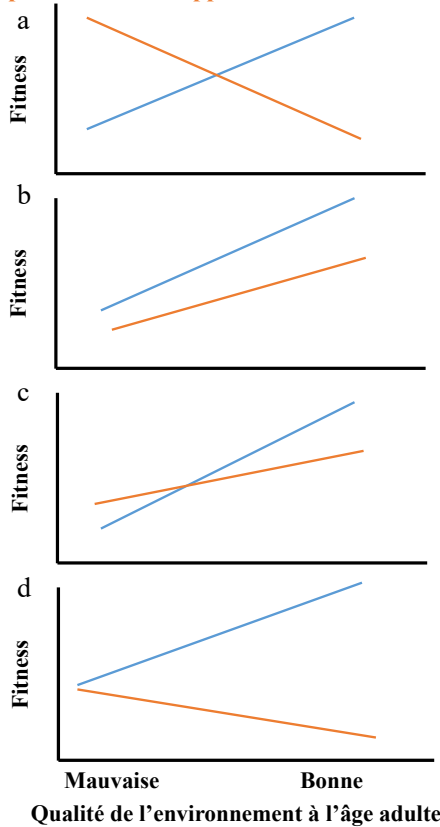
Les raisons qui sous-jacentes restent compliquées à expliquer. Pourtant, il est plausible que la variété des habitats rencontrés au cours de la vie quotidienne d'un individu puisse influencer la capacité des crapauds à répondre de manière optimale à des contraintes environnementales divergentes.

Par exemple, il est possible que les mares de reproduction (où les œufs et les têtards se développent) orientent les réponses développementales des embryons et des larves pour qu'ils se comportent de manière optimale dans le même type d'habitat plus tard dans leur vie (Lind & Johansson, 2011) (voir **figure 15** et encadré n°1). En conséquence, dans des types d'habitats très hétérogènes (mélangeant des zones forestières et agricoles à une très petite échelle spatiale), la probabilité pour un individu post-métamorphique de se disperser vers un type d'habitat contrasté est probablement importante (par exemple, un individu qui s'est développé dans un étang agricole et qui vit plus tard dans un habitat forestier ou vice versa, Kovar et al., 2009; Miaud & Sanuy, 2005) entraînant un « *environmental mismatching* ». Par conséquent, la qualité de l'habitat terrestre entourant l'étang de reproduction (auquel les crapauds sont relativement fidèles, Reading et al., 1991) peut être critique pour la survie (Unglaub et al., 2018).

Cela est particulièrement vrai pour les amphibiens terrestres qui présentent une agilité et des performances locomotrices comparativement faibles (Citadini et al., 2018) et chez qui l'évitement de micro-habitats sous-optimaux pourrait influencer la divergence des traits morphologiques (Janin et al., 2012; Trochet et al., 2019).

Encadré n°1

Environnement avantageux pendant le développement
 Environnement délétère pendant le développement



Adaptation à l'environnement adapté de Monaghan (2008). La qualité de l'environnement est déterminé par la quantité de ressource (faible, élevée) mais peut être lié à un stress faible ou élevé (Contamination par exemple). Les lignes représentent les **normes de réactions** des individus se développant aux extrémités basses (délétère) et hautes (avantageux) du spectre environnemental. **(a)** « **Environmental matching** » Pour ceux qui se développent dans des environnements à la fois bons et mauvais, la fitness est la plus élevée lorsque les environnements adulte et développemental correspondent. **(b)** Effets de « **silver spoon** ». Dans ce cas, il n'y a pas de correspondance environnementale. La fitness s'améliore toujours avec l'amélioration de l'environnement de l'adulte, et ceux qui se sont développés dans de mauvaises conditions ont toujours une fitness inférieure à ceux qui se sont développés dans de bonnes conditions. **(c)** La fitness augmente toujours avec la qualité de l'environnement adulte, mais lorsque la qualité est mauvaise, ceux qui se sont développés dans un tel environnement ont un avantage sur ceux qui ne se sont pas développés dans ces conditions. **(d)** Une combinaison de la « **silver spoon** » et de « **Environmental matching** ». Pour ceux qui se sont développés dans un bon environnement, la fitness s'améliore à mesure que la qualité de l'environnement adulte s'améliore ; pour ceux qui se sont développés dans un environnement mauvais, la fitness diminue à mesure que la qualité de l'environnement adulte augmentent ; cependant, quel que soit l'environnement adulte, ceux qui se sont développés dans un bon environnement ont toujours une meilleure fitness.

À l'inverse, les estimations des intercepts indiquaient clairement une similitude entre les individus issus d'habitats mixtes et de terres agricoles par rapport aux crapauds de l'habitat forestier (**Figure 17**). Ce résultat est convergent avec ceux mis en évidence par des études antérieures (Guillot et al., 2016; Zamora-Camacho & Comas, 2017) et qui ont montré que les crapauds des zones forestières divergeaient des individus provenant soit d'habitats agricoles intensifs, soit d'habitats intermédiaires avec des haies, les individus de ces deux dernières catégories étant similaires en termes de morphologie (Guillot et al., 2016).

Les contrastes d'intercept peuvent suggérer que les relations allométriques diffèrent par le biais d'un taux de croissance spécifique au cours de l'ontogénèse sous contraintes environnementales (Sanger et al., 2013). Une variété de contraintes environnementales pourrait expliquer cette similitude entre les individus originaires d'habitats intermédiaires et agricoles (Chopin et al., 2019). Ces habitats relativement ouverts peuvent influencer la disponibilité des proies, la pression de prédation et/ou les conditions microclimatiques qui devraient influencer les traits liés à la locomotion (taille des membres), à la recherche de nourriture (masse corporelle) et aux attributs anti-prédateurs (glandes parotoïdes) (Bókony et al., 2019; Guillot et al., 2016; Ingleby et al., 2016; Sumasgutner et al., 2019; Zamora-Camacho, 2018).

b) Comment l'habitat modifie les relations traits-physiologie

Contrairement aux réponses morphologiques mises en évidence ci-dessus, les mesures physiologiques (y compris les caractères sexuels secondaires) ont fourni des réponses plus directes avec des changements de coefficient de pente selon les différences attendues liées aux types d'habitats (habitats mixtes ayant une position intermédiaire entre la forêt et les terres agricoles). De tels effets ont été trouvés pour deux indices différents mais complémentaires de l'activité sexuelle (Testostérone et largeurs des avant-bras).

(i) Niveau de corticostérone en période de reproduction

Curieusement, aucune différence dans les niveaux de corticostérone n'a été détectée. En effet, ni la comparaison simple (**Figure 19**) ni les relations allométriques semblent être différentes selon l'habitat. Cela pourrait s'expliquer par les taux importants de CORT en période de reproduction chez cette espèce (Brischoux et al., 2018). Les niveaux de CORT et de T chez les crapauds épineux mâles reproducteurs sont principalement déterminés par la température environnementale. Cependant des taux importants de ces deux hormones sont retrouvés en début de période de reproduction et ces taux décroissent au long de la période (Brischoux et al., 2018; Brischoux & Cheron, 2019).

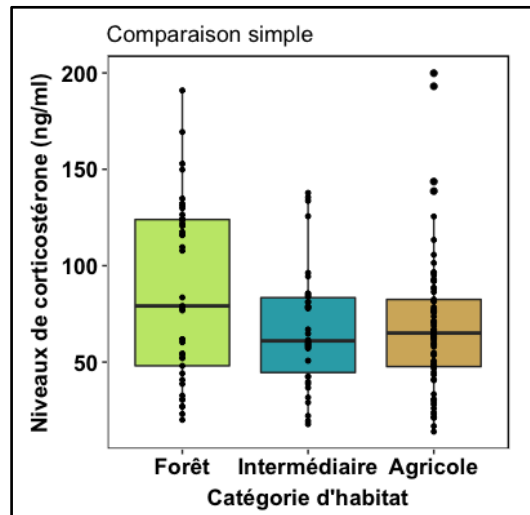


Figure 19 : Niveau de corticostérone (ng/ml) en fonction des catégories d'habitat. Les mesures ont été effectuées sur des mâles adultes reproducteurs. Les couleurs représentent des habitats différents (vert pour "Forêt", bleu pour milieu "Intermédiaire", jaune pour milieu "agricole").

(ii) Traits liés à la reproduction (T et caractères sexuels secondaires)

Concernant la testostérone, la relation entre les niveaux de testostérone et la taille corporelle était négative chez les individus provenant uniquement de l'habitat forestier (Figure 20). Ces résultats indiquent que les individus plus grands des habitats forestiers sécrètent des niveaux plus faibles de testostérone que les individus plus petits, alors qu'aucune relation de ce type n'a été trouvée chez les individus des autres types d'habitats.

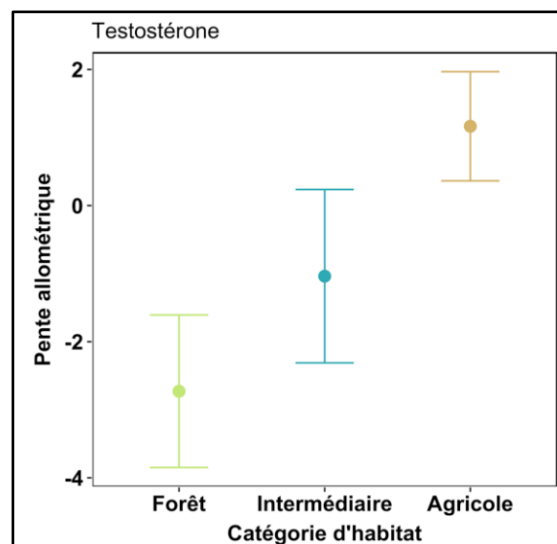


Figure 20 : Pentes allométriques des niveaux de testostérone (ng.ml⁻¹, SVL). Toutes les mesures sont exprimées en log10. Les mesures ont été effectuées sur des mâles adultes reproducteurs. Les couleurs représentent des habitats différents (vert pour "Forêt", bleu pour milieu "Intermédiaire", jaune pour milieu "agricole").

Dans un second temps, la condition corporelle était proportionnelle à la surface des callosités nuptiales (qui permettent aux mâles d'agripper les femelles pendant l'amplex), mais cette relation est plus marquée chez les individus provenant d'habitats forestiers. Trois hypothèses différentes peuvent expliquer ces résultats.

Structuration en âge

Tout d'abord, il est possible que dans les habitats agricoles, la mortalité accrue entraîne une structuration d'âge différente composée principalement d'individus plus jeunes (Zamora-Camacho & Comas, 2017) (Figure 21). De plus, dans les habitats agricoles, la taille corporelle est généralement plus grande à un âge plus jeune (Zamora-Camacho, 2018; Zamora-Camacho & Comas, 2017) (Figure 21), ce qui pourrait s'expliquer par un taux de croissance plus rapide dans les habitats agricoles et éventuellement par un compromis entre la durée de vie et la croissance (Lee et al., 2013; Lemaître et al., 2015; Olsson & Shine, 2002; Rollo, 2002; Zamora-Camacho & Comas, 2017). Comme le taux de testostérone diminue avec l'âge (Chen et al., 1994), les différences de structure d'âge entre les types d'habitat peuvent éclairer les relations divergentes entre le taux de testostérone et la taille corporelle.

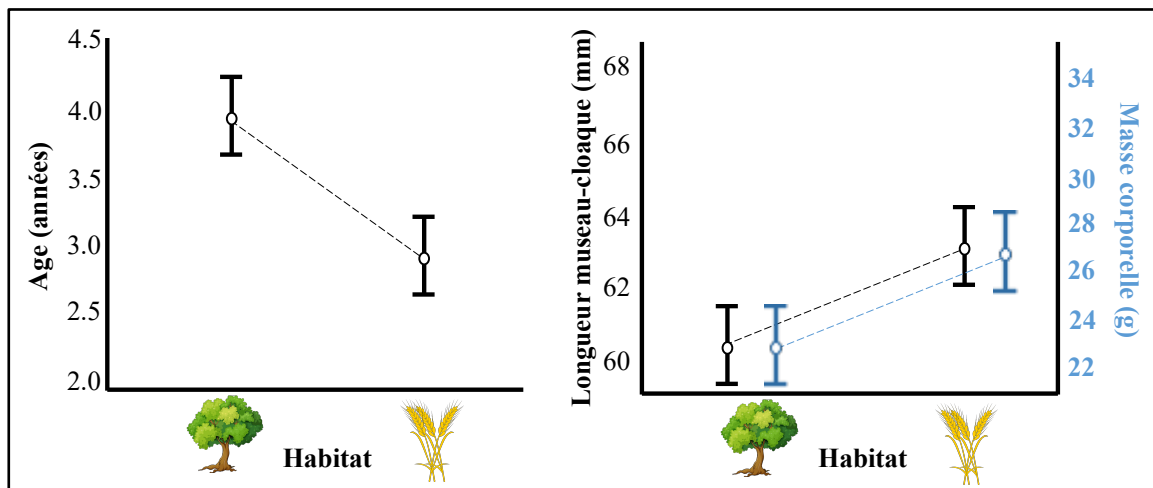


Figure 21 : Age des crapauds mâles (A), longueur museau-cloaque et masse corporelle (B). Les données sont issues de Zamora-Camacho & Comas (2017) et représentent les moyennes et erreurs standards.

Abondance en femelle

Les sites de reproduction agricoles étant caractérisés par une plus faible abondance de femelles reproductrices (Renoirt et al. 2021b) (Annexe III), la compétition pour l'accès aux femelles pourrait être plus intense dans ces habitats, influençant ainsi les niveaux de testostérone en tant que médiateur du succès de l'accouplement dans les étangs de reproduction (Cayuela et al., 2017) où une rivalité plus importante pourrait induire des niveaux de testostérone plus élevés (Burmeister & Wilczynski, 2001; Eikenaar et al., 2012; Hirschenhauser & Oliveira, 2006).

Cette dernière hypothèse s'accorde relativement bien avec la relation trouvée entre la condition corporelle et la surface des callosités nuptiales chez les individus provenant d'habitats forestiers. En effet, dans ce type d'habitat, les mâles plus grands ont une plus grande surface de callosités nuptiales qui leur permet de serrer étroitement les femelles pendant l'amplexus, diminuant ainsi le risque de perdre des opportunités d'accouplement à cause de la rivalité (Bowcock et al., 2009; Bruning et al., 2010; Orton et al., 2020). En effet, chez les anoues mâles, la taille des callosités nuptiales est positivement liée au succès reproducteur (Orton et al., 2020) et les mâles plus grands peuvent réussir à éliminer les concurrents plus petits déjà attachés à une femelle pendant l'amplexus (Bruning et al., 2010; Davies & Halliday, 1977). Dans notre contexte, une plus grande surface des callosités nuptiales chez les individus plus grands provenant d'habitats forestiers pourrait contribuer à sécuriser les opportunités d'accouplement (amplexus) et donc la fitness tout en diminuant les effets négatifs potentiels des niveaux élevés de testostérone (McGlothlin et al., 2010; Wingfield et al., 2001), une hormone dont il a été montré qu'elle atteint une concentration remarquablement élevée chez les mâles reproducteurs de notre espèce d'étude (Brischoux et al., 2018).

Perturbation endocrinienne

Il est également possible que le fait de vivre dans des sites soumis à des activités agricoles (terres agricoles ou types d'habitats intermédiaires) expose les individus à divers produits agrochimiques, dont certains sont reconnus comme des perturbateurs endocriniens (par exemple, Bókony et al., 2018; Hayes et al., 2006; Orton & Tyler, 2015).

Une telle hypothèse peut expliquer le contraste entre les habitats que nous avons trouvé dans les relations qui lient la taille corporelle à la testostérone et peut donc indiquer que certains composés peuvent perturber ces relations dans les zones agricoles. En effet, une augmentation de la sécrétion de testostérone en réponse à une contamination environnementale a déjà été documentée chez les crapauds (Hopkins et al., 1997). Une telle réponse peut être préjudiciable aux individus car il a été démontré que des niveaux élevés de testostérone circulante représentent des coûts énergétiques élevés, réduisent le stockage des graisses, augmentent la mortalité, altèrent le comportement ou suppriment le système immunitaire (McGlothlin et al., 2010 ; Wingfield et al., 2001). Si cette hypothèse se vérifie, un tel mécanisme peut influencer la condition physique individuelle (survie) et finalement affecter la persistance de la population (McGlothlin et al., 2010; Wingfield et al., 2001).

(iii) Longueur des télomères

Enfin, nous avons trouvé des relations divergentes entre la longueur des télomères et la condition corporelle entre les types d'habitats (**Figure 22**), ces relations étant négatives chez les individus des forêts, positives chez les crapauds des habitats agricoles et intermédiaires.

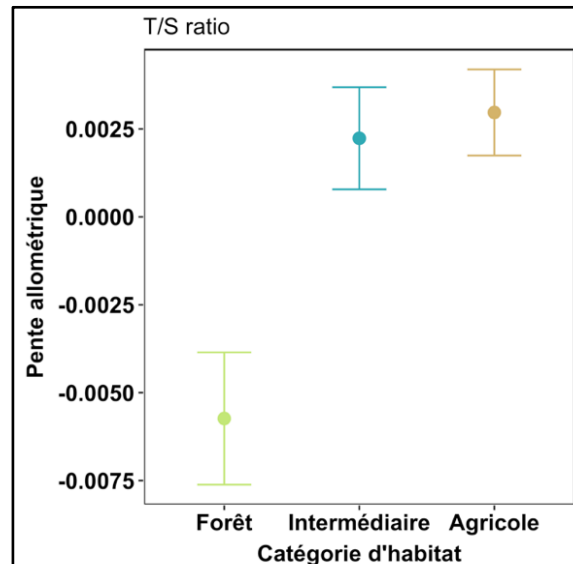


Figure 22 : *Pentes allométriques de la longueur des télomères (T/S ratio, BCI). Toutes les mesures sont exprimées en log10. Les mesures ont été effectuées sur des mâles adultes reproducteurs. Les couleurs représentent des habitats différents (vert pour "forestiers", bleu pour milieux "Intermédiaire", jaune pour milieux "agricole").*

La longueur des télomères est liée à la sénescence car ils raccourcissent généralement avec l'âge (Bichet et al., 2020; Sánchez-Montes et al., 2020, mais voir Bize et al., 2009). Si les individus en meilleure condition corporelle sont également des individus plus âgés, le schéma que nous avons trouvé dans les sites forestiers était attendu (Rollings et al., 2017). De manière surprenante, nous avons trouvé la relation inverse dans les zones agricoles. Deux hypothèses différentes peuvent expliquer ce résultat. Premièrement, ce résultat pourrait être une conséquence de la divergence de la structuration de l'âge entre les types d'habitat, comme suggéré ci-dessus (Zamora-Camacho & Comas, 2017), avec des individus de grande taille mais plus jeunes - donc avec des télomères plus longs - dans les habitats agricoles (**Figure 21**).

Une seconde hypothèse est lié au fait de vivre dans des zones agricoles qui peut exposer les individus à divers produits agrochimiques, dont certains peuvent perturber l'attrition naturelle de la longueur des télomères en activant la télomérase (une enzyme qui empêche l'attrition des télomères, Blackburn, 1991, 2005; Hatakeyama et al., 2016). Un tel résultat a déjà été décrit dans un contexte différent avec des composés perfluorés (PFAS) induisant une élongation des télomères avec l'âge chez les espèces d'oiseaux (Blévin et al., 2017; Sebastiano et al., 2020). Il est clair que les composés présents dans les

zones agricoles sont différents de ceux inclus dans ces études, mais l'effet de différents contaminants sur la dynamique d'attrition des télomères a déjà été décrit dans différents contextes (Chatelain et al., 2020; Louzon et al., 2019).

c) Différence de comportement entre habitats contrastés

L'étude de comportement présentée dans cette section est issue des résultats préliminaires toujours en cours d'analyse. L'étude n'a pas montré de différence statistique de comportement entre catégories d'habitat ($p > 0.06$, **Figure 23**). L'étude du comportement a mis en évidence des différences entre site pour la mobilité ($X^2 = 23.61$, $df = 7$, $p = 0.001$), le type de position ($X^2 = 18.01$, $df = 7$, $p = 0.029$) et la variable « Out » ($X^2 = 16.30$, $df = 7$, $p = 0.023$) qui n'est pas expliqué par la catégorie d'habitat. D'autres facteurs déterminent le comportement des crapauds sur nos sites d'études. Les individus « shy » aillant tendance à préférer les habitats présentant plus de refuges (Kelleher et al., 2018).

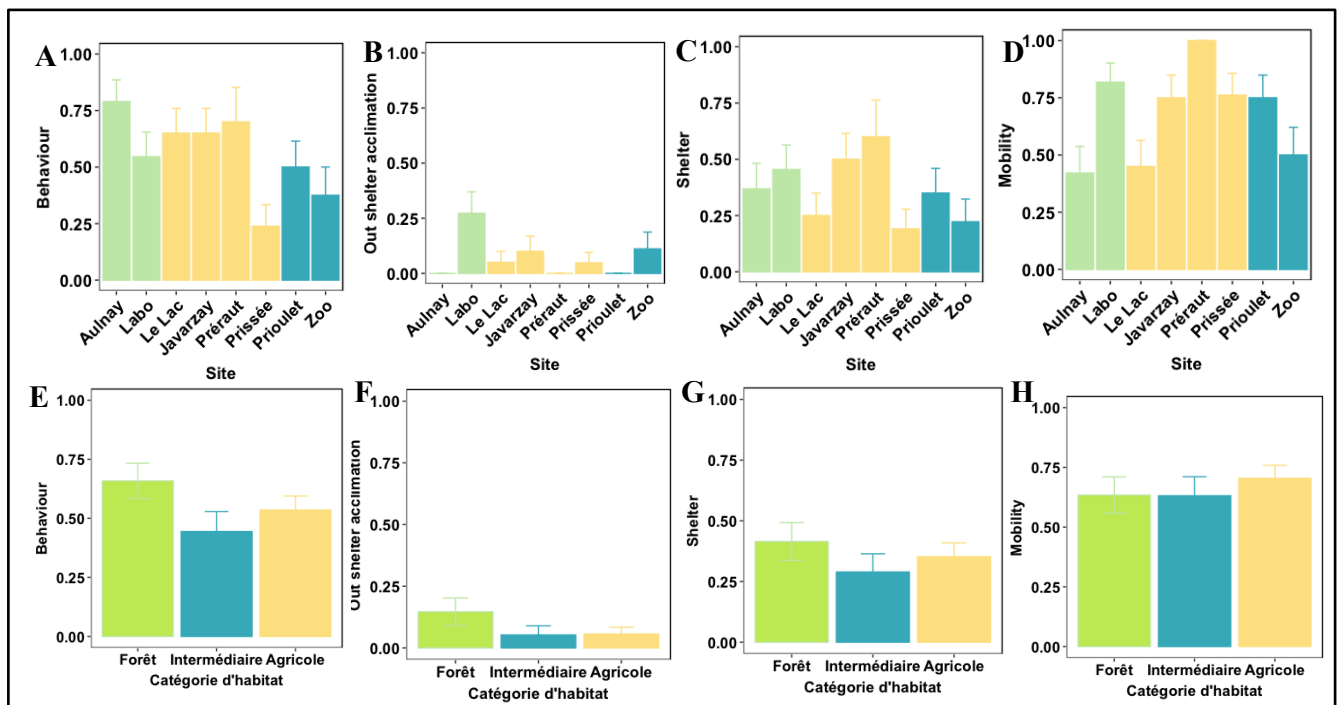


Figure 23 : Différence de comportement entre sites (A, B, C, D) ou catégories d'habitat (E, F, G, H) chez *Bufo spinosus*. A, E/ "Behavior" (Proportion de mâles observés en position de type "body raising" quand l'abri a été retiré) B, F/ "Out" (Proportion de mâles qui sont sortis de l'abri pendant l'acclimation), C, G/ "Shelter" (Proportion de mâle dans le second abri pendant les 30 minutes de vidéos), D, H/ "Mobility" (Proportion de mâle qui se sont déplacés dans l'arène plus de 3 minutes pendant les 30 minutes). Les mesures ont été faites sur des mâles en reproduction. Les données représentent la proportion \pm SE. Les couleurs représentent des habitats différents (vert pour "forestier", jaune pour milieu "agricole", bleu pour milieu "Intermédiaire").

Indépendamment de la catégorie d'habitat, notre étude de comportement a mis en évidence une relation entre le type de position « Body-raising » des crapauds mâles en soulevant l'abri et le niveau de corticostérone ($X^2=4.82$, $df=1$, $p=0.028$) (Figure 24). On sait

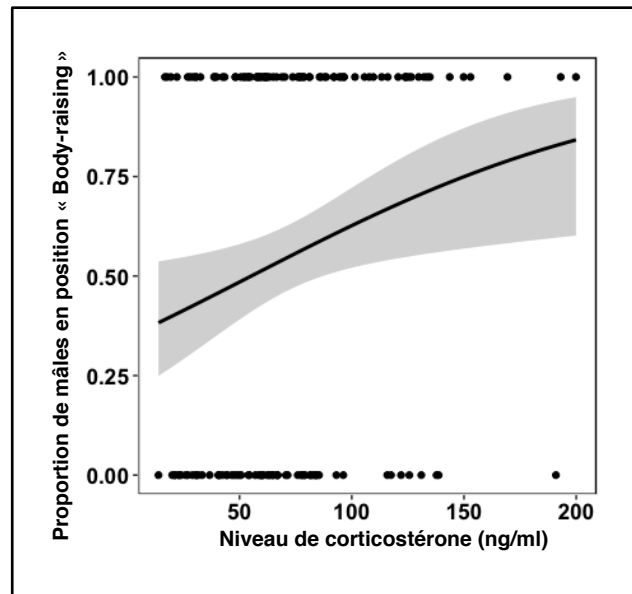


Figure 24 : Relation entre la proportion de mâles observés en position de type "body raising" et le niveau de corticostérone (ng/ml). Les mesures ont été faites sur des mâles en reproduction. La bande grise représente l'intervalle de confiance à 95%.

Les amphibiens du genre *Bufo* synthétisent des composés anti-prédateurs appelés bufotoxines. Ces bufadiénolides sont synthétisés à partir du même précurseur que les hormones de stress glucocorticoïdes et les hormones sexuelles stéroïdiennes, en passant par les mêmes étapes initiales (Fedorova et al., 2015) ce qui pourrait expliquer cette relation entre position de défenses et niveau de corticostérone. Des niveaux élevés de bufadiénolides peuvent être des sous-produits du stress physiologique et/ou des effets de perturbation endocrinienne connus pour être provoqués par de nombreux polluants anthropiques.

III) Plasticité phénotypique ou divergence génétique ?

Pour déterminer si les différences observées résultent d'une divergence génétique des crapauds de nos différentes catégories, nous avons conduit des études de génétiques des populations. Ces études sont toujours en cours d'analyse.

Les études sur la génétique de population des crapauds des zones d'études ne détectent pas de structuration génétique significative globale ($K=1$). De même que l'AFP (analyse factorielle principale) qui ne détecte pas de structuration génétique marquée. Cependant, l'analyse structuration

génétique fine révèle des différences entre populations pour les valeurs de *Fst* corrélées aux distances géographiques euclidiennes (**Tableau 8**).

	Aulnay	Javarzay	Labo CEBC	Le lac Ensigné	Prissé	Prerault	Prioulet	Zoodyssée
Aulnay	-	NS	NS	NS	*	NS	NS	NS
Javarzay	0,0096	-	NS	NS	*	NS	*	NS
Labo CEBC	0,0175	0,0165	-	NS	*	NS	NS	NS
Le lac Ensigné	0,0051	0,006	0,0192	-	*	NS	*	*
Prissé	0,0451	0,0344	0,0261	0,0361	-	*	*	*
Prerault	0,0111	0,0032	0,0168	0,0157	0,0399	-	NS	NS
Prioulet	0,0173	0,0149	0,0206	0,0189	0,0497	-0,0145	-	NS
Zoodyssée	0,0139	0,0088	0,0145	0,0216	0,026	0,0038	0,0018	-

Tableau 8: Comparaison multiples des valeurs de *FST* pour chaque population (diagonale du bas) et leur niveau de significativité (diagonale du haut). En vert sont représentés les sites *forestiers*, en jaune les milieux *agricoles* et en bleus les milieux *intermédiaires*. Les seuils de *p*-value ont été ajustés à l'aide de la correction de Bonferroni, $P = 0.0018$.

Une structuration génétique dite en "isolement par la distance" qui montrent des populations suivant les deux bassins versants différents orientés Ouest (*Prissé, Labo, Prioulet, Zoodyssée*) et Est (*Aulnay, Javarzay, Lelac, Prerault*). Ces études sont encore en cours d'analyse mais ce manque de structuration génétique important semble indiquer qu'il n'y a pas de divergence génétique entre les populations et que les résultats sur la morphologie et la physiologie relèvent de la plasticité phénotypique plus qu'une divergence génétique.

La façon la plus empirique de déterminer si les différences structurelles observées relève d'une divergence génétique ou de la plasticité phénotypique, serait une expérience de jardin commun. Des individus de chaque site sont placés dans des conditions similaires à leur habitat naturel mais également dans des habitats considérés comme non naturels où on étudie leur fitness. Un test usuel pour étudier la divergence adaptative d'une population est une expérience de transplantation réciproque. Si les individus qui montrent la meilleure fitness dans un site sont ceux qui proviennent de ce site, alors les différences entre populations sont connues pour avoir évolué sous la sélection naturelle dans le but d'augmenter la fitness sous les conditions locales. Cependant, on ne peut pas savoir quelles caractéristiques de l'habitat entraînent cette adaptation locale. Une expérience de jardin commun peut répondre à ces questions quand on s'intéresse à un trait connu pour avoir une importance sur la fitness. Si les phénotypes individuels varient dans les populations sources dans la direction indiqués par les traits fonctionnels étudiés, on peut conclure que la sélection imposée par les conditions environnementales connues a mené la population à diverger. La divergence au sein des populations existe car la sélection est suffisamment forte pour outrepasser l'homogénéisation du flux de gènes. Par exemple, chez les têtards d'anoures, on connaît les facteurs de divergence liés aux variations d'habitats, on connaît également quels traits phénotypiques influencent la fitness dans certains habitats (*Van Buskirk & Arioli, 2005a*).

IV) Conclusion

Nous avons constaté que les types d'habitat influencent la morphologie et la physiologie des crapauds mâles adultes. Il est intéressant de noter que ces différences se sont produites par le biais de variations subtiles mais significatives des relations allométriques des différents traits morphologiques et physiologiques que nous avons étudiés. Notre étude souligne la nécessité d'englober un large éventail de tailles corporelles ainsi qu'un large éventail de traits morphologiques et physiologiques si nous voulons évaluer de manière exhaustive l'influence des pratiques agricoles modernes sur la faune sauvage. Les conditions vécues au début de la vie peuvent avoir des effets permanents sur les phénotypes individuels (Lindström, 1999 ; Monaghan, 2008). De tels effets peuvent diminuer ou augmenter la fitness, selon que les conditions du début de la vie étaient relativement mauvaises ou bonnes. Les variations de morphologie entre les types d'habitat suggèrent clairement des réponses complexes liées à la structure de l'habitat qui nécessiteront des études approfondies englobant les habitats aquatiques et terrestres afin d'évaluer la contribution relative des conditions de développement par rapport à la croissance post-métamorphique pour évaluer de manière exhaustive l'influence de la structure de l'habitat sur la morphologie chez des espèces généralistes aussi répandues. Des expériences d'élevage croisé à grande échelle *in natura* (par exemple, la translocation d'individus post-métamorphiques) et/ou des expériences de jardin commun dans des conditions semi-contrôlées peuvent s'avérer des voies de recherche utiles afin de déchiffrer les effets que nous avons détectés (Bachmann & Van Buskirk, 2021; Lind & Johansson, 2011; Van Buskirk & Arioli, 2005b). De plus, les sources des contrastes que nous avons mis en évidence (par exemple, la structure physique ou chimique de l'environnement, ou une combinaison des deux) restent à évaluer.

Chapitre II

Effets de la contamination environnementale pendant l'embryogénèse



“You have a grand gift for silence, Watson. It makes you quite invaluable as a companion.”
— Arthur Conan Doyle, *The Complete Sherlock Holmes*

Cette partie résume les **articles III, IV, V et VI** portant sur les effets de l'AMPA et du nicosulfuron sur le développement embryonnaire de *Bufo spinosus*. Pour plus d'informations, voir les articles associés à la fin du manuscrit.

I) Contexte

A) Effets de l'AMPA ou du nicosulfuron sur le développement embryonnaire

Dans le cadre des études des effets de la contamination environnementale sur le développement du crapaud épineux, nous nous sommes intéressés à deux contaminants en particulier, l'AMPA et le Nicosulfuron (voir méthodes).

a) Contamination historique et métabolisation : le cas de l'AMPA

Les herbicides à base de glyphosate figurent actuellement parmi les produits chimiques agricoles les plus utilisés dans le monde. Vendus sous le nom commercial Roundup® (RU), les herbicides à base de glyphosate étaient le 17^e pesticide le plus utilisé à la fin des années 1980, en termes de matière active appliquée, mais en 2002, il s'agissait de l'herbicide le plus utilisé aux États-Unis (Gianessi & Reigner, 2007). L'impact de cette seule classe d'herbicides sur les pratiques agricoles modernes est indéniable (Gilbert, 2013); la combinaison de la nature herbicide à large spectre du glyphosate et du développement de variétés de cultures résistantes a élevé les herbicides à base de glyphosate au rang des produits chimiques agricoles les plus vendus de tous les temps.

En outre, de nombreuses molécules nominales sont rapidement métabolisées dans des conditions naturelles (Giesy et al., 2000). Par exemple, le glyphosate a une demi-vie très courte (1 à 4 jours) et est rapidement métabolisé en acide aminométhylphosphonique (AMPA) (Annett et al., 2014). L'AMPA est détecté plus fréquemment en raison de sa demi-vie plus longue (Grandcoïn et al., 2017). Il est le principal contaminant détecté dans les eaux de surface dans le monde avec un taux presque deux fois supérieur à celui du glyphosate (Battaglin et al., 2014; Bonansea et al., 2017; Silva et al., 2018).

Pourtant, les études écotoxicologiques ne s'intéressent généralement pas aux métabolites des produits agrochimiques (Schusterova et al., 2019) alors que l'AMPA est l'un des contaminants les plus abondants dans les environnements aquatiques dans le monde. Par exemple, bien que l'AMPA soit considéré comme moins toxique pour les organismes aquatiques (FAO, 1997, Carey et al, 2008), certains auteurs ont montré une toxicité significative pour les algues vertes, certaines espèces d'oiseaux, les mammifères, et dans une moindre mesure pour les poissons (Giesy et al., 2000). Une des conséquences de l'utilisation intensive du glyphosate est la contamination des rivières par la substance active et ses métabolites, l'acide aminométhylphosphonique (AMPA), induisant l'eutrophisation des rivières (Carles et al., 2019). La persistance du glyphosate dans ces écosystèmes est soupçonnée d'être principalement influencée par les processus de biodégradation microbienne. Les fluctuations

saisonniers des concentrations de glyphosate et d'AMPA sont corrélées, atteignant un pic au printemps/été peu après l'épandage des pesticides.

La toxicité du glyphosate est relativement bien étudiée dans la littérature et montre des effets comme des altérations des activités d'enzymes antioxydantes, ainsi qu'un déséquilibre biochimique chez des espèces de poissons (*Oreochromis niloticus* et *Prochilodus lineatus* ; Cavalcante et al., 2008; Langiano & Martinez, 2008). Chez les amphibiens, des taux de mortalité importants sont relevés (Relyea, 2005), de même que des modifications morphologiques, de durée de développement ou de comportement (Cattaneo et al., 2011; Jones et al., 2010). L'exposition aux herbicides augmente également la quantité de bufadiénolides chez les têtards de crapauds communs (*Bufo bufo*) (Bókony et al., 2017). Pourtant, son métabolite principale montre un nombre de publication bien moins important mais des effets sublétaux significatifs sur les espèces non-cibles (de Brito Rodrigues et al., 2019; Domínguez et al., 2016; Guilherme et al., 2014; Matozzo et al., 2018, 2019)

b) Contaminant émergent: le cas du Nicosulfuron

Parmi les différents nouveaux pesticides régulièrement employés, l'utilisation des herbicides à base de sulfonilurées a fortement augmenté (de Lafontaine et al., 2014; Food and Agriculture Organization, 2018). Ce groupe d'herbicides est utilisé pour lutter contre les « mauvaises » herbes dans une grande variété de cultures, notamment le blé, l'avoine, le soja ou le maïs. Malgré leur importance croissante dans l'agriculture (de Lafontaine et al., 2014), les études détaillées sur leurs effets potentiels sur les espèces non ciblées à des concentrations pertinentes pour l'environnement sont rares. Par exemple, les herbicides à base de sulfonilurées sont très toxiques pour les plantes sensibles et peuvent être toxiques pour les microalgues et le phytoplancton à de faibles concentrations (Seguin, Leboulanger & Rimet, 2001). Bien que les herbicides à base de sulfonilurées soient généralement considérés comme peu toxiques pour les espèces non cibles (Centers for Disease Control and Prevention, 2017; Kamrin, 1997), il existe de plus en plus de preuves de leur rôle potentiel dans l'induction d'un développement anormal, d'effets tératogènes, de perturbations biochimiques et d'altérations comportementales (Breteau et al., 2000; Park et al., 2020; Pretto et al., 2011; Saglio, Olsén & Bre, 2001).

B) Susceptibilité du stade embryonnaire chez l'amphibien

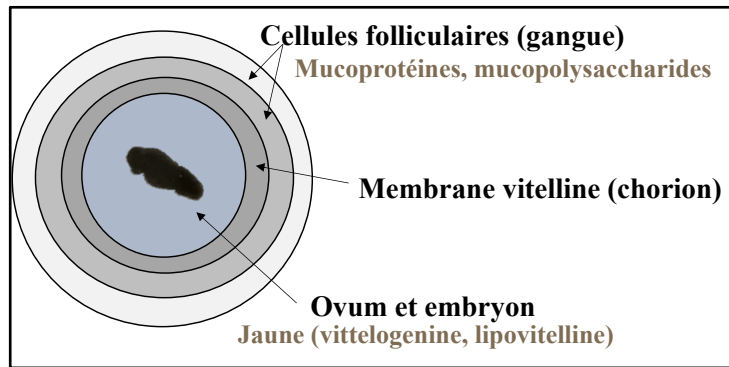


Figure 25 : Représentation de l'anatomie d'un œuf d'amphibien anamniotique avec la composition protéique des couches (en marron). Adapté selon Vitt & Caldwell, 2014).

Les embryons d'amphibiens sont considérés comme étant moins sensibles à la contamination environnementale pour plusieurs raisons. Les oocytes sont protégés par une membrane protectrice sous forme de plusieurs couches d'apparence gélatineuses composées de polysaccharides, glycoprotéines et d'une membrane périvitelline (Figure 25, Vitt & Caldwell, 2014). La composition de ces couches sont synthétisées par les cellules de l'oviducte qui joue un rôle important dans la fertilisation et la protection des embryons (Maës et al., 1997). La composition, l'épaisseur et la viscosité dépend des espèces et agit comme une barrière à un bon nombre de produits chimiques (Berrill et al., 1994, 1998; Brodeur et al., 2009; Edginton et al., 2007; Wagner et al., 2015). Cependant des réactions entre cette matrice et des substances ont été reportées résultant en un cocktail plus toxique pour les embryons (Marquis et al., 2006). De plus, la membrane peut représenter une protection insuffisante contre les contaminants comme sous l'effet de l'insecticide α -cyperméthrin qui provoque des effets tératogènes après exposition (Greulich & Pflugmacher, 2003). Les embryons peuvent également être moins sensibles aux polluants qui affectent le système nerveux, car le système nerveux à ce stade ontogénique précoce n'est pas encore complètement développé (Ortiz-Santaliestra et al., 2017). C'est également le cas pour les nitrates, qui sont transformés en nitrites et en nitrosamines, plus toxiques, par les bactéries intestinales. Ainsi, la différenciation incomplète de l'intestin aux premiers stades de la vie peut avoir un effet protecteur (Egea-Serrano et al., 2012).

II) Résultats et discussion

A) Traits de développement et qualité de la descendance

Le succès d'éclosion chez les espèces de crapauds est connu pour être très variable (Bishop et al., 2010; Bókony et al., 2018; Häkkinen et al., 2001; Rowe, Hopkins & Coff, 2001). Bien que les conditions environnementales puissent influencer le succès d'éclosion (Bishop et al., 2010; Chivers et al., 2001; Delia et al., 2019), des études ont montré que le transfert maternel (c'est-à-dire les hormones glucocorticoïdes : Saino et al., 2005 ; contaminants : Bergeron et al., 2010; Metts et al., 2013; Unrine et al., 2006, 2007) peut également affecter le succès d'éclosion. Cet effet suggère que le succès d'éclosion est sous une forte influence parentale et pourrait être utilisé comme un indicateur de la qualité de la ponte. Cependant, ces traits sont souvent des traits liés au développement post-éclosion. Or pendant la phase embryonnaire les traits développementaux et d'histoire de vie sont intimement liés et varient possiblement en fonction de stressseurs environnementaux.

L'article III examine la relation entre la qualité de la ponte (évaluée par le succès d'éclosion) et la qualité de la descendance. Notre expérience de jardin commun dans des conditions contrôlées nous a permis d'évaluer le succès d'éclosion indépendamment des facteurs naturels affectant le développement embryonnaire (Chivers et al., 2001; Egea-Serrano et al., 2014; Gomez-Mestre et al., 2013). La qualité de la descendance a été évaluée en utilisant deux ensembles complémentaires de marqueurs connus pour être liés à la fitness. Le but de cette étude était de déterminer des traits de développement représentatif des traits d'histoire de vie de cette espèce pouvant être affectés par la contamination environnementale.

Dans un premier temps, nous avons utilisé deux traits de la descendance, à savoir la **durée du développement embryonnaire** et la **taille corporelle des embryons éclos**, car ces traits peuvent être utilisés comme marqueurs de la qualité de la descendance chez les amphibiens (Delia et al., 2019; Van Buskirk, 2002). Nous avons constaté que les pontes avec un succès d'éclosion plus faible étaient caractérisées par des durées de développement embryonnaire plus rapides pour les individus qui ont éclos (**Figure 26**).

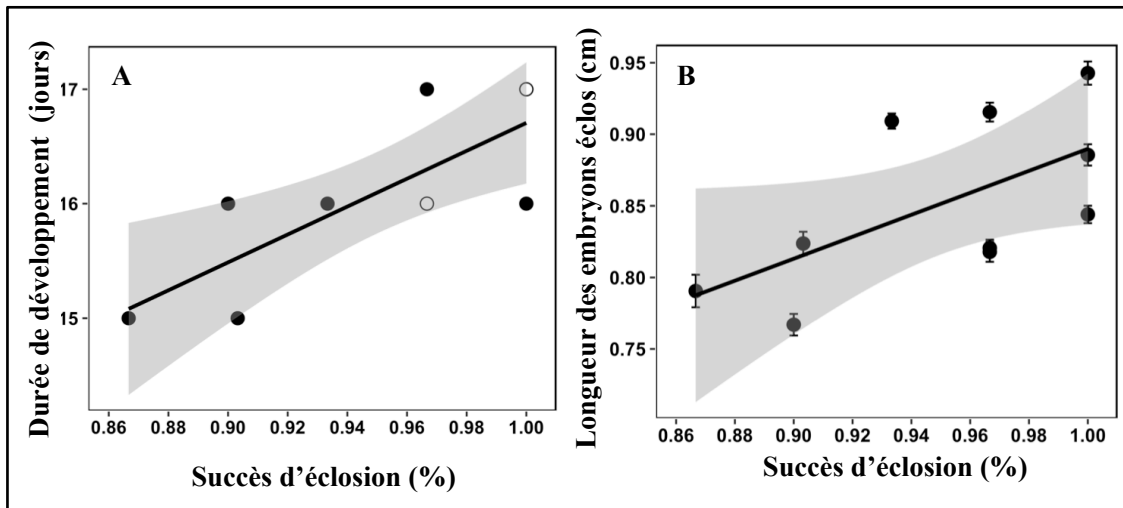


Figure 26 : A : Durée du développement embryonnaire (jours) en fonction du succès d'éclosion de la ponte (%). Les points noirs représentent tous les individus d'une même ponte. Comme deux pontes présentaient un succès d'éclosion et une durée de développement similaires, nous les avons représentées par des points blancs. B : Taille des embryons éclos (moyenne±se) par rapport au succès d'éclosion (%). Les bandes grises indiquent les intervalles de confiance à 95%.

Une telle relation pourrait être le résultat d'une caractéristique intrinsèque de l'éclosion, selon laquelle certains éclosions se développent plus rapidement que d'autres (Magrath, 1990; Nager et al., 2000). A son tour, un développement embryonnaire rapide pourrait conduire à une accumulation de dommages cellulaires par surproduction d'espèces réactives de l'oxygène (ROS), entraînant une baisse du succès d'éclosion (Lee et al., 2013). Alternativement, une telle relation pourrait être une réponse adaptative des embryons aux signaux environnementaux. Les embryons peuvent répondre à des signaux chimiques sécrétés par des embryons morts voisins et accélérer leur développement afin d'échapper à des conditions environnementales néfastes (Delia et al., 2019; Lee et al., 2013). La plasticité de l'éclosion est connue chez les amphibiens et a été supposée être une réponse adaptative aux risques de prédation (Sih & Moore, 1993; Touchon & Wojdak, 2014; Warkentin, 1995).

Nous avons également constaté que les embryons qui ont mis moins de temps à se développer ont éclos plus petits. Ce résultat était attendu étant donné la relation négative bien connue entre la durée du développement embryonnaire et la taille à l'éclosion chez les vertébrés ectothermes (Gillooly & Dodson, 2000). Par exemple, on sait que les ectothermes se développent plus rapidement et atteignent la maturité à une taille plus petite lorsqu'ils sont exposés à une température plus élevée (Zuo et al., 2012). Étant donné que les espèces ont évolué en fonction des contraintes de leur milieu et donc les mécanismes de détoxification ou de réparation lors de dommages oxydatifs différents. Par exemple chez les vertébrés, il a été montré que une accélération du développement peut mener à des dommages oxydatifs ce qui suggère un compromis liant les équilibres redox aux stratégies de croissance (Smith et al., 2016).

Enfin nous avons étudié **la longueur des télomères** des embryons éclos (**Article III**), car ce marqueur a été suggéré comme un outil moléculaire prometteur pour évaluer la qualité individuelle (Angelier et al., 2019; Bichet et al., 2020; Eastwood et al., 2019). Les télomères sont de courtes séquences d'ADN non codantes et répétitives situées à l'extrémité des chromosomes eucaryotes. L'attrition des télomères peut entraîner la mort cellulaire programmée (Campisi & d'Adda di Fagagna, 2007). La longueur des télomères peut varier selon les conditions environnementales (Angelier et al., 2018; Blévin et al., 2016; Chatelain et al., 2020), les stades ontogénétiques (Burraco, Valdés, et al., 2020) et les espèces (Whittemore et al., 2019). Ainsi, les télomères peuvent donner un aperçu des réponses phénotypiques et physiologiques aux conditions environnementales pendant les premiers stades de la vie (Heidinger et al., 2012). Comme chez la plupart des vertébrés, les télomères raccourcissent tout au long de la vie adulte chez les amphibiens (Sánchez-Montes et al., 2020), et la période de développement est considérée comme une période critique pour la longueur des télomères des éclosions (Burraco, Comas, et al., 2020; Eastwood et al., 2019; Foote et al., 2011; Stier et al., 2020). Il est important de noter que la longueur des télomères de l'éclosion est également liée à des paramètres de la fitness, tels que la survie et la longévité, chez de multiples espèces de vertébrés (Boonekamp et al., 2014; Heidinger et al., 2012; Wilbourn et al., 2018), soulignant la pertinence de cette mesure pour évaluer la qualité des éclosions.

En conséquence, nous avons prédit que la descendance des pontes de moindre qualité (c'est-à-dire avec une plus grande mortalité embryonnaire) aurait des tailles corporelles et des longueurs de télomères réduites. Dans l'ensemble, nous avons constaté que le succès d'éclosion était lié à la durée du développement embryonnaire et à la taille des éclosions (**Figure 27**). Parmi le groupe d'individus pour lesquels la longueur des télomères a été évaluée, nous avons constaté que la longueur des télomères était également liée au succès d'éclosion de la ponte. Les éclosions dont le succès d'éclosion était plus faible ont produit des embryons éclos avec des télomères plus courts.

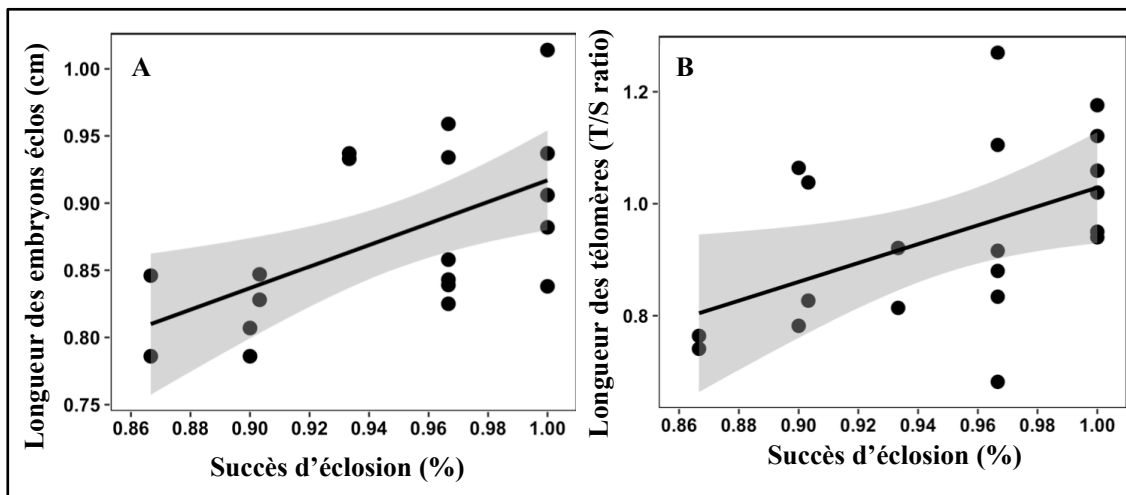


Figure 27 : Taille des embryons éclos (A, cm) et longueur relative des télomères (B, T/S ratio) en fonction du succès d'éclosion (%) pour les individus pour lesquels la longueur des télomères a été évaluée. Les bandes grises indiquent les intervalles de confiance à 95%.

Il est connu que le taux de réplication cellulaire pendant le développement embryonnaire est négativement corrélé avec la taille des télomères à l'éclosion (Foote et al., 2011; Jennings et al., 2000; Stier et al., 2020). Étant donné que des télomères plus courts au début de la vie sont liés à une fitness plus faible plus tard dans la vie (par exemple, la longévité : Boonekamp et al., 2014; Heidinger et al., 2012; Stier et al., 2020; Wilbourn et al., 2018), nos résultats suggèrent que la descendance des pontes de moindre qualité pourrait connaître des performances globales relativement moins bonnes plus tard dans la vie, d'autant plus si la ponte subit des stress environnementaux tels que la contamination environnementale.

Pour conclure, cette étude nous a permis de montrer que des traits de développement intrinsèques à la descendance étaient représentatifs de la qualité de la ponte. On peut s'attendre à ce que des stressés environnementaux puissent affecter ces traits de développement altérant la fitness à long terme des individus.

B) Deux contaminants, deux concentration-réponses différentes

a) AMPA et non monotonie

Cette partie résume les **articles IV** et **V** portant sur les effets de l'AMPA sur le développement embryonnaire de *Bufo spinosus*.

Sur la base d'un échantillon de très grande taille (>2000 œufs issus de 20 pontes), nos résultats montrent que l'exposition chronique à des concentrations faibles et pertinentes pour l'environnement

d'AMPA, un contaminant primaire détecté dans les eaux de surface (Bonansea et al., 2017; Coupe et al., 2012; Grandcoin et al., 2017) altère la qualité du développement embryonnaire des crapauds épineux. Plus spécifiquement, nos résultats montrent que l'AMPA influence la mortalité embryonnaire, la durée du développement et la morphologie de manière complexe (Articles IV). De plus, l'AMPA modifie le statut oxydatif, mais pas la longueur des télomères, pendant le développement embryonnaire (Article V). Ces effets physiologiques se sont produits à des concentrations pertinentes pour l'environnement, représentatives des concentrations réelles de ce contaminant dans la nature.

Il est intéressant de noter que les modèles de réponse de tous les traits mesurés divergent des relations concentration-réponse linéaires classiques.

La mortalité embryonnaire était 2-3 fois plus élevée dans les œufs qui se sont développés avec les concentrations d'AMPA basse et moyenne (Figure 28).

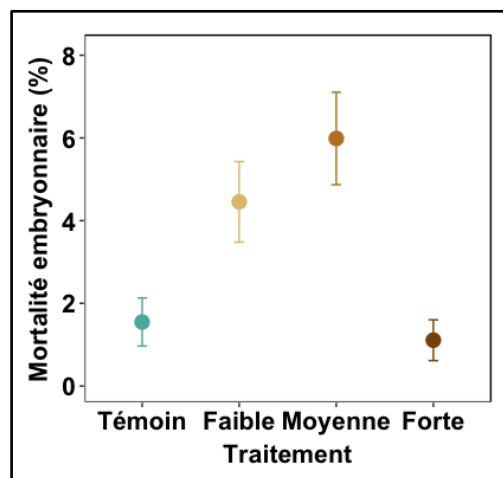


Figure 28 : Mortalité embryonnaire (%) en fonction des différents traitements d'AMPA

En se concentrant uniquement sur les différences entre les embryons de contrôle et les embryons élevés aux deux concentrations inférieures d'AMPA, le modèle que nous avons trouvé pourrait suggérer un effet létal direct de l'AMPA sur les embryons, probablement par une toxicité accrue entraînée par une concentration accrue d'AMPA (Baier, Gruber, et al., 2016; Baier, Jedinger, et al., 2016; Broomhall & Shine, 2003; García-Muñoz et al., 2010). Cependant, les résultats obtenus à partir de la plus forte concentration d'AMPA n'appuient pas l'hypothèse d'une augmentation des concentrations d'AMPA entraînant une augmentation de la mortalité. En effet, les embryons qui ont été exposés à la plus forte concentration d'AMPA se sont développés et ont survécu aussi bien que ceux du traitement témoin. Une mortalité embryonnaire accrue pourrait diminuer le succès reproductif

des crapauds qui pondent leurs œufs dans un étang contaminé (Hayes et al., 2006; Richter et al., 2003).

(i) Mortalité sélective

En étudiant le stress oxydant, l'exposition à de faibles concentrations d'AMPA a influencé les niveaux de thiols (Figure 29A) mais n'a pas influencé les niveaux de catalase, bien que les deux marqueurs aient montré des tendances similaires. Pour ces marqueurs, les niveaux étaient plus élevés aux concentrations d'AMPA les plus faibles ou moyenne testées, ce qui suggère des réponses à faible concentration et une non-monotonie (Hill et al., 2018; Shi et al., 2016; Vandenberg et al., 2012). Fait intéressant, bien que nous n'ayons pas détecté d'influence des concentrations d'AMPA sur la SOD et la GPx, nous avons constaté que les ratios pertinents de ces enzymes antioxydantes (c'est-à-dire SOD/(GPx+CAT)) étaient influencés par les traitements AMPA (Figure 29B).

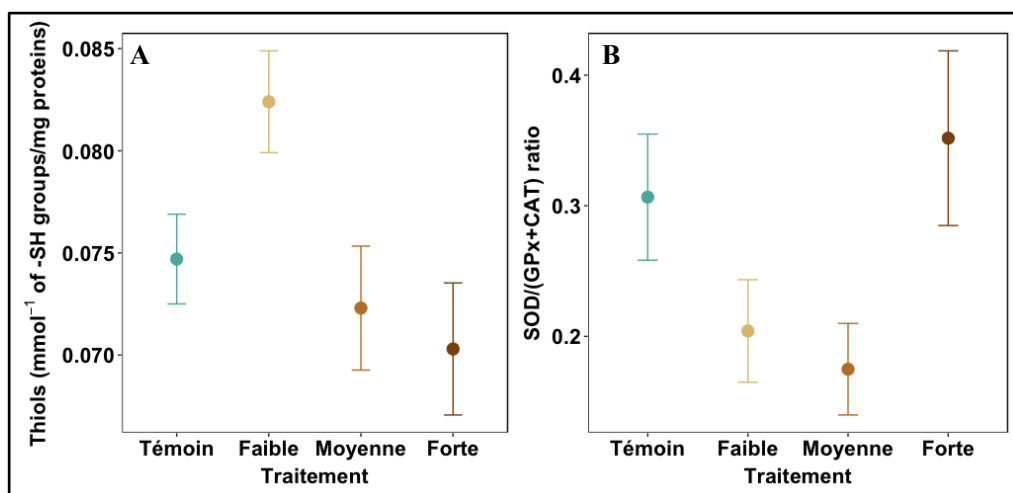


Figure 29 : Niveaux de thiols (A, mmol⁻¹ of -SH groups/mg proteins) et ratio de SOD/(CAT+GPx) (B) en fonction des différents traitements d'AMPA.

Les ratio SOD/(GPx+CAT) ont montré une réponse à l'exposition à l'AMPA et une non-monotonie, avec les valeurs les plus basses obtenues pour les concentrations d'AMPA faibles et moyennes. Ces résultats suggèrent un possible effet stimulant à faible dose de l'AMPA avec une stimulation de la catalase et des thiols.

Le stress oxydant est défini comme un déséquilibre entre pro et anti oxydant chez les organismes (Halliwell & Gutteridge, 2015). C'est un mécanisme important de toxicité des contaminants environnementaux. La présence de POP (polluants organiques persistant) dans les eaux de surface peuvent entraîner l'induction de espèces réactives de l'oxygène (ROS) et donc du stress oxydant chez

les organismes aquatiques (Lushchak, 2011). L'utilisation du stress oxydant comme biomarqueurs de l'exposition à des contaminants représente un outil puissant pour évaluer la qualité d'un environnement aquatique. Sous stress oxydant, une réponse cellulaire peut avoir lieu pour pallier aux effets délétère du stress oxydant (Lushchak, 2011).

La réponse oxydative est modérée par un facteur de transcription Nrf2 médié par une séquence (antioxydant response element, ARE). Lorsque le signal est généré, Nrf2 déclenche la transcription d'enzyme antioxydant comme la glutathione S-transferase (GST), la glutathione peroxidase (GPx), la glutathione reductase (GR), la superoxide dismutase (SOD), la catalase (CAT), la NAD(P)H oxidase, la NADPH quinone oxidoreductase (NQO-1), la glutamate-cystéine ligase (GCL) et tout le système **thiolrédoxine**. Tout ce système représente la réponse antioxydante (ARE). CAT et SOD sont les premières enzymes intervenant dans le contrôle des niveaux intracellulaires de ROS (Figure 30)(Halliwell & Gutteridge, 2015).

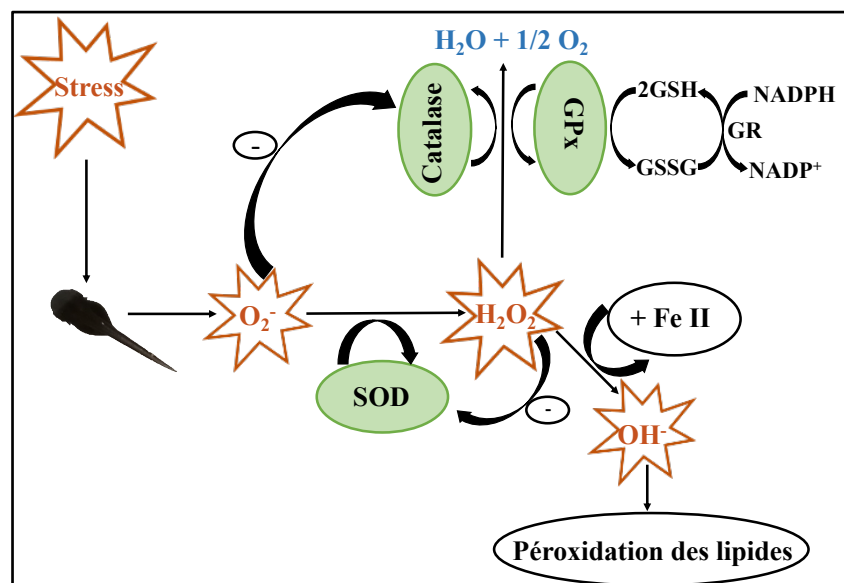


Figure 30 : Représentation des processus de détoxification et de peroxydation des lipides sous l'effet des ROS (en orange) avec l'action des enzymes antioxydantes (en vert, SOD, GPx, Catalase) à la suite d'un stress environnemental. Adapté selon Halliwell & Gutteridge, 2015.

L'activité des enzymes antioxydantes, la quantité de groupes de protéines et les marqueurs de dommages oxydatifs (dommages par les ROS) aux biomolécules (lipides, vitamine, ADN) sont souvent utilisés pour surveiller la présence de substances pro-oxydantes dans les environnements aquatiques (Trevisan et al., 2013). Les marqueurs de dommages oxydants aux biomolécules, comme la peroxydation des lipides peuvent être induits par l'environnement chez les organismes aquatiques. Les ROS sont produits à travers le métabolisme intracellulaire et ont des effets physiologiques à bases concentrations. La présence de stressseurs environnementaux peut donc entraîner un déséquilibre qui peut se répercuter au niveau de l'organisme. A contrario, si les ROS sont produits en grandes quantités

après une contamination à doses environnementales, l'oxydation peut avoir des effets importants sur l'intégrité des structures cellulaires, les protéines, l'ADN ou les lipides. Les enzymes antioxydantes agissent pour limiter cette augmentation de ROS mais elles peuvent également être inactivées sous leur effet.

(i) Une augmentation des niveaux de CAT et SOD en présence de xénobiotiques peut être interprétée comme une réponse des organismes pour survivre en présence de stress oxydatifs comme montré dans la littérature (Clasen et al., 2012).

(ii) La GPx est une enzyme catalysant la réduction de l'eau oxygénée par la déshydrogénation du glutathion. Elle neutralise l'eau oxygénée et réduit les acides gras oxydés. Le long de l'ontogénie chez les amphibiens, l'activité des enzymes glutathione décroît.

(iii) Le niveau de thiols, aussi bien en protéines (PSH) ou en composés non protéiques (NPSH) est indicateur de la capacité antioxydante de l'organisme (Reischl et al., 2007). Les thiols sont des marqueurs de stress oxydant sous la forme de structures protéiques ou non protéiques. Lors du stress oxydant ils rétablissent l'équilibre redox en éliminant les radicaux libres. En éliminant les radicaux libres ils s'oxydent et sont éliminés. Et les taux effondrés de thiols montrent un stress oxydant ancien ou chronique. Les thiols sont de bons indicateurs de la capacité antioxydante d'un organisme (Lushchak, 2011), de sorte que leur concentration diminue en cas de fortes perturbations du statut oxydant (Baba & Bhatnagar, 2018; Ulrich & Jakob, 2019).

Le stress oxydant n'est pas forcément délétère pour l'organisme car il peut jouer un rôle important dans les processus biologiques. Le système d'équilibre redox diffère selon le stade, le taxon, le sexe, le génotype ou sous l'effet de facteurs abiotiques surtout dans le niveau de protéines, enzymes générées, ou le type d'antioxydant (Halliwell & Gutteridge, 2015). Un rapport équilibré ou déséquilibré des activités des marqueurs est connu pour altérer l'intégrité des cellules (de Haan et al., 1992, 1996). Par conséquent, en plus des marqueurs seuls, nous avons choisi d'afficher le rapport entre la superoxyde dismutase et la glutathion peroxydase (SOD/GPx) et le rapport entre la superoxyde dismutase et les activités GPx et catalase (SOD/(GPx+CAT)). Un rapport déséquilibré de (SOD/(GPx+CAT)) peut conduire à une prolifération de radicaux délétères (de Haan et al., 1992) et un rapport déséquilibré de (SOD/GPx) peut conduire à une prolifération de H₂O₂ entraînant une sénescence cellulaire accélérée et une morphologie altérée (de Haan et al., 1996).

Chez les têtards, le stress oxydant joue un rôle dans la régression de la queue et la remodelisation des tissus pendant la métamorphose. Une altération de l'activité enzymatique peut donc impacter le stockage d'énergie et le développement (Menon & Rozman, 2007).

À notre connaissance, seules quelques études ont étudié la concentration pertinente de l'AMPA sur les paramètres biochimiques (de Brito Rodrigues et al., 2019; Guilherme et al., 2014; Matozzo et al., 2018, 2019) et la plupart des études ont porté sur son composé parent, le glyphosate (de Brito

Rodrigues et al., 2019). Par exemple, chez le têtard d'une autre espèce d'amphibiens (*Pelobates cultripes*), l'exposition à une concentration sublétales de glyphosate a induit une altération de l'activité antioxydante en diminuant l'activité de la glutathion réductase mais n'a pas affecté les niveaux de SOD ou de catalase (Burraco & Gomez-Mestre, 2016). Chez *Anguilla anguilla*, les formulations à base de glyphosate ont augmenté la catalase dans le foie, les reins (Lushchak et al., 2009) et dans les branchies (Guilherme et al., 2012).

Il est intéressant de noter qu'étant donné le succès d'éclosion plus faible dans les concentrations Faible et Moyenne (Figure 23), une mortalité sélective des embryons sensibles au stress oxydant peut avoir eu lieu dans ces deux groupes avant l'éclosion. Selon cette hypothèse, les embryons survivants peuvent être les individus qui sont plus résistants au stress oxydant médié par l'AMPA. Cette hypothèse concorde avec l'absence de relation entre les caractéristiques du développement et les marqueurs du statut oxydant (Article V). En effet, le développement embryonnaire peut être limité par le stress oxydant, notamment l'oxydation du groupe thiol des protéines (Goto, 1992), et un développement accéléré ou des mécanismes compensatoires peuvent améliorer le métabolisme et le stress oxydant cellulaire (Burraco et al., 2020 ; Smith et al., 2016).

(ii) Limitations des dommages ADN

Ce processus pourrait également expliquer pourquoi nous n'avons pas détecté d'effet sur les télomères (Article V) (Figure 31).

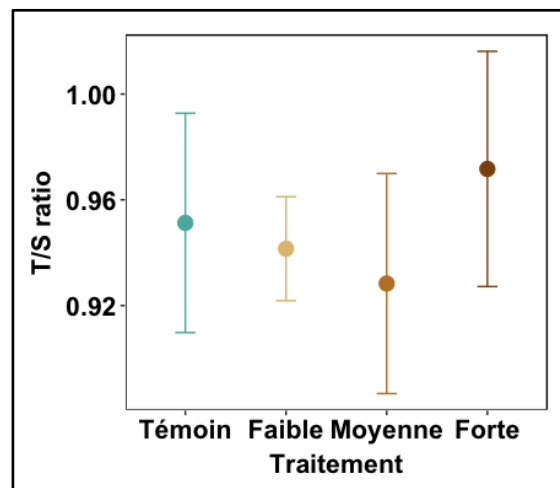


Figure 31 : Longueur des télomères (T/S ratio) en fonction des différents traitements d'AMPA.

De plus, bien que le raccourcissement des télomères se produise normalement lors de la division cellulaire, ce processus peut être amplifié dans des conditions de stress, notamment lorsqu'elles sont associées à un stress oxydant (Reichert & Stier, 2017). Ainsi, l'attrition des télomères induite par le

stress peut être liée à un stress oxydant cellulaire intense (Chatelain et al., 2020, **Figure 32**) car la guanine (le nucléotide principal des télomères) est particulièrement sensible à l'oxydation (Bjelland, 2003).

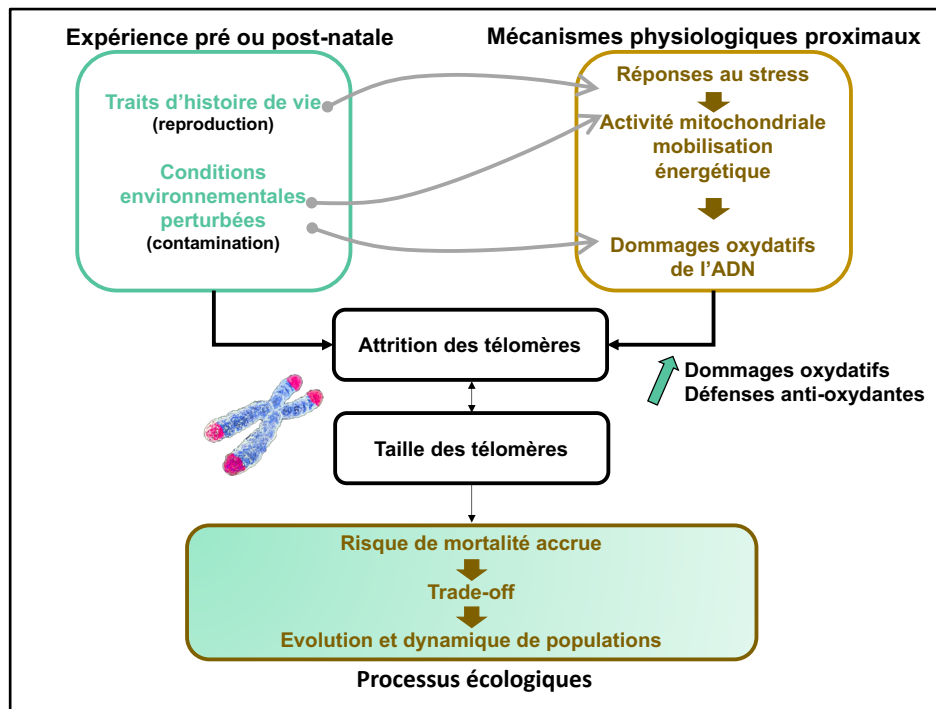


Figure 32 : Effets en cascade attendus déclenchés par l'expérience pré ou post-natale individuelle sur les processus écologiques en suivant la dynamique des télomères et les mécanismes physiologiques proximaux.

Adapté de Chatelain et al, 2020,

Ces résultat était attendu car des travaux antérieurs ont montré que l'exposition à des concentrations d'AMPA pertinentes pour l'environnement pouvait endommager l'ADN (de Brito Rodrigues et al., 2019 ; Guilherme et al., 2014). Dans notre contexte, la longueur des télomères peut être influencée par une production accrue de pro-oxydants ou une diminution des défenses antioxydantes (Chatelain et al., 2020 ; Reichert et Stier, 2017). Cependant, si les embryons survivants étaient plus aptes à faire face au stress oxydant, les dommages à leur ADN pourraient être réduits (Coluzzi et al., 2014). Étant donné que la prolifération de H_2O_2 induit la sénescence cellulaire (de Haan et al., 1996) ou l'attrition des télomères dans les fibroblastes primaires humains (Coluzzi et al., 2014), une mortalité sélective pourrait sélectionner les individus ayant la capacité de préserver la longueur de leur télomères. Le développement précoce constitue une phase où les niveaux de stress oxydant sont élevés en raison des besoins métaboliques présumés importants. Cependant, nos résultats sur l'exposition à l'AMPA suggèrent une altération du compromis entre le stress oxydant et les traits de développement, et donc la possibilité pour l'AMPA d'affecter l'expression des traits d'histoire de vie.

(iii) Augmentation des durées de développement

La durée du développement a également été influencée de manière significative par une exposition chronique à l'AMPA. En effet, l'exposition chronique à l'AMPA semble retarder l'éclosion car la durée du développement était plus longue dans tous les groupes exposés à l'AMPA, indépendamment de la concentration. Les deux concentrations inférieures d'AMPA ont produit des durées de développement similaires, tandis que la concentration la plus élevée d'AMPA a produit des durées de développement qui étaient significativement plus courtes que celles des deux concentrations inférieures d'AMPA, mais plus longues que celles des embryons témoins (**Figure 33A**).

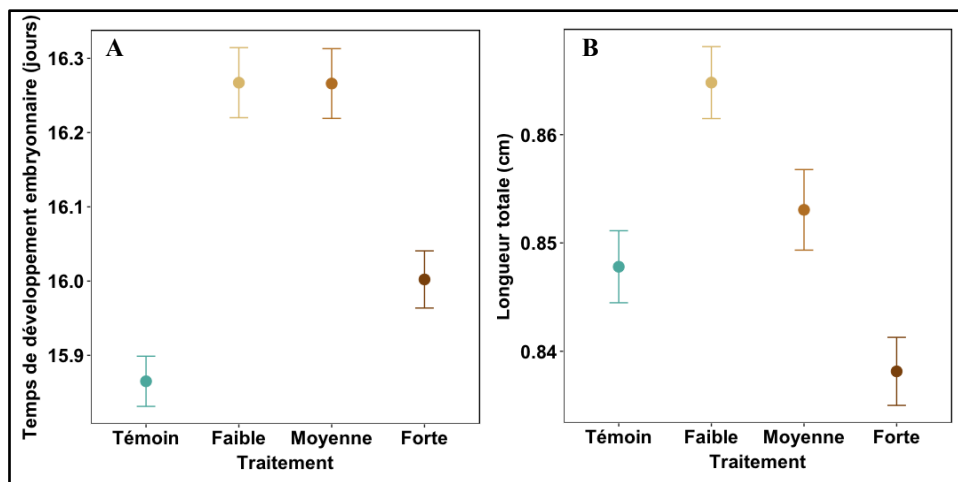


Figure 33 : Temps de développement embryonnaire (A, jours) et longueur totale des embryons (B, cm) en fonction des différents traitements d'AMPA.

Encore une fois, ce modèle semble plus complexe que les réponses classiques de concentration et les études futures devraient utilement examiner les résultats d'approches similaires avec des concentrations plus élevées d'AMPA. Nous avons trouvé que l'exposition chronique à l'AMPA influence non seulement la taille des têtards à l'éclosion, mais aussi leurs proportions corporelles (**Figure 33B**). Les embryons soumis à la plus faible concentration d'AMPA ont produit des têtards plus longs, avec une longueur de corps similaire à celle des autres groupes mais des queues plus longues (**Article IV**). Inversement, les embryons soumis à la plus forte concentration d'AMPA ont produit des têtards plus courts, avec des queues plus courtes. De la même manière que les paramètres précédents du développement embryonnaire que nous avons étudié, ces modèles de réponse de la morphologie contrastent avec les réponses classiques de concentration, et semblent indiquer un effet concentration-dépendant de l'AMPA sur l'architecture du corps. Une telle réponse mérite clairement des investigations futures afin de clarifier les mécanismes par lesquels l'exposition à l'AMPA pendant le développement embryonnaire influence la morphologie globale. Si l'altération du développement embryonnaire que nous avons mis en évidence a des conséquences sur le développement des têtards

dans les étangs naturels, cela reste compliqué à évaluer directement avec notre conception expérimentale. Une éclosion retardée pourrait potentiellement augmenter la vulnérabilité des embryons immobiles aux prédateurs (Zamudio et al., 2016). En outre, le retard du développement embryonnaire peut également induire des effets en cascade sur la durée du développement ultérieur des larves (têtards), une conséquence potentiellement délétère si l'éclosion a lieu dans des plans d'eau éphémères.

Enfin, une taille corporelle réduite peut diminuer la mobilité et donc la capacité de recherche de nourriture tout en augmentant la susceptibilité à la prédation (Broomhall & Shine, 2003; Hoff & Wassersug, 2000). Bien que, pris ensemble, tous ces éléments tendent à suggérer des conséquences écologiques pour les embryons (et peut-être les têtards) se développant dans l'eau contaminée par des concentrations environnementales d'AMPA, ces hypothèses doivent être prises avec prudence et nécessiteront des tests approfondis, en particulier parce que la survie post-embryonnaire peut être plus importante pour la persistance de la population que la survie des œufs (Vonesh & De la Cruz, 2002).

En outre, il est important de souligner le fait que la magnitude absolue des effets que nous avons trouvés semble relativement faible. En effet, bien que la mortalité embryonnaire ait été multipliée par deux ou trois dans certaines de nos concentrations expérimentales, la durée du développement a augmenté de ~2,5 % et la taille du corps a été soit augmentée de ~2,1 %, soit diminuée de ~1,2 %. Bien que nous ne puissions pas entièrement exclure que l'importance écologique de ces effets soit insignifiante, ils pourraient également avoir des conséquences sur les traits liés à la fitness tels que le succès reproductif des parents (mortalité embryonnaire) et la survie de la descendance (durée de développement, morphologie du têtard) et pourraient donc finalement affecter la persistance des populations (Hayes et al., 2010; Orton & Tyler, 2015). De tels résultats sont clairement importants à prendre en compte à une époque où les amphibiens sont en fort déclin (Berger et al., 2013; Grant et al., 2016; Hayes et al., 2010; Wake, 2012). De manière importante, nos résultats contrastent avec ceux trouvés avec le composé parent sur des espèces similaires (Baier et al., 2016a,b) suggérant que l'AMPA peut être au moins aussi toxique que le glyphosate ou sa formulation contenant des surfactants (Giesy et al., 2000 ; Relyea, 2009 ; Moore et al., 2012). Pourtant, nous manquons clairement d'études concernant sa toxicité à des concentrations écologiquement pertinentes chez les espèces non ciblées (Matozzo et al., 2018, 2019). En se concentrant sur le métabolite primaire du glyphosate, notre étude renforce le potentiel de cet herbicide largement utilisé et de son métabolite AMPA (les principaux contaminants détectés dans les eaux de surface dans le monde, Battaglin et al., 2014; Medalie et al., 2020; Okada et al., 2020; Silva et al., 2018) à altérer le statut oxydant de la faune sauvage.

b) Nicosulfuron et effet tératogènes

Cette partie résume l'article VI portant sur les effets du nicosulfuron sur le développement embryonnaire de *bufo spinosus*.

Dans l'ensemble, nous avons constaté que des concentrations de nicosulfuron pertinentes pour l'environnement modifiaient le développement embryonnaire de *Bufo spinosus*. Plus particulièrement, l'exposition au nicosulfuron a ralenti le développement embryonnaire et a influencé la morphologie des embryons éclos (l'article VI). En outre, l'exposition au nicosulfuron a entraîné une diminution des niveaux de thiols et une augmentation marginale de l'activité de la catalase. Il est intéressant de noter que des niveaux plus faibles de thiols étaient liés à une mortalité embryonnaire plus élevée, à une proportion plus importante d'individus déformés et à une durée de développement embryonnaire plus faible ; et une activité catalase plus élevée était également liée à une proportion plus importante d'individus déformés, ce qui suggère que les effets de l'exposition au nicosulfuron ont induit des effets tératogènes.

(i) Effets développementaux

La plus forte concentration de nicosulfuron que nous avons testée ($0,83\mu\text{g}\cdot\text{l}^{-1}$) est très similaire aux concentrations trouvées dans les eaux de surface (Cessna et al., 2015). Bien que sublétale, cette concentration a influencé le développement embryonnaire de *Bufo spinosus*, ce qui est illustré par des durées de développement accrues et une taille corporelle plus importante (Figure 34).

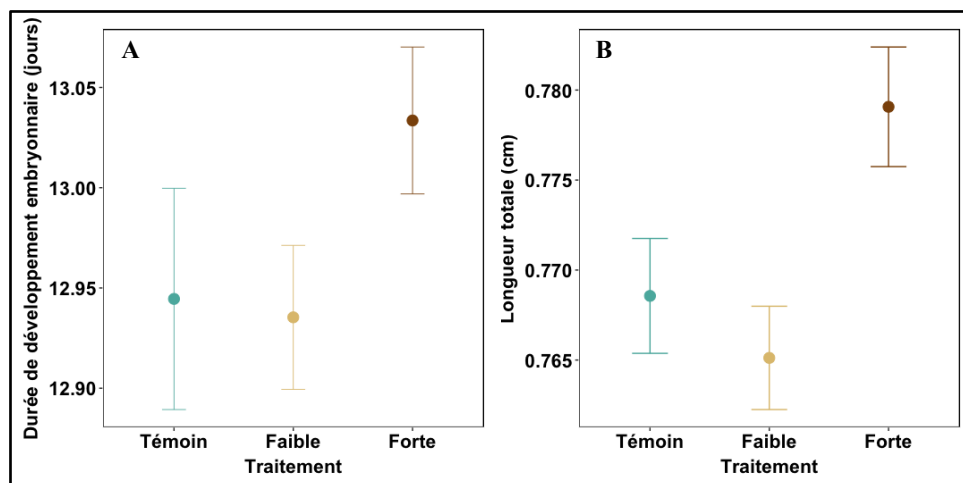


Figure 34 : Temps de développement embryonnaire (A, jours) et longueur totale des embryons (B, cm) en fonction des différents traitements de nicosulfuron.

Il est intéressant de noter que le modèle de covariation entre ces deux traits était attendu étant donné la relation généralisée entre la durée du développement embryonnaire et la taille du corps (Gillooly et Dodson, 2000). Il est compliqué d'évaluer si ces réponses peuvent avoir des conséquences pour les têtards se développant dans des conditions naturelles.

Une éclosion retardée peut potentiellement accroître la vulnérabilité des embryons immobiles aux prédateurs (Zamudio et al., 2016) ou augmenter la vulnérabilité des têtards à la dessiccation si la ponte a lieu dans des plans d'eau temporaires (Lindgren et al., 2018; Székely et al., 2017). À l'inverse, l'augmentation de la taille du corps peut améliorer la mobilité et donc la capacité de recherche de nourriture tout en diminuant la susceptibilité à la prédation (Broomhall et Shine, 2003 ; Hoff et Wassersug, 2000). Étonnamment, ces effets étaient détectables pour notre traitement le plus haut, ce qui suggère uniquement que la concentration inférieure que nous avons testée n'a pas déclenché d'effets visibles sur le développement embryonnaire.

(ii) Perturbation du statut oxydant

Quelle que soit la concentration testée, le nicosulfuron a influencé les niveaux de thiols avec des niveaux plus faibles - donc une oxydation plus élevée des thiols - chez les individus exposés pendant leur développement embryonnaire (Figure 35). En conséquence, nos résultats suggèrent que l'exposition au nicosulfuron a modifié le statut oxydant. Nous avons également constaté une tendance à l'augmentation de la catalase.

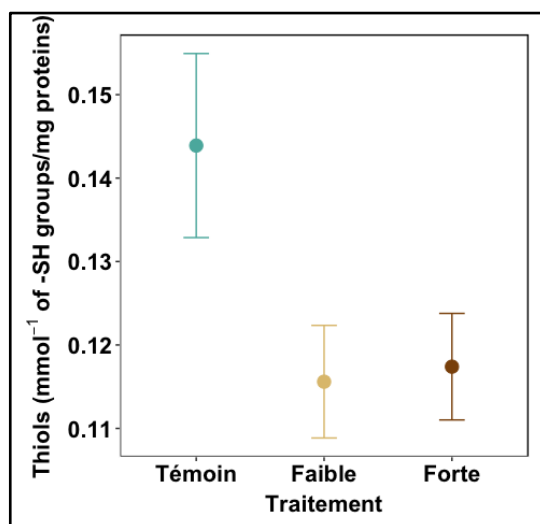


Figure 35 : Niveaux de thiols (mmol^{-1} of -SH groups/mg proteins) en fonction des différents traitements de nicosulfuron.

En accord avec les résultats sur la durée du développement (voir ci-dessus), il convient de noter qu'un développement rapide peut augmenter les dommages oxydants, avec de possibles effets durables (Janssens & Stoks, 2018). Dans ce contexte, le ralentissement du développement embryonnaire, tel qu'observé chez les individus exposés au traitement le plus élevé de nicosulfuron pourrait constituer une stratégie pour éviter une augmentation disproportionnée des dommages oxydatifs liés à la fois à l'exposition au nicosulfuron et au développement rapide.

(iii) Effets tératogènes

Enfin, nous avons constaté que les caractéristiques liées au développement (c'est-à-dire la durée du développement embryonnaire, le nombre d'individus déformés, le succès d'éclosion) sont liées à certains marqueurs du statut oxydatif (SOD, thiols et catalase). La relation négative entre la durée du développement embryonnaire et la SOD ou les thiols est cohérente avec d'autres études qui ont suggéré que le métabolisme accru lié au développement accéléré peut augmenter le stress oxydant cellulaire (Burraco et al., 2020 ; Smith et al., 2016). Les niveaux de thiol étaient négativement liés à la proportion d'éclosions déformées, et l'activité catalase était positivement liée à une proportion plus élevée d'individus déformés (Figure 36).

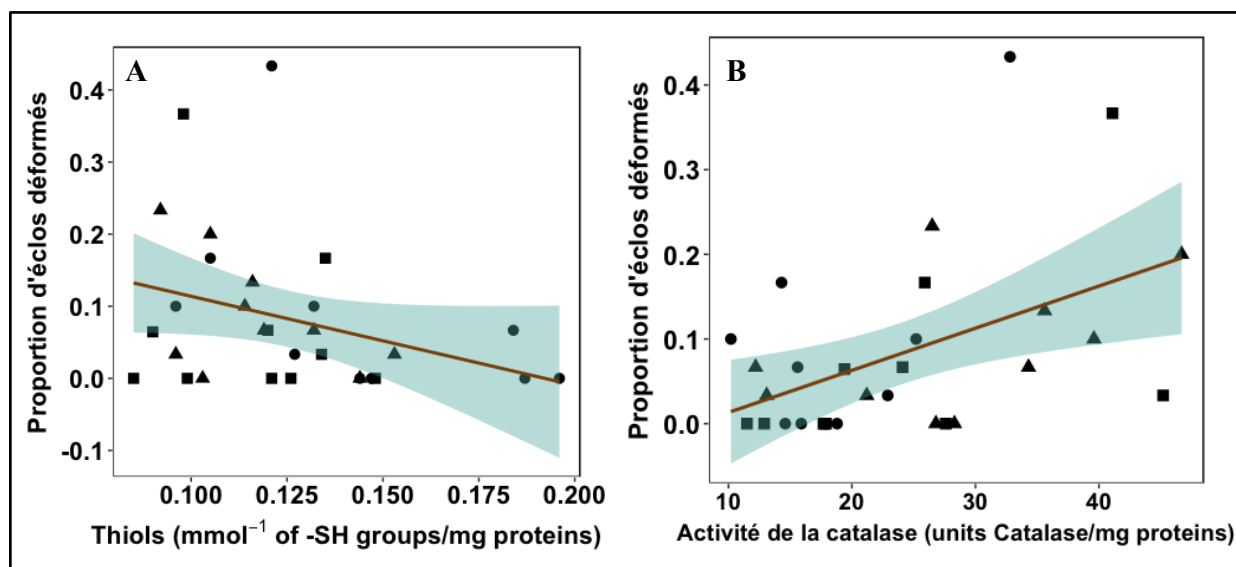


Figure 36 : Proportion d'embryons éclos déformés en fonction des niveaux de thiols (A, mmol⁻¹ of -SH groups/mg proteins) et de l'activité de la catalase (B, units catalase/mg proteins) en fonction des différents traitements de nicosulfuron (• Témoin, ■ Faible, ▲ Forte)

Ces résultats suggèrent fortement que la perturbation du statut oxydant pourrait être liée aux effets tératogènes (Wu et al., 2017; Xie et al., 2016). En effet, l'apparition de malformations est connue pour

être liée au stress oxydant induit par les pesticides (Liendro et al., 2015; Rutkoski et al., 2020; Wells et al., 2005), ce qui suggère que, dans notre étude, ces effets tératogènes pourraient être liés au déséquilibre oxydant induit par le nicosulfuron (**Figure 37**).

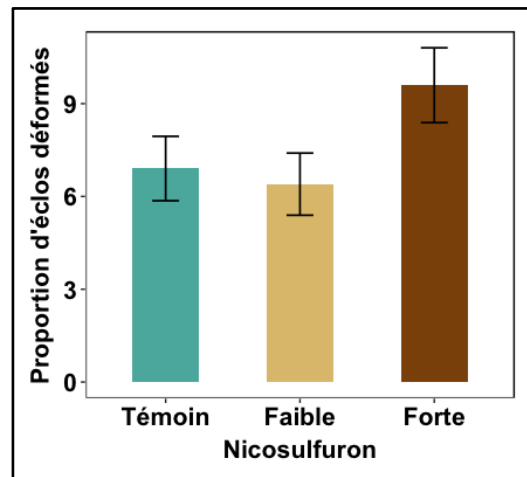


Figure 37 : Proportion d'embryons éclos déformés en fonction des différents traitements de nicosulfuron. Les données sont représentées à titre informatif et ne reflètent pas de différence significative.

L'apparition de malformations physiques chez les têtards d'anoues est fréquente dans les habitats agricoles (Lajmanovich et al., 2003; Mann et al., 2009; Taylor et al., 2005), et il a également été démontré que ces malformations sont préjudiciables à la mobilité et aux performances (Sotomayor et al., 2012), à la croissance et à la survie (Herek et al., 2020; Ruiz et al., 2010; Schuyttema & Nebeker, 1998). De plus, nous avons trouvé une tendance à induire des effets tératogènes du nicosulfuron (**Figure 37**). L'exposition au glyphosate et au Roundup est connue pour induire des malformations sur les poussins d'oiseaux (Paganelli et al., 2010). Jayawardena et al. (2016) ont trouvé près de 60% de malformations chez les têtards de grenouille arboricole *Polypedates cruciger* traités avec une concentration environnementale de Roundup. Les effets comprenaient cyphoses, scolioses et œdèmes. Dans les populations naturelles de grenouilles, les individus morphologiquement malformés constituent généralement une petite fraction de moins de 2% (Ouellet 2000). Cependant, une incidence beaucoup plus élevée (jusqu'à 60 %) de spécimens malformés a été documentée dans les étangs contaminés (Meteyer 2000).

III) Conclusion

Dans nos études impliquant différents contaminants (AMPA ou nicosulfuron), on a pu montrer que le stade embryonnaire constituait un stade sensible à la contamination environnementale chez les

amphibiens. Les propriétés chimiques de ces deux contaminants pouvant passer outre les couches de la gangue protégeant les embryons. Ces effets allant de la perturbation de la balance du statuts oxydant, d'altération de traits de développement et effet tératogènes (Figure 38). Chose intéressante, pour les deux herbicides, l'augmentation du stress oxydant a entraîné une stratégie d'augmentation de la durée de développement chez les embryons. L'accélération ou le ralentissement du développement ou de la croissance peut avoir des conséquences importantes chez de nombreux groupes taxonomiques allant de changements dans le turnover de protéines aux changements de trajectoires allométriques (Gomez-Mestre et al., 2010; Kulkarni et al., 2011). De plus, les deux herbicides ne montrent pas la même relation concentration-effet, l'AMPA montrant une non-monotonie et le nicosulfuron une relation linéaire classique.

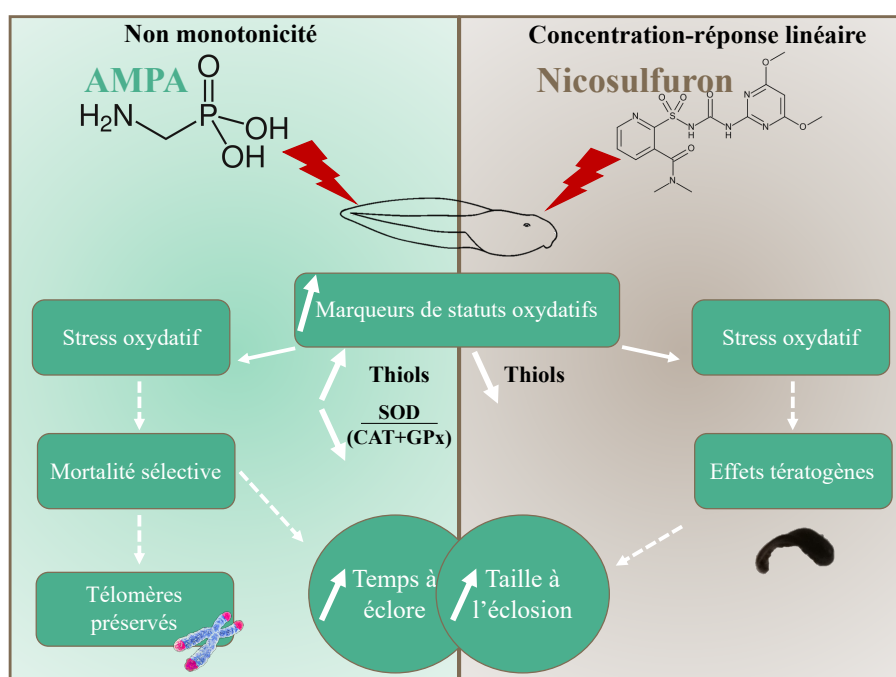


Figure 38 : Schéma récapitulatif des effets de deux herbicides sur le développement embryonnaire du crapaud épineux (*Bufo spinosus*).

Chapitre III

Effets de la contamination environnementale sur le développement larvaire



Collecte d'une « *bufonite* », illustré dans *Hortus sanitatis*, publiée dans Mainz en 1491.

“Sweet are the uses of adversity which, like the toad, ugly and venomous, wears yet a precious jewel in his head”. **William Shakespeare** - *Comme il vous plaira* - 1599

Ce chapitre résume les **articles VII, VIII et IX** portant sur les effets de l'AMPA et du nicosulfuron sur le développement post-embryonnaire et la métamorphose chez *Bufo spinosus*. Pour plus d'informations, voir les articles en fin de manuscrit.

D) Contexte

A) Développement post-éclosion sensible à la contamination environnementale chez les anoues

Les premiers stades larvaires des amphibiens sont généralement considérés comme le stade de vie le plus sensible lors de l'évaluation des effets de la pollution environnementale (Anguiano et al., 1994 ; Berrill et al., 1998 ; Greulich et Pflugmacher, 2003 ; Ortiz-Santaliestra et al., 2006). Le développement des tissus et des organes commence à la fécondation et se poursuit jusqu'à la métamorphose ; cette différenciation incomplète des organes et des tissus pourrait rendre les premiers stades larvaires plus sensibles à la contamination chimique (Egea-Serrano et al., 2012). Les différences de sensibilité des stades larvaires peuvent également être dues à des changements dans les rapports surface/volume, la perméabilité de la peau et le métabolisme (Ralph et Petras, 1998). Cependant, dans une méta-analyse aucune différence de sensibilité aux polluants entre les embryons et les larves n'a été trouvée (Egea-Serrano et al., 2012), les auteurs déclarant que cela pouvait être dû à un manque de « carry-over effect ». En effet, il est prouvé qu'une exposition plus précoce aux polluants au stade embryonnaire du développement entraîne des effets néfastes, tels que la différenciation sexuelle et des déformations qui peuvent n'apparaître qu'au moment de la métamorphose (Bridges, 2000 ; Orton et Routledge, 2011 ; Orton et Tyler, 2014).

Les stades larvaires plus tardifs des têtards, c'est-à-dire les têtards pro-métamorphiques avec des pattes arrière émergées, peuvent être plus tolérants aux polluants que les stades antérieurs. Par exemple, les têtards de *Rhinella arenarum* étaient plus sensibles à l'exposition à l'atrazine au stade Gosner 25 par rapport à ceux exposés au stade 38-39 (Brodeur et al., 2009). Cette sensibilité différentielle peut être due à une plus grande capacité de détoxification métabolique chez les têtards à un stade de développement plus avancé (Bucciarelli et al., 1999). Cependant, certaines études démontrent une sensibilité accrue des stades larvaires plus tardifs par rapport aux stades larvaires plus précoces après exposition à certains contaminants. Par exemple, Howe et al. (1998) ont exposé des larves de *Rana pipiens* et *Bufo americanus* au début (stade Gosner 29) et à la fin (stade Gosner 40) de leur développement aux herbicides alachlore et à l'insecticide atrazine, et ont constaté que les deux espèces étaient plus sensibles aux deux produits chimiques lorsqu'elles étaient exposées au dernier stade de leur développement. Il a été suggéré que cela était dû aux changements complexes qui se produisent à ce stade, le têtard entrant en métamorphose, ce qui ajoute un stress physiologique et développemental supplémentaire (Howe et al., 1998). Une étude récente a démontré une sensibilité différentielle chez

les larves de *Hyla intermedia* exposées aux fongicides tébuconazole et pyriméthanil tout au long de la période de développement, les faibles concentrations (5 µg/L) étant associées à des effets plus importants sur la survie et les incidences de déformation que les fortes concentrations (50 µg/L) si l'exposition avait lieu avant la métamorphose. Pendant la métamorphose, il y avait une relation concentration-réponse claire (Bernabò et al., 2016). Par conséquent, il est probable qu'il y ait une sensibilité différentielle entre les stades de vie ainsi qu'entre les espèces, en fonction des molécules auxquelles les larves d'amphibiens sont exposées.

B) L'utilisation du comportement pour montrer les effets de la contamination

De nombreuses espèces répondent aux facteurs de stress environnementaux en modifiant leur comportement (Tuomainen et Candolin 2011, Wong et Candolin 2015). Ces changements comportementaux, intentionnels ou non, jouent un rôle central dans la détermination de la capacité des animaux à faire face à un environnement changeant. Cela s'explique par le fait que de nombreux comportements influencent directement les indicateurs de fitness tels que la survie, la croissance et la reproduction, et parce que les altérations comportementales ont tendance à se manifester en réponse à des niveaux plus faibles de changement environnemental que ces indicateurs de fitness (Zala et Penn 2004, Melvin et Wilson 2018). Ainsi, les réponses comportementales sont de plus en plus utilisées pour évaluer les impacts des facteurs de stress environnementaux, y compris la contamination.

Les pesticides peuvent perturber le comportement de vertébrés aquatiques en agissant sur la vitesse de nage, le temps d'activité (dans la recherche de nourriture par exemple) ou la personnalité (Ford et al., 2021). Les différentes familles chimiques induisent des réponses variées ce qui reflète probablement les processus physiologiques sous-jacents. Shuman-Goodier et al. (2016), ont étudié une grande partie des classes de pesticides et leurs effets sublétaux sur le comportement. Les pyréthroïdes, les carbamates et les organophosphates ont tous produit une forte diminution de la vitesse de nage, alors que les phosphonoglycines et les triazines n'ont montré aucun effet global.

Les changements de comportement, y compris la vitesse de nage et le niveau d'activité, peuvent être directement liés à la survie, la croissance et la reproduction des vertébrés aquatiques. Par exemple, une espèce de cyprinidé exposés à un insecticide pyréthroïde, ont montré des niveaux d'activité réduits, ce qui a entraîné une augmentation de la prédation et une réduction des taux de croissance (Floyd et al., 2008). De même, les réductions comportementales de la vitesse de nage et de l'activité chez les saumons juvéniles exposés aux pesticides organophosphorés et carbamates ont été liées à une réduction de la croissance, de l'alimentation et de la taille lors de la migration (Baldwin et al., 2009). Relyea et Edwards (2010) ont constaté que la réduction des niveaux d'activité des têtards d'anouère suite à une exposition sublétaux aux pesticides réduisait les taux de prédation par les prédateurs des tritons. Bridges (1997) a rapporté un compromis où les têtards avec des niveaux d'activité réduits ont connu des taux de prédation plus faibles par les tritons, mais aussi une croissance réduite et un développement retardé. La direction de ces effets interactifs n'est pas toujours intuitive et reflète le

besoin urgent d'intégrer la complexité écologique dans les études toxicologiques (Köhler et Triebkorn, 2013).

Les changements de comportement produisent également des effets indirects sur l'ensemble de la structure de la communauté en modifiant les interactions entre espèces (Boone et Semlitsch, 2002 ; Fleeger et al., 2003 ; Söffker et Tyler, 2012).

Cependant, les manières dont les contaminants environnementaux communs influencent le comportement des amphibiens n'ont pas été systématiquement évaluées. En effet, de plus en plus d'études démontrent que les contaminants influencent le comportement des amphibiens (Shuman-Goodier et Propper 2016) et les auteurs mesurent de nombreux paramètres comportementaux pour comprendre ces effets. Certains des paramètres comportementaux les plus courants mesurés chez les larves d'amphibiens sont la nage erratique, la nage en surface, l'alimentation et les niveaux d'activité générale, l'alimentation et le niveau d'activité général, bien qu'une série d'autres paramètres aient été utilisés (Moore et al. 2015, Miko et al. 2017, Sievers et al. 2018). Par exemple, les contaminants tels que le cuivre augmentent le temps que les têtards de grenouille des bois (*Lithobates sylveticus*) passent à nager à la surface de l'eau (Hayden et al. 2015), l'herbicide glyphosate diminue l'activité et augmente la dissimulation chez les têtards de grenouilles agiles (*Rana dalmatina*) (Miko et al. 2017), et les contaminants dans les zones humides d'eaux pluviales réduisent la capacité des têtards de grenouille (*Limnodynastes tasmaniensis*) à détecter les signaux olfactifs et donc interfèrent avec le comportement anti-prédateur normal (Sievers et al. 2018).

II) Résultats et discussion

A) Description du comportement à travers l'ontogénie chez *Bufo spinosus*

L'article VII nous a permis de décrire précisément le comportement selon l'ontogénie et d'identifier des stades de développement clefs. Nous avons montré que les niveaux d'activité, la locomotion et le comportement subissent des changements massifs au cours du développement du têtard. Ces changements ne sont pas linéaires, mais plutôt curvilignes au cours du développement, avec une phase d'activité croissante suivie d'un plateau et/ou d'une réduction des valeurs de ces paramètres. Ces deux phases différentes coïncident relativement bien avec les changements morphologiques associés à la métamorphose (c'est-à-dire une phase de croissance intense suivie de changements morphologiques liés à la métamorphose, Figure 39; Brown & Cai, 2007).

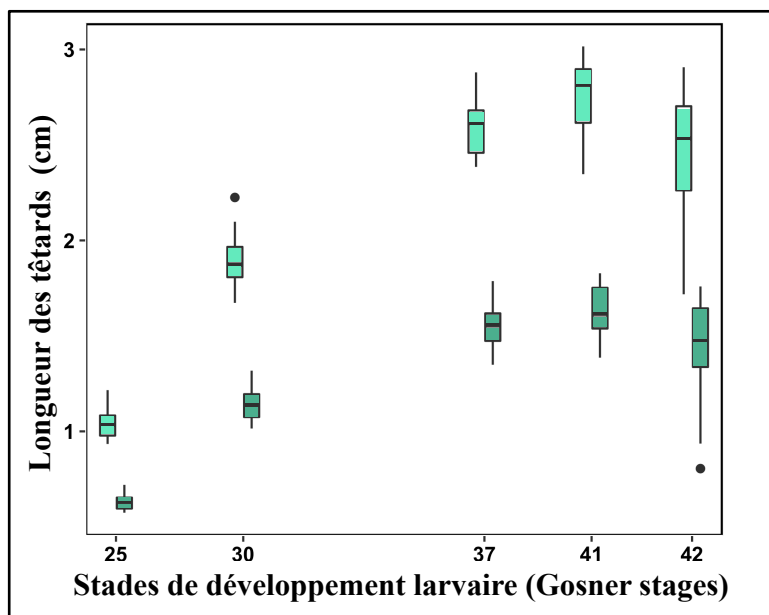


Figure 39 : Longueur du corps (bleu clair) et longueur de la queue (bleu foncé) le long de l'ontogénie chez les têtards de *Bufo spinosus*. Les intervalles entre les stades de développement de Gosner ont été calculés en utilisant le nombre moyen de jours entre deux stades de Gosner pour tous les individus.

a) Locomotion et activité

Les deux phases observées du développement (croissance somatique et initiation de la métamorphose) du têtard ont fortement influencé toutes les métriques que nous avons étudiées. Les valeurs les plus élevées des niveaux d'activité, de la distance parcourue, des vitesses de nage ont été atteintes entre GS 30 et GS 37 (**Figure 40**), qui représentent les derniers stades de développement au début des changements les plus intenses liés à la métamorphose.

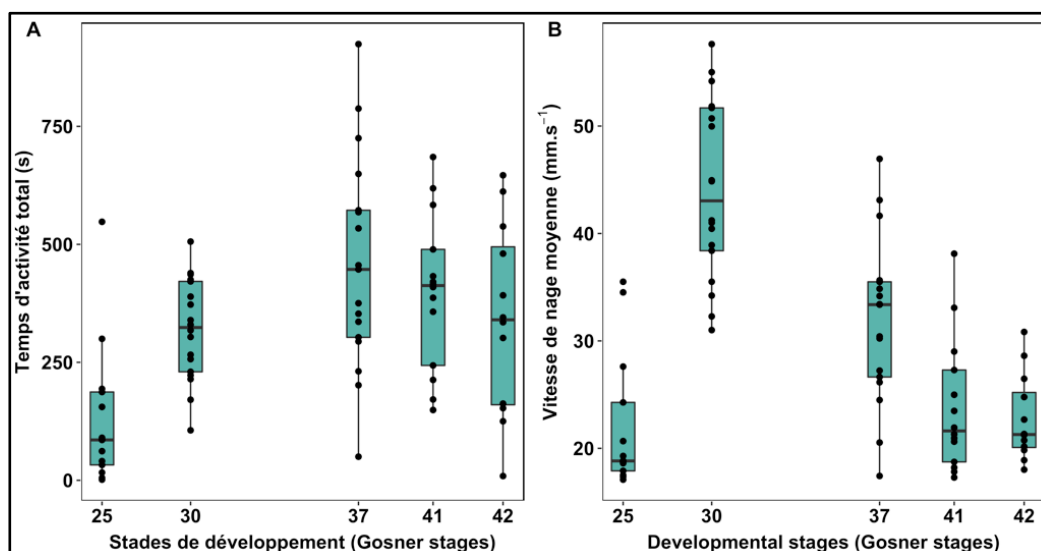


Figure 40 : Temps d'activité total (*s*, *A*) et vitesse de nage moyenne ($\text{mm}\cdot\text{s}^{-1}$, *B*) le long de l'ontogénie chez les têtards de *Bufo spinosus*. Les intervalles entre les stades de développement de Gosner ont été calculés en utilisant le nombre moyen de jours entre deux stades de Gosner pour tous les individus.

Nos résultats suggèrent que, afin de soutenir la croissance intensive qui se produit entre GS 25 et GS 37, les têtards ont augmenté leurs niveaux d'activité et leur mobilité, probablement pour optimiser la recherche de nourriture tout en évitant la prédation (Van Buskirk & Saxer, 2001). En retour, une plus grande longueur du corps et de la queue est susceptible d'améliorer la locomotion (Wilson & Franklin, 2000 ; Wilson et al., 2005 ; Johnson et al., 2015 ; Lindgren et al., 2018).

Au cours des stades ultérieurs du développement (GS 41), la longueur totale des têtards est restée à peu près constante (Figure 39), tandis que les traits liés à la locomotion ont diminué de manière significative (Figure 40).

Ces stades sont caractérisés par de forts changements morphologiques liés à la métamorphose, avec la fin de la croissance des membres postérieurs (GS 41), l'émergence des membres antérieurs de la cavité corporelle et le début de l'atrophie de la queue (GS 42). Il est intéressant de noter qu'une étude théorique antérieure a suggéré que la forme des têtards, ainsi que les grandes oscillations latérales, compensent la réduction de l'hydrodynamisme (augmentation de la traînée) liée à la présence des membres postérieurs (Liu et al., 1996). En conséquence, il a été suggéré que les membres postérieurs restent attachés au côté du corps pour améliorer l'hydrodynamisme (Stehouwer & Farel, 1984). En revanche, nos résultats ont montré que la locomotion était fortement réduite à GS 41, lorsque les membres postérieurs sont pleinement développés mais probablement pas encore utiles et/ou utilisés pour la propulsion (Wilson & Franklin, 2000).

Ces membres pourraient avoir une influence négative sur l'hydrodynamisme et/ou empêcher des mouvements ondulatoires efficaces du corps et de la queue (par exemple, la présence de membres postérieurs des deux côtés de la base de la queue pourrait empêcher les ondulations de celle-ci).

En conséquence, il a été démontré que la présence de membres postérieurs influence les changements posturaux et directionnels et l'évitement de la prédation (Stehouwer & Farel, 1984 ; Brown & Taylor, 1995). Le GS 41 est également caractérisé par la croissance des membres antérieurs qui se produisent dans les chambres atriales où se trouvent les branchies (Wells, 2007). Cependant, une activité comparativement plus faible a également été observée à GS 42, lorsque les membres postérieurs et antérieurs sont présents et que l'atrophie de la queue commence, ce qui semble limiter davantage la locomotion aquatique des têtards (Calsbeek & Kuchta, 2011).

Alternativement, nous pouvons émettre l'hypothèse qu'après GS 37 l'activité n'est plus dédiée à la croissance aiguë (comme le suggère le plateau de la taille corporelle), mais plutôt vers le maintien de l'organisme et la métamorphose. A cet égard, en diminuant les coûts de la locomotion (baisse des niveaux d'activité et des vitesses de nage), les têtards consacrent plus d'énergie à la métamorphose (Beck & Congdon, 2003; Ruztzwath et al., 2020). En outre, d'autres changements majeurs liés à la

métamorphose devraient également influencer négativement la locomotion ; par exemple, le passage de l'herbivorie à la détritivorie et la carnivorie, est caractérisée par des modifications anatomiques importantes du système digestif et de la bouche (Vitt & Caldwell, 2014). Le disque oral disparaît, et les altérations de l'épithélium intestinal empêchent une absorption efficace des nutriments (Hourdry et al., 1996). Cela pourrait empêcher les têtards à des stades de développement ultérieurs de rechercher efficacement de la nourriture (par exemple, incapacité à acquérir et/ou digérer la nourriture), limitant ainsi la nécessité d'afficher des performances locomotrices bien développées. En conséquence, il a été suggéré que pendant les derniers stades de développement, les têtards deviennent aphagiques et dépendent, en partie, de la résorption de la queue pour alimenter les demandes énergétiques (Gonçalves et al., 2015 ; Bouchard et al., 2016 ; Ruthsatz et al., 2018).

b) Complexité comportement : l'analyse des séries chronologiques (DFA)

Chez les têtards, les valeurs les plus élevées des niveaux d'activité, de la distance parcourue, des vitesses de nage ont été atteintes entre GS 30 et GS 37, qui représentent les derniers stades de développement au début des changements les plus intenses liés à la métamorphose (Figure 40). Des changements similaires dans l'indice de complexité comportementale confirment ces hypothèses (Figure 41) : l' α DFA était nettement plus faible aux stades GS 30 et GS 37, indiquant des niveaux plus élevés de complexité comportementale (stochasticité) à ces stades (MacIntosh, 2014).

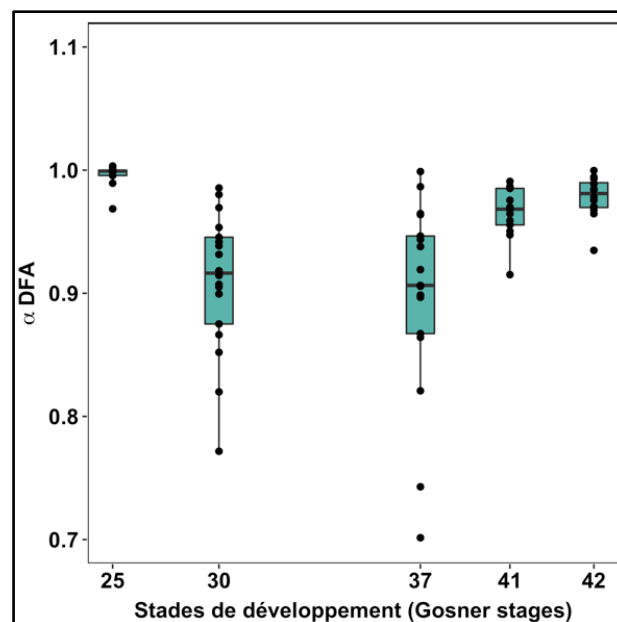


Figure 41 : Score de DFA (sans unité) le long de l'ontogénie chez les têtards de *Bufo spinosus*. Les intervalles entre les stades de développement de Gosner ont été calculés en utilisant le nombre moyen de jours entre deux stades de Gosner pour tous les individus.

En conclusion, notre étude montre que l'ontogénie du têtard est caractérisée par de fortes variations de la locomotion et du comportement en fonction de deux phases de développement différentes (par ex., croissance somatique vs. changements morphologiques liés à la métamorphose).

Toutes les mesures que nous avons étudiées indiquent que le pic d'activité et le comportement associé se situent à un moment charnière où la croissance somatique diminue et où des changements morphologiques significatifs se produisent (i.e. croissance des membres postérieurs). De plus, l'utilisation d'outil tel que l'analyse de série chronologique (DFA) peut nous permettre de déterminer si l'organisation temporelle de séquences comportementales des têtards de *Bufo spinosus* diffère sous l'effet de contamination environnementale (AMPA ou nicosulfuron).

B) Effets des deux contaminants sur le développement larvaire de *Bufo spinosus*

a) Développement post-éclosion et AMPA

Nos résultats suggèrent que de faibles concentrations d'AMPA pertinentes pour l'environnement influencent le développement embryonnaire et larvaire des têtards de *Bufo spinosus* (Article VII). La plupart des effets sur la morphologie des têtards ont été liés à l'exposition pendant le développement embryonnaire, conformément aux résultats d'une étude précédente (Article IV, V). Pourtant, cette étude montre que ces effets chez les individus exposés pendant le développement embryonnaire se produisaient encore jusqu'aux derniers stades larvaires, au début de la métamorphose, chez les individus qui ont été élevés dans des conditions témoins après l'éclosion. Enfin, nous avons trouvé de forts effets de l'exposition à l'AMPA sur la survie (Figure 42). La proportion de têtards morts pendant l'expérience est plus importante chez les têtards exposés seulement pendant le stade larvaire et cet effet semble plus fort chez les têtards exposés à des concentrations importantes. De plus, cet effet sur la survie semble continuer en période post-métamorphose avec une survie moins importante chez les individus exposés en période larvaire seulement à des concentrations fortes (bleu foncé sur la figure 42).

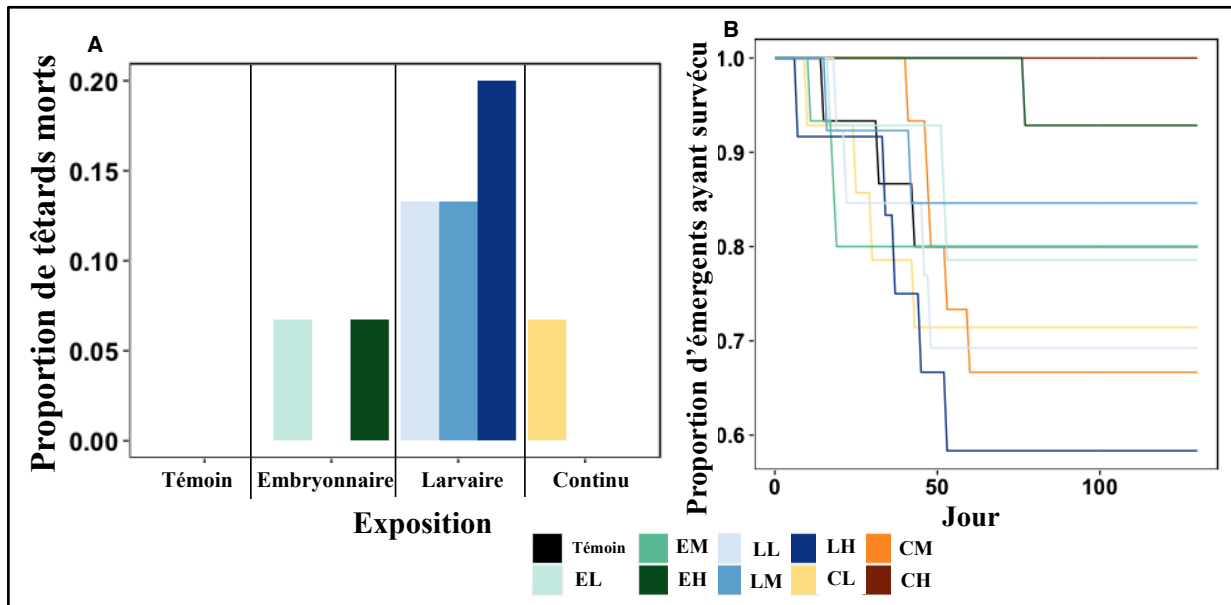


Figure 42 : Proportion de têtards morts pendant le stade larvaire en fonction des traitements (A), Courbe de survie des émergents jusqu'à la fin de l'expérimentation (B, J+130 post métamorphose). Les couleurs représentent les traitements. **EL** : Embryonnaire-Faible, **EM** : Embryonnaire-Médium, **EH** : Embryonnaire-Forte, **LL** : Larvaire-Faible, **LM** : Larvaire-Médium, **LH** : Larvaire-Forte, **CL** : Continu-Faible, **CM** : Continu-Médium, **CH** : Continu-Forte.

(i) Exposition au stade embryonnaire

Pour les têtards exposés seulement au stade embryonnaire, on remarque que les têtards exposés ne présentent pas de différence de taille au stade GS25 mais une augmentation au stade GS42 pour les têtards à la concentration la plus forte (**Figure 43**). Ces résultats soulèvent des questions importantes concernant les effets durables possibles d'une exposition précoce et temporaire à la contamination chez les vertébrés en développement.

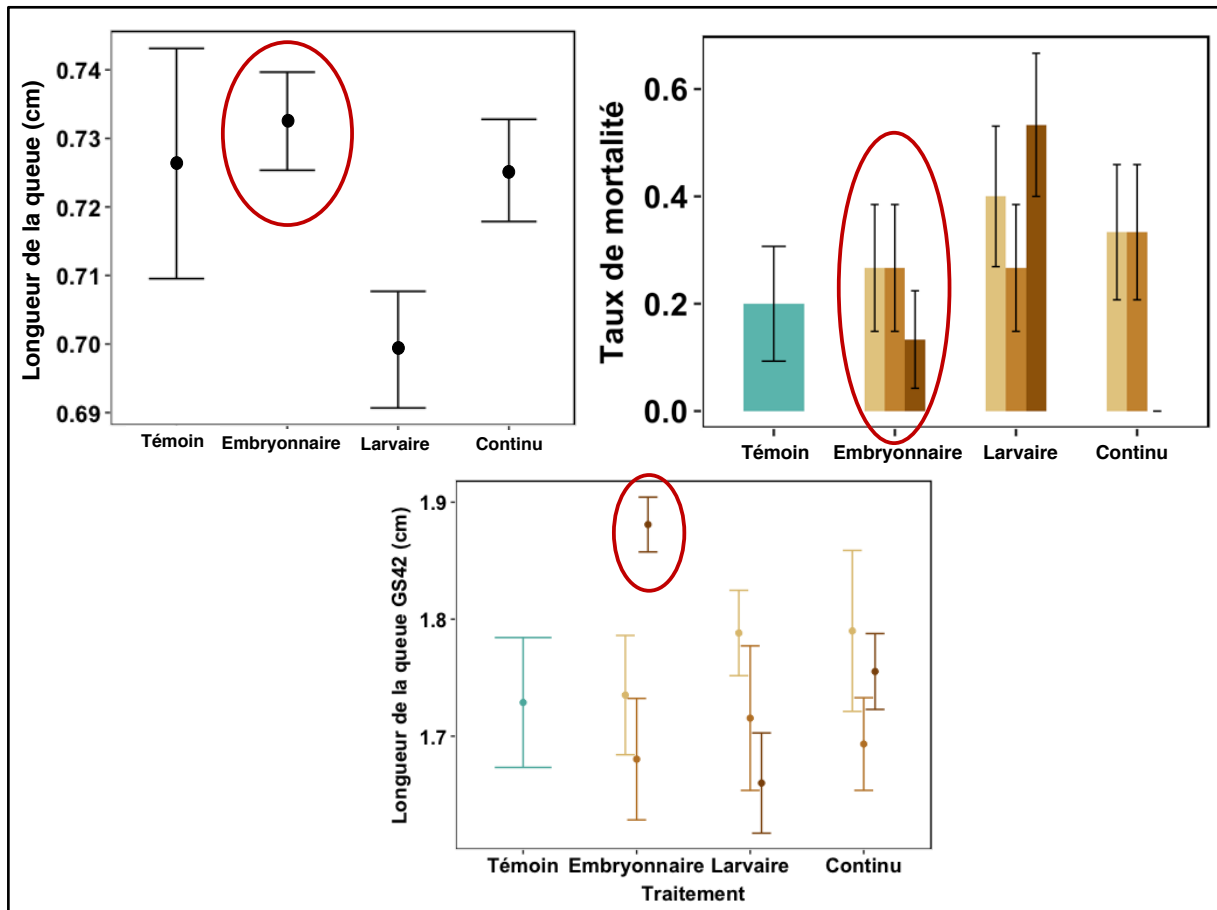


Figure 43 : Longueur de la queue au stade GS25 (cm, en haut à gauche), taux de mortalité chez les têtards (en haut à droite) et longueur de la queue au stade GS42 soumis à l'AMPA. Les couleurs montrent les concentrations. En vert « témoin », en marron clair « Faible », en marron « Moyenne », en marron foncé « Forte ». Les cercles rouges sont concentrés autour de l'exposition embryonnaire.

En effet, bien que ces individus aient été exposés à l'AMPA pendant le développement embryonnaire ($16,10 \pm 0,02$ jours), ils ont été élevés dans des conditions contrôlées pendant tout le développement larvaire ($74,29 \pm 0,71$ jours). Les effets de l'AMPA pendant le développement embryonnaire comprennent la modification de la durée du développement, du succès d'éclosion et du statut oxydant et ont été suggérés pour induire une mortalité sélective des embryons sensibles (**Articles IV, V**). Les derniers stades larvaires auxquels les effets d'une exposition précoce à l'AMPA étaient détectables correspondent à de forts changements morphologiques et physiologiques associés à la métamorphose (Beck & Congdon, 2003 ; Brown & Cai, 2007 ; Wright et al., 2011). Plus précisément, à ce stade, les têtards cessent de se nourrir alors que leur appareil alimentaire et digestif se modifie pour passer du broutage et de la détritivorie sous l'eau à l'alimentation de proies animales mobiles sur terre (Hourdry et al., 1996 ; Vitt & Caldwell, 2014). Le système respiratoire passe également de la respiration branchiale sous l'eau à la respiration pulmonaire sur terre (Vitt & Caldwell, 2014). Enfin, les modes d'utilisation de l'énergie comprennent la résorption de la queue qui sert à alimenter, du moins en partie, les changements morphologiques associés à la métamorphose et au maintien de l'organisme (Bouchard

et al., 2016 ; Gonçalves et al., 2015). Tous ces changements morphologiques pourraient être affectés par une exposition précoce à la contamination, et vraisemblablement par les mécanismes associés qui visent une faible survie dans un environnement contaminé (par exemple, mécanismes de détoxification, statut oxydant altéré, mortalité sélective, **Article IV**).

Une hypothèse pourrait être liée aux résultats trouvés dans le chapitre précédent. Dans **l'Article V**, on a pu montrer que l'AMPA induisait une mortalité sélective avec une sélection de têtards qui présentent des défenses oxydantes plus efficaces.

Cependant, cette mortalité sélective ne concerne pas les têtards traités à la concentration la plus forte. De plus, **l'Article IV** ne montre pas d'effet de l'AMPA sur le succès d'éclosion et **l'Article V** ne montre pas d'induction de stress oxydant par l'AMPA pour ces têtards traités à la concentration « Forte ». On peut faire l'hypothèse que l'AMPA n'a pas d'effet dans des stades pré éclosion et pendant l'éclosion cependant, placer les têtards dans un environnement sans AMPA à des stades plus tardifs entraîne une stimulation de la croissance.

(ii) Exposition au stade larvaire

L'exposition « larvaire » signifie donc que les têtards sont pour la première fois soumis à la contamination par l'AMPA pendant leur phase de développement larvaire. Plus précisément, nous avons constaté que les individus traités pendant une très courte période de leur développement larvaire (c'est-à-dire entre GS22 et GS25, ce qui correspond à 5 jours) présentaient une longueur de queue plus petite que leurs homologues de tous les autres moments d'exposition (**Figure 43**). Ce résultat indique que l'exposition à l'AMPA juste après l'éclosion peut rapidement affecter les taux de croissance et les investissements énergétiques relatifs dans les différentes parties du corps. Il est important de noter que ces effets étaient transitoires et n'étaient pas détectables à des stades ultérieurs du développement, ce qui suggère que les têtards ont pu compenser ces taux de croissance précoces altérés et/ou se sont acclimatés à l'exposition à l'AMPA.

On peut faire également faire l'hypothèse que l'exposition à un stress (AMPA) peut amener à un compromis entre quantité d'énergie allouée à la maintenance (détoxification avec augmentation des défenses oxydantes ou immunité, **Figure 44**) pour faire face à l'AMPA au détriment de la croissance et du comportement (recherche de nourriture car on ne détecte pas d'impact sur la durée de développement) ce qui peut être vu comme une **stratégie de compensation**.

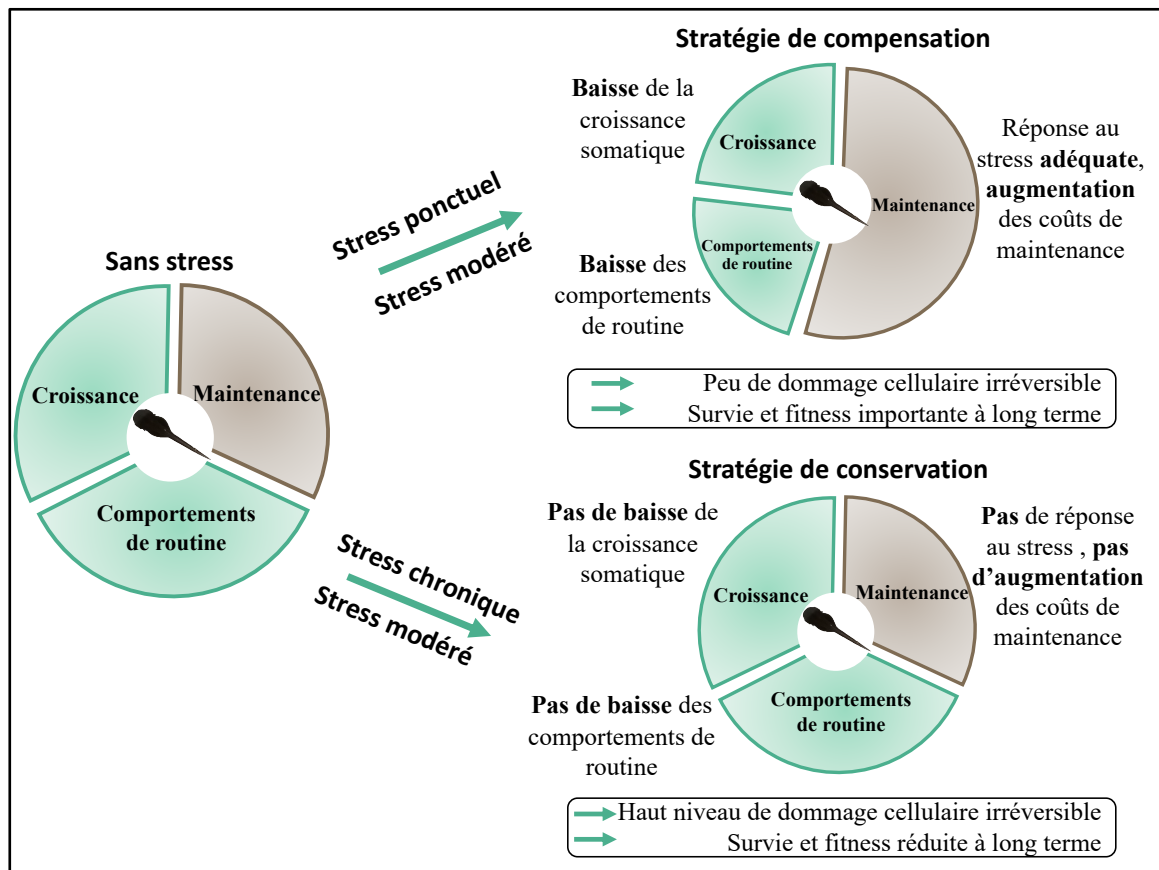


Figure 44 : Potentiels mécanismes de réponse à la contamination environnementale à des concentrations sublétales. Deux stratégies sont possibles en réponse à un stress ponctuel ou chronique : une stratégie de compensation, l'énergie allouée à la maintenance au détriment des comportements de routine et de la croissance, limitant les dommages cellulaires et les effets sur la fitness à long terme. Sous un stress chronique, la capacité métabolique est dépassée, la réponse au stress bloquée ne modifiant pas l'allocation d'énergie mais entraîne des dommages oxydants irréversibles et un impact à long terme sur la survie.

Adapté de Petitjean et al., 2019.

Par exemple, les oiseaux compensant les réductions de croissance subissent un stress oxydatif et une immunocompétence réduite (Alonso-Alvarez, et al., 2007), et des réponses similaires ont été détectées chez les insectes (De Block & Stoks, 2008) et les poissons (Kim et al., 2019). Les réponses compensatoires peuvent également affecter la physiologie plus tard dans la vie, comme chez les individus qui grandissent plus vite après une période de mauvaises conditions nutritionnelles, mais qui conservent des taux métaboliques plus élevés à l'âge adulte (Criscuolo et al., 2008). Des mécanismes de croissance compensatoire ont été montrés chez les amphibiens en réponse à différents facteurs de stress environnementaux (par exemple, la dessiccation, Altwegg & Reyer, 2003, la densité, Jasienski, 2008, la prédation, Vonesh & De la Cruz, 2002), probablement par l'expression du comportement alimentaire (Broomhall & Shine, 2003). Nous pouvons émettre l'hypothèse qu'à l'instar de ces autres facteurs de stress environnementaux, la contamination environnementale peut potentiellement affecter

plusieurs traits liés à la croissance chez les têtards, leur permettant d'atteindre une taille corporelle similaire à celle des individus témoins. Bien que nous n'ayons pas quantifié la quantité de nourriture ingérée au cours de notre expérience, il est possible que le taux d'alimentation soit l'une des variables qui permettrait une compensation chez les têtards exposés pendant les stades larvaires.

Cependant, on expose de manière chronique pendant toute la phase larvaire donc s'oriente possiblement vers **stratégie de conservation (Figure 44)**. Ce qui pourrait expliquer que l'on n'observe pas de différence plus loin dans le développement. La demande énergétique suite à l'exposition aux stress est trop importante, donc on peut supposer une augmentation de dommages oxydants, cellulaires et une survie à long terme impactée (mortalité plus importante en larvaire).

Alternativement, les amphibiens peuvent présenter des mécanismes de détoxification qui leur permettent de faire face à l'exposition à la contamination (Pašková et al., 2011). Dans notre contexte, il est possible que l'exposition à l'AMPA pendant le développement larvaire ait induit de tels mécanismes qui ont permis à ces individus de faire face à la contamination de l'eau à des stades ultérieurs du développement. Quels que soient les mécanismes impliqués dans ces réponses compensatoires (c'est-à-dire un comportement alimentaire accru, des mécanismes de détoxification ou une combinaison des deux), ils semblent se produire avec un coût pour les têtards en développement, car la survie semblait compromise dans ce groupe d'individus. Des études futures sont nécessaires afin d'évaluer les coûts de survie de l'exposition aux contaminants environnementaux en fonction des stades de développement des amphibiens.

(iii) Exposition tout au long du développement

Enfin, les têtards exposés tout le long du développement ne montrent aucune réponse à l'AMPA (Figure 44). On peut faire l'hypothèse que les réallocations d'énergie permettent d'augmenter la quantité d'énergie allouée à la maintenance. La réponse au stress de contamination est adéquate et limite les dommages cellulaires et n'influence pas la survie (Article V). Cela peut s'expliquer par la mortalité sélective des embryons en amont chez les individus de la concentration « Faible » et « Moyenne » (Article IV). Chez les têtards de la concentration « Forte » l'AMPA ne semble pas induire d'effets délétères mais on peut s'attendre à des dommages irréversibles (érosion des télomères) et une baisse de la survie à long terme.

En écotoxicologie, l'acclimatation est définie comme une tolérance accrue d'un toxique, résultant d'une exposition chronique à une concentration sublétales de ce toxique (McDonald et Wood 1993). Cependant, l'exposition à long terme à un polluant n'induit pas systématiquement une résistance accrue chez les organismes exposés. Par exemple, Tate-Boldt et Kolok (2008) ont montré que chez une espèce de poisson, les *Pimephales promelas*, une tolérance accrue au cuivre ne se développera que

chez les poissons qui ont connu un cycle prononcé et relativement long de dommages et de réparation branchiale.

D'autre part, au-delà de la fenêtre d'acclimatation, le seuil de sensibilisation définit une zone dans laquelle les mécanismes compensatoires ne peuvent plus maintenir l'homéostasie et, si le stress persiste, les organismes mourront (DiGiacopo & Hua, 2020).

Au cours de l'acclimatation, la zone dans laquelle les organismes sont stressés, leurs mécanismes physiologiques compensent la situation stressante (Silvestre et al., 2012). Dans ce nouvel état, une exposition continue au même toxique n'induit plus d'état pathologique dans l'organisme et les mécanismes physiologiques sont maintenant compensés. Cette résistance accrue à un toxique améliore l'aptitude de l'organisme dans l'environnement pollué. Cependant, les mécanismes, qu'ils soient physiologiques, biochimiques ou moléculaires, qui sont déclenchés au cours du processus d'acclimatation, nécessitent généralement de l'énergie. Ce coût métabolique explique que lorsque les animaux acclimatés retournent dans un environnement sans contamination, leur fitness est réduite par rapport aux organismes non acclimatés (Xie et Klerks 2004 ; Kwok et al. 2009).

(iv) Compromis d'allocation d'énergie face à la contamination

Les hypothèses présentées mériteraient des études approfondies notamment via l'étude des dommages moléculaires et cellulaires à tous les stades de développement (stress oxydant, longueur des télomères) et un suivi des individus post-métamorphique à l'âge adultes. De plus, les études futures devraient examiner l'influence de l'AMPA sur les taux d'alimentation, le métabolisme, et/ou les taux de conversion de la nourriture en tissu.

Les conditions pendant le développement peuvent affecter le comportement, la fitness, la physiologie plus tard dans la vie (Monaghan, 2007) et c'est particulièrement le cas pour des espèces avec stratégies de développement complexes comme les amphibiens (métamorphose). La fitness et la pérennité de la population dépend fortement du temps de développement, de la durée et la taille à la métamorphose. Grandir dans des conditions sub-optimales (température, salinité, contamination...) peut affecter cette fitness à long terme. Les hormones activent une cascade de voies cataboliques qui peuvent déséquilibrer le statut redox des organismes et surproduire des espèces réactives de l'oxygène (ROS), endommageant des biomolécules essentielles comme les protéines ou l'ADN (Monaghan, Metcalfe, & Torres, 2009). L'altération de ces voies métaboliques peut réduire la santé et la fitness des individus (Costantini, 2014, 2019 ; Crespi et al., 2013).

Face à la contamination environnementale dans le cadre de notre étude, certains concepts de stratégie métabolique de tolérance au stress peuvent être intéressants à évaluer. L'énergie peut être allouée aux fonctions de maintenance (détoxification, réparations et défenses des dommages cellulaires), aux comportements de routine (locomotion, nourrissage) ou à la croissance somatique ou gonadale (croissance et reproduction) (Figure 44). Dès lors que l'organisme se retrouve face à un stress

ponctuel ou modéré, une stratégie de compensation peut se mettre en place. L'énergie disponible va donc être réallouée dans des fonctions différentes. Les compromis d'allocation permettent des d'augmenter la quantité d'énergie allouée à la maintenance pour faire face au stress au détriment des autres fonctions (comportement de routine et croissance somatique) (Kooijman, 2009; Sokolova et al., 2012).

Lors d'une exposition à un stress à long terme (exposition chronique à un contaminant), une stratégie de conservation peut se mettre en place. La demande énergétique devient trop importante pour faire face efficacement au budget total de l'organisme menant à un blocage de la réponse (arrêt métabolique). Les compromis sont bloqués au prix de dommages importants cellulaires (apoptoses) et moléculaires (érosions des télomères et dommages oxydatifs). La survie à long terme est impactée avec une baisse importante de la fitness (Monaghan et al., 2009 ; Petitjean et al., 2019 ; Sokolova, 2013).

b) Développement post-embryonnaire et nicosulfuron

Nous avons évalué les altérations du développement en utilisant des traits morphologiques (taille et forme du corps), développementaux (durée des stades de développement) et comportementaux pertinents (**Article VII**). Dans l'ensemble, nous avons constaté que l'exposition à des concentrations de nicosulfuron pertinentes pour l'environnement modifiait plusieurs traits morphologiques et comportementaux des têtards en développement.

(i) Effet de la concentration mais pas de l'exposition

Le résultat le plus marquant de notre étude est l'absence d'effet du moment de l'exposition par rapport aux effets des concentrations testées. Ces résultats suggèrent que l'exposition au nicosulfuron peut déclencher des effets similaires indépendamment des stades de développement auxquels cette exposition a lieu (par exemple, le développement embryonnaire uniquement, le développement larvaire uniquement, ou les deux), ce qui indique que les mécanismes par lesquels le nicosulfuron a modifié le développement sont similaires entre les embryons et les larves. Plus important encore, ce résultat suggère qu'une exposition limitée dans le temps (par exemple, le développement embryonnaire uniquement) peut induire des effets durables tout au long du développement larvaire jusqu'à la métamorphose. Nous n'avons pas mis en lumière de stratégie de compensation alors même qu'on a pu constater que les embryons présentaient des dommages oxydants (**Article VI**).

(ii) Modification des proportions corporelles

Les concentrations pertinentes pour l'environnement que nous avons testées étaient sublétales car elles n'ont pas induit de mortalité élevée et, de plus, elles n'ont pas modifié les durées de développement.

Pourtant, ces concentrations ont influencé la morphologie des têtards à des stades de développement qui sont cruciaux entre la fin de la croissance somatique et le début de la métamorphose (**Article VII**).

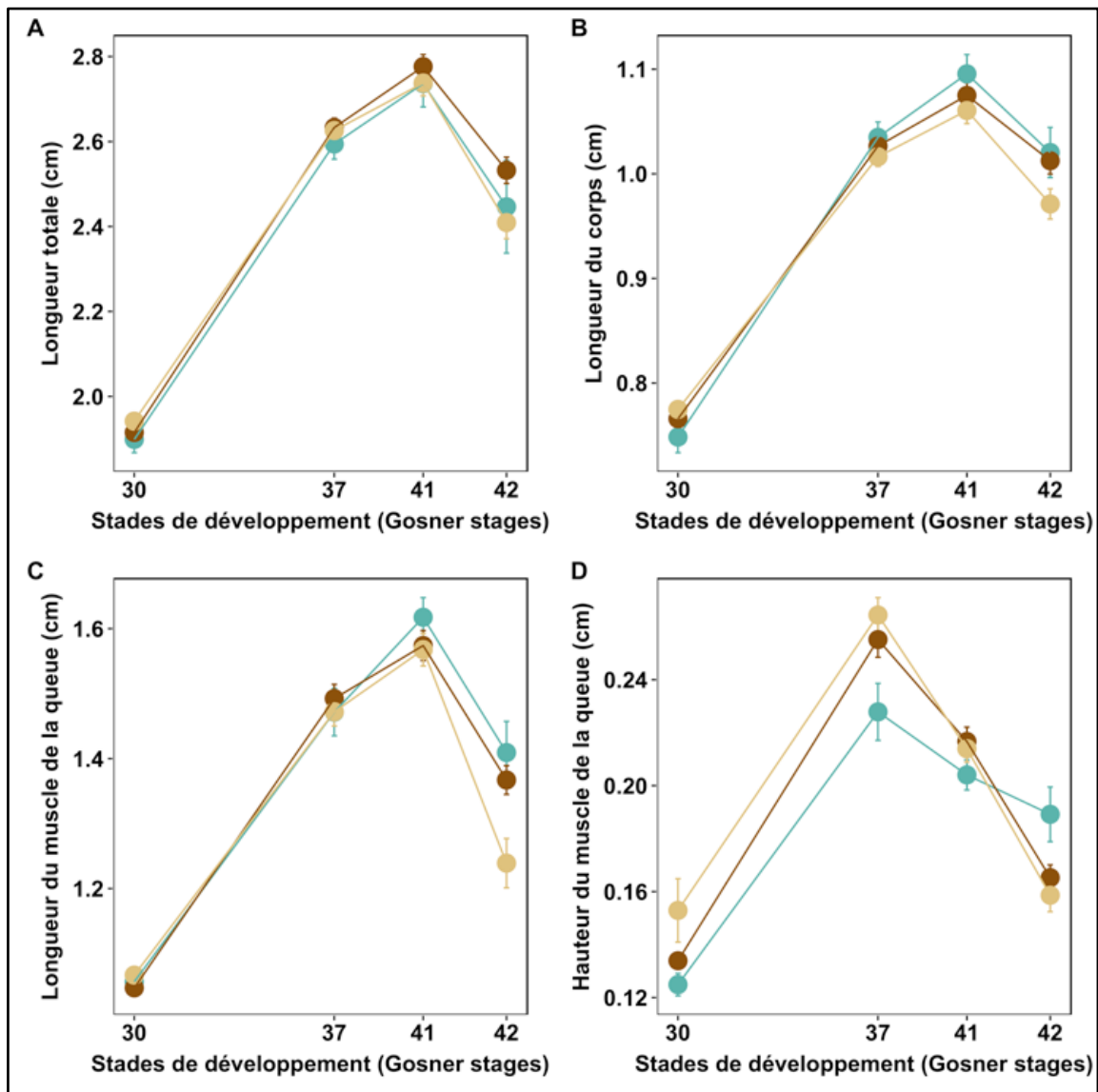


Figure 46 : Longueur totale (en cm ; A), longueur du corps (en cm ; B), longueur du muscle de la queue (en cm ; C) et hauteur du muscle de la queue (en cm ; D) par rapport aux stades de développement Gosner chez les têtards de *Bufo spinosus*. Les données représentent la moyenne \pm SE. Les intervalles entre les stades de développement de Gosner ont été calculés en utilisant le nombre moyen de jours entre deux stades de Gosner pour tous les individus. Les couleurs représentent les différentes concentrations (Témoin = Bleu, Faible = marron clair, Fort = marron foncé).

À ces stades, les individus du groupe témoin étaient plus petits, mais avec un corps plus grand, et avaient des muscles caudaux plus longs mais plus fins (**Figure 46**).

De même, nous avons constaté que ces effets morphologiques dépendaient de la concentration testée, les individus de la concentration la plus faible étant différents des individus de la concentration la plus élevée. Ces résultats suggèrent que l'exposition au nicosulfuron a influencé les patrons de croissance - et donc les investissements énergétiques - des différentes parties du corps (corps *versus* queue), et a notamment influencé la musculature de la queue, qui est critique pour la locomotion des têtards, les têtards avec des muscles caudaux plus petits et/ou plus fins devant fournir un effort de nage plus important (Touchon & Wojdak, 2014).

Enfin, nous avons constaté que l'exposition au nicosulfuron avait tendance à persister pendant la métamorphose, les individus post-métamorphiques du groupe à forte concentration présentant une masse corporelle plus importante que leurs homologues du groupe à Faible concentration ou du groupe Témoin (Figure 47).

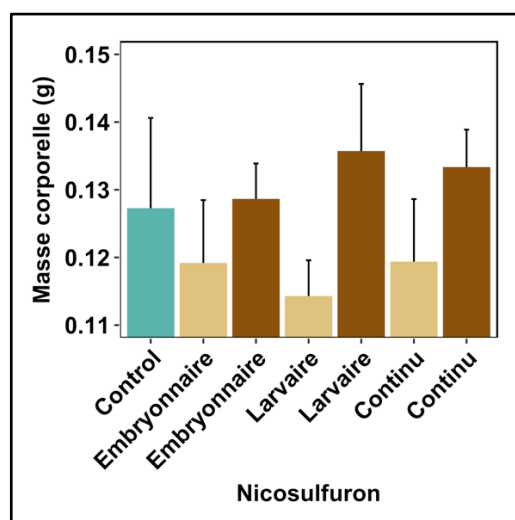


Figure 47 : Masse à la métamorphose (g) des individus traités au nicosulfuron. L'exposition était différente selon la phase de développement, les couleurs montrent les différentes concentrations (« Témoin » en bleu, « Faible » en marron clair ou « Forte » en marron foncé)

Une fois encore, ce résultat peut être lié à des investissements différents dans les parties du corps (le corps par rapport à la queue), à des modifications du comportement (qui peuvent affecter l'efficacité de la recherche de nourriture et donc l'acquisition d'énergie), ou à une combinaison de ces éléments. Une autre hypothèse, non mutuellement exclusive, serait liée au schéma de mobilisation énergétique qui se produit à la fin de la métamorphose. En effet, à ce stade, les têtards deviennent aphagiques et dépendent, au moins en partie, de la résorption de la queue pour alimenter les besoins énergétiques des stades ultimes de la métamorphose (Bouchard et al., 2016; Gonçalves et al., 2015; Ruthsatz et al., 2018). Dans notre étude, aux derniers stades de développement correspondant à l'atrophie et à la résorption de la queue (GS42), les têtards du groupe témoin présentent des muscles caudaux à la fois

plus longs et plus hauts, ce qui suggère que la dynamique de résorption de la queue et la récupération énergétique associée peuvent fortement diverger entre les individus exposés ou non au nicosulfuron. Quel que soit le mécanisme sous-jacent, ce résultat renforce l'idée que l'exposition au nicosulfuron, même temporaire et précoce dans le développement, peut avoir une influence durable sur les amphibiens en développement.

(iii) Comportement erratique et rôle de l'Acétylcholinestérase

L'exposition au nicosulfuron a fortement influencé tous les paramètres comportementaux examinés dans notre étude (Figure 48). Ce résultat était attendu étant donné les influences bien connues des herbicides à base de sulfonylurées sur le comportement d'autres espèces (Bretaud et al., 2000; Saglio, Olsén, Bre, 2001), effets qui sont probablement médiés par la perturbation de l'acétylcholinestérase (AChE, Bretaud et al., 2000; Pretto et al., 2011).

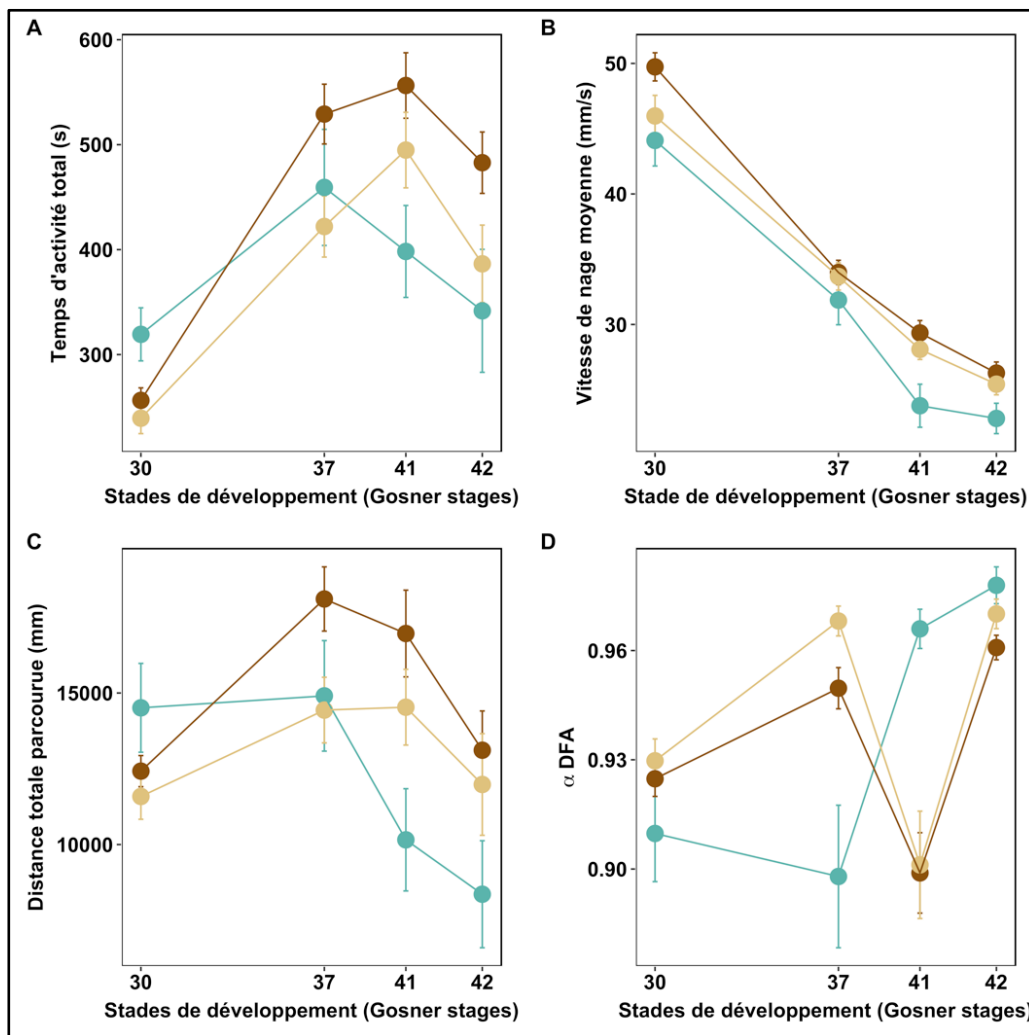


Figure 48 : Temps d'activité total (en secondes ; A), vitesse de nage moyenne (en mm.s⁻¹ ; B)), distance totale parcourue (en millimètres ; C) et α DFA (indice d'échelle sans unité inversement lié à la complexité du comportement ; D) par rapport aux stades de développement (Gosner stages) chez les têtards de *Bufo spinosus*.

Les données représentent la moyenne \pm SE. Les intervalles entre les stades de développement de Gosner ont été calculés en utilisant le nombre moyen de jours entre deux stades de Gosner pour tous les individus. Les couleurs représentent les différentes concentrations (Témoin = Bleu, Faible = marron clair, Fort = marron foncé).

Les têtards exposés au nicosulfuron étaient globalement plus actifs, nageaient plus vite et parcouraient de plus longues distances. De plus, la complexité comportementale (évaluée par la méthode DFA, [Article VII](#)) était également affectée par l'exposition au nicosulfuron. Nous avons constaté que le pic de complexité comportementale est survenu plus tôt dans le développement des individus du groupe témoin (GS 37), tandis que chez les individus exposés, ce pic est survenu plus tard au cours du développement (GS 41). Comme pour la morphologie, nous avons constaté que ces effets comportementaux dépendaient de la concentration testée, les individus de la concentration Faible étant différents des individus de la concentration Forte. De tels effets comportementaux devraient avoir des conséquences sur les traits liés à la locomotion tels que l'efficacité de la recherche de nourriture ([Browne & Moore, 2014](#)) et la capacité d'évitement et de fuite des prédateurs ([Moreira et al., 2019](#)). Dans notre étude, il est plausible qu'une perturbation de l'activité AChE dans le cerveau des têtards, dont on sait qu'elle augmente tout au long du développement ([Franceschini et al., 1987](#) ; [Sasaki et al., 1985](#)), soit provoquée par le nicosulfuron.

Parmi les divers effets sublétaux mis en évidence ci-dessus, les altérations du comportement sont intéressantes car il a été démontré que ces modifications sont des indicateurs écologiquement pertinents du stress physiologique ([Ford et al., 2021](#) ; [Scott & Sloman, 2004](#)). Il est important de noter que les changements de comportement et d'activité liés à l'exposition aux produits phytosanitaires peuvent être particulièrement utiles dans la mesure où ils peuvent fournir des alertes précoces plus sensibles que les méthodes de tests standards ([Scott & Sloman, 2004](#)). Dans ce contexte, il a été démontré que les herbicides à base de sulfonylurée perturbent l'activité de l'acétylcholinestérase (AChE) et les marqueurs du statut oxydant chez les poissons, ce qui affecte à son tour leurs réponses comportementales ([Bretaud et al., 2000](#) ; [Saglio, Olsén & Bre, 2001](#)).

Comme on a pu le voir, les pesticides ont un rôle de perturbateur endocrinien ou perturbateur de médiateur chimique. L'acétylcholinestérase est la principale enzyme responsable du métabolisme hydrolytique du neurotransmetteur acétylcholine en choline et acétate. On la retrouve dans les tissus nerveux. Des études précédentes chez *Carassius auratus* exposé au nicosulfuron ont mis en avant l'implication de l'acétylcholinestérase (AChE) sur le comportement ([Bretaud et al., 2000](#); [Saglio, 2001](#)). Chez *Gambusia affinis*, l'exposition à des concentrations sublétales de chloryrifos, un insecticide, réduit le comportement locomoteur comme la distance parcourue et la vitesse de nage. Ce comportement locomoteur altéré est due à une accumulation d'acétylcholine (ACh) suite à l'inhibition de l'activité enzymatique de l'AChE ([Rao et al., 2005](#)), de même chez *Danio rerio* une relation entre le comportement de natation et la concentrations d'AChE non musculaire a été démontrée ([Tilton et al., 2011](#)). L'atrazine modifie les comportements défensifs de *Danio rerio* conjointement avec une

baisse de l'activité enzymatique de l'AChE dans le cerveau (Schmidel et al., 2014). Des liens entre le comportement locomoteur et la cholinestérase ont été mis en évidence chez la truite arc-en-ciel, une diminution de l'activité cérébrale de l'AChE étant liée à une diminution du comportement de nage (Beauvais et al., 2001). Chez les amphibiens comme *Hypsiboas pulchellus*, l'exposition à un herbicide à concentrations sublétales inhibe les activités AChE et butyrylcholinestérase (BChE, une enzyme ayant le même rôle qu'AChE mais retrouvée dans le foie) et à la même concentration, les auteurs montrent une augmentation significative de la vitesse de nage et la distance moyenne (Peltzer et al., 2013).

Des dosages supplémentaires sont donc nécessaires pour déterminer si le comportement erratique et l'augmentation de l'activité chez les têtards traités est due à une perturbation de l'activité enzymatique. Il reste à vérifier si ces altérations comportementales sont des conséquences de morphologies divergentes (voir ci-dessus), de la perturbation de mécanismes physiologiques liés au comportement (c'est-à-dire l'AChE) ou d'une combinaison de ces éléments.

III) Conclusion : Conditions expérimentales et absence de contraintes

Enfin, il est également important de noter que les têtards expérimentaux ont été élevés dans des conditions optimales avec de la nourriture disponible *ad libitum*, des conditions thermiques constantes et l'absence de prédateurs et de compétiteurs. De telles conditions optimales sont clairement différentes de ce qui est observé *in natura*, où la compétition, la prédation et/ou le manque de nourriture peut se produire (Broomhall & Shine, 2003 ; Goater, 1994 ; Jones et al., 2011). Il est possible que les conditions que nous avons offertes aux têtards pendant leur croissance fussent suffisamment optimales pour qu'ils puissent compenser les effets de la contamination (Capellán & Nicieza, 2007). En accord avec cette idée, il est important de souligner que les crapauds de nos expériences étaient ~1,4 fois plus grands et ~3 fois plus lourds que les crapauds sauvages capturés au même stade (**Figure 49**, AMPA : SVL=1,15±0,08 cm ; Masse=0,194±0,03 g ; Nicosulfuron : SVL=1,03±0,08 cm ; Masse=0,125±0,03 g pour les métamorphes expérimentaux contre SVL=0,78±0,07 cm ; Masse=0,05±0,01 g pour n=16 crapauds capturés sur le terrain).

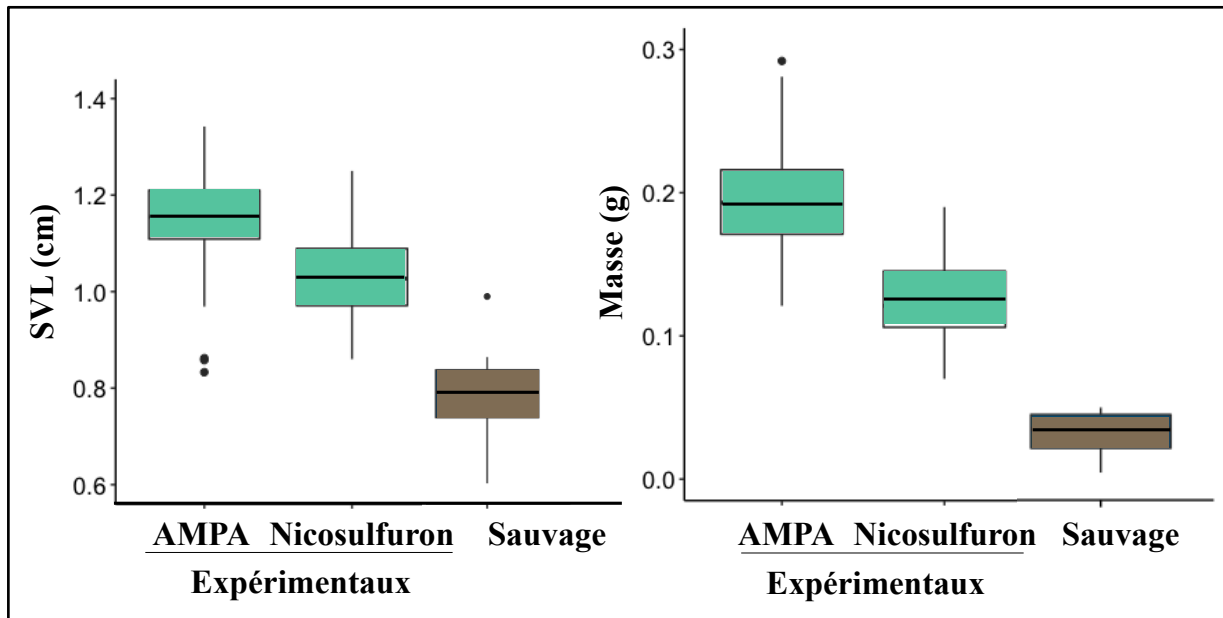


Figure 49 : Comparaison entre individus post-métamorphique issus de conditions contrôlées (« Expérimentaux », Bleu) et du milieu naturel (« Sauvage », Marron) pour la taille museau-cloaque (SVL) et la masse corporelle (Masse).

Ces fortes différences suggèrent que les têtards qui se développent dans des conditions naturelles produisent des crapauds plus petits et plus maigres. Nous pouvons également émettre l'hypothèse que dans des conditions naturelles relativement difficiles, l'influence des contaminants anthropogéniques peut être beaucoup plus prononcée que dans des conditions expérimentales. Les études futures devraient viser à évaluer l'influence de la contamination environnementale sur les têtards en développement dans des conditions environnementales contraignantes en manipulant la disponibilité de la nourriture, la compétition, la prédation et leur combinaison (Hua et al., 2017 ; Relyea, 2001).

Perspectives



Crédit photo : Matthias Renoirt

“The temptation to form premature theories upon insufficient data is the bane of our profession.”

— Sir Arthur Conan Doyle
dans *The Valley of Fear* (1914), 43

I) Exploration de l'effet de la contamination environnementale chez les adultes

A) Exposition directe pendant la phase terrestre

Comme on a pu le montrer dans les **Article IV, V, VI, VIII et IX**, les phases de développement aquatiques sont particulièrement sensibles à l'exposition aux herbicides étudiés et montrent des réponses développementales altérées. Les organismes aquatiques ou terrestres sont rarement confrontés à une contamination aiguë par des substances toxiques. En général, ils sont confrontés à des concentrations relativement faibles sur une longue période. Le stress chronique et aigu présente des défis écologiques différents. Les organismes sont susceptibles de présenter des réponses moléculaires, biochimiques, physiologiques ou morphologiques différentes à ces deux types de stress. Les conséquences d'une exposition chronique aux contaminants pour la survie des populations et des espèces pourraient être délétères, à moins que le développement de la plasticité phénotypique au sein d'une génération, ou l'adaptation au fil des générations, ne produisent des individus plus tolérants à un environnement pollué (DiGiacopo & Hua, 2020). Les crapauds épineux, de par leur capacité de migration limitée et leur peau perméable, sont soumis au risque de la contamination pendant leur développement et leur stade adulte. Les amphibiens terrestres post-métamorphiques, juvéniles et adultes, peuvent absorber des pesticides à partir de sols contaminés (Storrs Méndez et al. 2009), lors de leur migration à travers le paysage agricole (Leeb et al. 2020 ; Lenhardt et al. 2013).

C'est dans ce contexte que nous avons donc capturé des mâles et des femelles *Bufo spinosus* en automne 2020 dans le but d'effectuer une exposition chronique aux deux contaminants étudiés pendant cette thèse, l'AMPA et le nicosulfuron (**Figure 50**). L'exposition aux deux contaminants a commencé en avril 2021 et finira en novembre 2021.

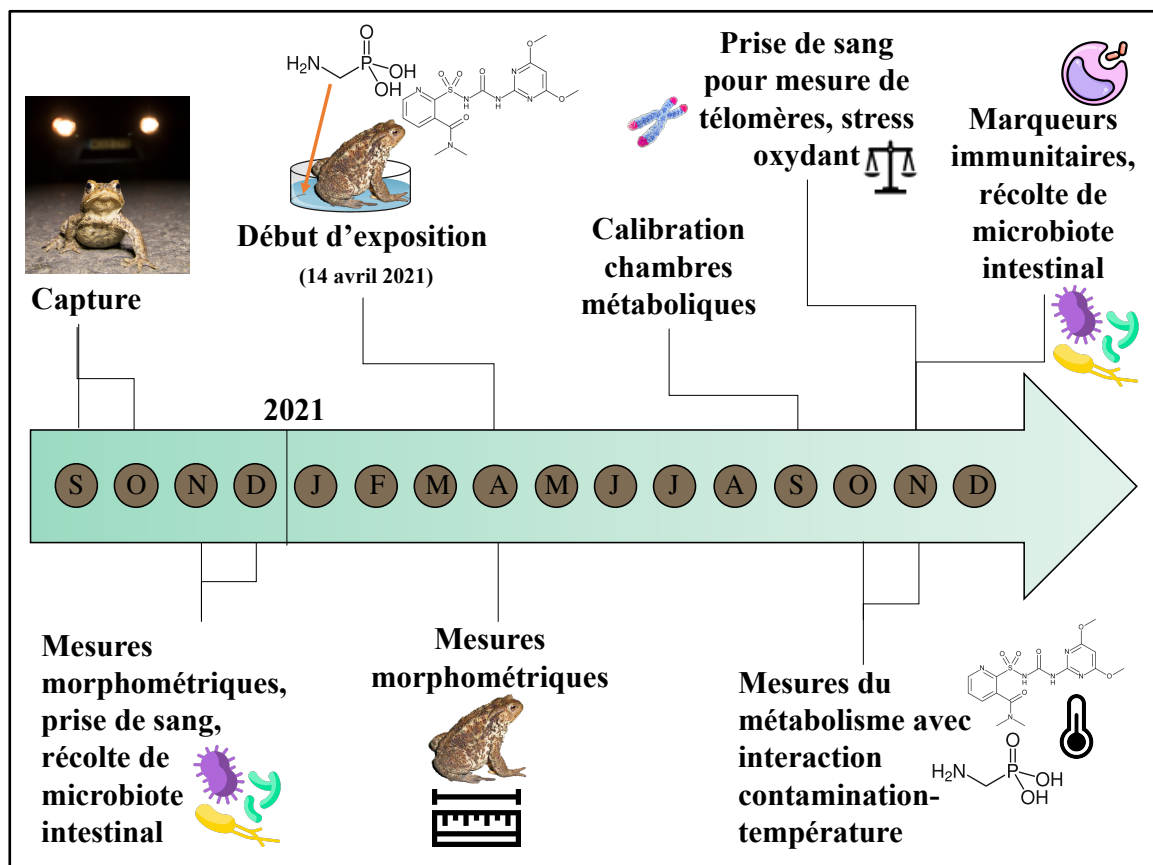


Figure 50 : Chronologie et planning de l'expérimentation sur l'exposition chronique des adultes (mâles et femelles) de *Bufo spinosus* à deux contaminants (AMPA et nicosulfuron). Les analyses et mesures sont faites conjointement avec une post-doctorante (Sabrina Tartu).

Les variables mesurées pendant cette expérimentation seront la croissance, le microbiote intestinal, la longueur des télomères, le stress oxydant, des marqueurs immunitaires et le taux métabolique standard.

Dans un contexte de réchauffement climatique, cette mesure de taux métabolique standard se fera à deux températures différentes car les contaminants sont connus pour agir différemment selon la température. Le réchauffement climatique a un impact complexe sur la faune et la flore sauvages par le biais d'interactions synergiques avec d'autres facteurs de stress environnementaux qui proviennent soit de l'activité anthropique, soit de sources naturelles (Noyes et al. 2009 ; Narayan 2016). Plusieurs espèces d'amphibiens ont montré des schémas de toxicité dépendant de la température avec l'insecticide méthomyl (Lau et al., 2015). Un défi majeur auquel les ectothermes sont confrontés dans des environnements thermiques variables est le maintien d'un métabolisme énergétique approprié (par exemple, le taux métabolique standard, SMR) (Angilletta et al. 2002 ; Little et Seebacher 2016) qui est déterminé en mesurant les taux de consommation d'O₂ au repos et représente l'énergie

nécessaire pour couvrir les fonctions physiologiques minimales (Rowe et al. 1998 ; Beck et Congdon 2003).

Ce taux métabolique représente un coût métabolique fixe auquel chaque organisme est confronté. Il correspond au coût énergétique de la maintenance de l'organisme, y compris le maintien du gradient H⁺ mitochondrial, le renouvellement des protéines, le transport des ions, la production d'hormones, la circulation sanguine et la ventilation (Bennett, 1988 ; Hulbert, 2000). Un faible SMR peut permettre à un individu de consacrer plus d'énergie à d'autres processus, comme la croissance (Blackmer et al., 2005) ou la reproduction (Jackson, Trayhurnt & Speakman, 2001 ; Ketola & Kotiaho, 2009). De même, si l'apport énergétique changeait, un SMR faible impliquerait une dépense énergétique quotidienne plus faible, ce qui se traduirait par une diminution du temps de recherche de nourriture et donc du risque de prédation (Steyermark et al., 2005).

Un SMR élevé est généralement associé à des organes proportionnellement plus grands, comme les intestins, le foie, les reins et le cœur (Speakman & McQueenie, 1996 ; Chappell, Bech & Buttemer, 1999 ; Hammond et al., 2000). Ainsi, un individu avec un SMR plus élevé peut être capable d'allouer plus d'énergie à différents processus qu'un individu avec un SMR plus faible. Cette hypothèse est soutenue par diverses études rapportant une corrélation positive entre le SMR et la masse corporelle (anoures : Steyermark et al., 2005 ; oiseaux : Piersma, Cadee & Daan, 1995), la taille des organes ajustée à la masse corporelle (anoures : Steyermark et al., 2005), et la taille des cellules (anoures : Hermaniuk, Rybacki & Taylor, 2016).

La future étude se concentrera sur la variation physiologique du taux métabolique standard (SMR) sous la contamination environnementale en interaction avec la température.

B) Plasticité phénotypique

L'article II nous a donc montré que les caractéristiques de l'habitat pouvaient entraîner des variations phénotypiques chez les mâles de crapauds épineux. De plus, l'étude de l'allométrie peut laisser à penser que ces différences s'inscrivent pendant le développement avec des décalages possibles entre environnements aquatiques pendant le développement embryonnaire et larvaire et environnements terrestres pendant les phases juvénile et adulte.

Les facteurs de stress subis au cours des premiers stades de la vie d'un animal peuvent avoir un impact sur son développement et sa croissance, mais peuvent également avoir des

conséquences sur d'autres caractéristiques de l'histoire de vie, telles que la reproduction et/ou la longévité, et donc sur la fitness.

Par exemple il a été montré que le stress oxydant subi pendant le développement peut affecter le phénotype d'un individu (Romero-Haro & Alonso-Alvarez, 2020). En manipulant le niveau de stress oxydant subi par les diamants mandarins (*Taeniopygia guttata*) au cours de leur développement, les auteurs ont montré que le stress oxydant altère le phénotype de la descendance et les soins parentaux des parents. Les articles V et VI nous ont montré que la contamination pouvait induire une altération du stress oxydant chez les têtards de *Bufo spinosus*. On peut donc se demander si ce décalage dans les différents phénotypes observés chez les adultes est une résultante d'un stress de contamination pendant le développement. Les mécanismes évolutifs (c'est-à-dire, adaptation génétique ou plasticité) sont également susceptibles d'aboutir à de l'adaptation locale (c'est-à-dire, meilleure fitness des individus dans leur environnement local que dans un nouvel environnement)

C) Adaptation locale

Pour aller plus loin avec l'analyse de la structuration génétique de nos populations, l'exploration de l'adaptation locale est une voie de recherche à étudier. La plupart des espèces habitent des environnements hétérogènes et montrent des changements graduels dans leur morphologie, comportement et composition génétique, qui coïncident souvent avec les variations environnementales.

L'adaptation à un nouvel habitat implique non seulement un changement phénotypique, mais aussi un avantage fonctionnel qui se traduit par une meilleure fitness. Ce paradigme phénotype-performance-fitness fournit un cadre pour comprendre la différenciation phénotypique adaptative. L'adaptation locale unique se produit lorsque les génotypes sont adaptés au site spécifique où ils se trouvent.

La persistance *in situ* des populations dans un environnement changeant dépend largement de leur capacité à s'adapter aux nouvelles conditions environnementales. L'adaptation locale se produit lorsque la sélection favorise l'association non aléatoire entre le succès reproductif des individus (c'est-à-dire la fitness) et les caractéristiques des environnements qui maximisent ce succès reproductif (Anderson et al., 2014; Hereford, 2009; Kawecki & Ebert, 2004; Savolainen et al., 2007), et elle pourrait se produire par une combinaison de plasticité et d'évolution génétique (Anderson et al., 2014; Ashander et al., 2016; Burggren, 2020; Lowry et al., 2013). Par conséquent, les populations adaptées localement ont tendance à présenter

une survie et une fitness plus élevées dans leur propre environnement " d'origine " que dans un environnement " étranger " et vice versa (Hereford, 2009; Kawecki & Ebert, 2004). Cette prédiction a été testée à plusieurs reprises par des expériences de transplantation réciproque et de jardin commun (Gomez-Mestre & Tejedo, 2003; Hereford, 2009) dans des environnements naturels (Gomez-Mestre & Tejedo, 2003; Leimu & Fischer, 2008; Savolainen et al., 2007) et modifiés par l'homme (Rolshausen et al., 2015). Une expérience de jardin commun avec des crapauds issus d'habitats contrastés est envisageable pour discriminer si les mécanismes retrouvés résultent de l'adaptation locale et l'expression de nouveaux traits fonctionnels.

Ces axes de recherches sur l'adaptation locale chez cette espèce sous l'effet de l'herbicide étudié dans cette thèse (AMPA) sont en cours au sein de l'équipe pour des résultats fin 2021.

II) Exploration de l'effet de la contamination environnementale pendant le développement

Nos études montrent donc des effets physiologiques des concentrations d'AMPA et de nicosulfuron pertinentes pour l'environnement sur les embryons d'amphibiens mais des réponses plus subtiles à des stades ultérieurs du développement. Ces études ont donc montré que les contaminations environnementales à des intrants chimiques pouvaient être un facteur de variabilité dans les populations. Par exemple, cette différenciation de taille que l'on retrouve dans les environnements agricoles peut s'expliquer par une sensibilité accrue à la contamination. De plus, la mortalité post métamorphique peut avoir un impact sur le recrutement et la reproduction. On a notamment pu montrer une absence de reproduction et de présence de femelles sur les sites agricoles (**Annexe III**).

A) Effets sublétaux

Nos études ont pu montrer que des concentrations faibles, bien en dessous des normes gouvernementales avaient des effets sur le développement de notre modèle d'étude.

Il reste crucial d'étudier les effets des composés parents comme on a pu le voir avec le nicosulfuron, mais aussi, de leurs métabolites à des concentrations pertinentes pour l'environnement sur des espèces non-modèles, afin d'évaluer de manière exhaustive les impacts des contaminants anthropiques sur l'environnement, comme le montre l'AMPA.

Peut-être plus important que l'ampleur des effets en soi, nous soulignons les patrons complexes des courbes concentration-réponse que nous avons trouvés. En effet, ces modèles contrastent avec d'autres études qui se sont concentrées sur des concentrations d'essai beaucoup plus élevées (FAO, 2013), et peuvent indiquer des effets à faible concentration et des réponses de concentration non-monotones (Vandenberg et al., 2012). De telles réponses non-monotones n'étaient pas attendues, et nous n'avons donc pas la puissance nécessaire pour tester cette hypothèse de manière approfondie, car la vérification de la non-monotonie nécessite au moins deux concentrations de part et d'autre du point d'inflexion (OCDE, 2014). Cependant, il est important de souligner que les effets des faibles concentrations et les réponses non monotones semblent être fortement liés à la perturbation endocrinienne (Vandenberg et al., 2012), un mécanisme sous-jacent possible aux réponses complexes que nous avons trouvés pour tous les paramètres étudiés. Si cette hypothèse est vraie, le décryptage des cibles de cette perturbation endocrinienne sera essentiel pour évaluer de manière exhaustive l'impact du métabolite primaire du glyphosate. En outre, cette hypothèse viendrait s'ajouter aux preuves récemment croissantes qui suggèrent que, pour les contaminants anthropiques, les courbes de réponse non-monotones à faibles concentrations sont beaucoup plus répandues qu'on ne le soupçonnait auparavant (Hill et al., 2018). Pourtant, la non-monotonie, bien que fondamentale pour la prise de décision réglementaire, se produit à des concentrations infimes qui sont généralement ignorées par la réglementation. Il est clair que la prise de décision doit aller au-delà de l'utilisation d'études à fortes doses pour déduire les concentrations officiellement sans effet (Hayes et al., 2010 ; Hill et al., 2018). Compte tenu de la variation temporelle du devenir des produits phytosanitaires due aux variations locales des conditions climatiques (Cabrerizo et al., 2018), et compte tenu de la coïncidence temporelle entre cette application et la reproduction des amphibiens et d'autres espèces non-cibles dans les zones tempérées (Berger et al., 2013), nos résultats renforcent la nécessité pour la toxicologie réglementaire de prendre en compte les effets de faibles concentrations de produits phytosanitaires pertinentes pour l'environnement sur la faune non-cible présente dans les zones agricoles.

B) Altérations cellulaires et hormonales

L'intégration de marqueurs du statut oxydant dans nos études permet de suggérer des mécanismes physiologiques potentiels responsables des effets sur les traits liés au développement. Ces effets pourraient indiquer une stimulation de la balance antioxydante pour limiter tout effet pro-oxydant lié à la contamination.

Le stress oxydant est souvent déterminé dans le sang mais cela peut se révéler problématique parce que les enzymes antioxydantes et les ROS sont compartementalisés et très dynamique donc différents selon les tissus. Les stressseurs pouvant impacter le stress oxydant peuvent avoir des effets différentiels selon le tissu étudié (Speakman et al., 2015). Il est important de mettre le stress oxydant en rapport avec des traits d'histoire de vie car la réponse moléculaire peut être différentielle selon le tissu (Costantini, 2014) ou alors effectuer des approches plus intégratives en mettant en relation avec des fonctions endogènes.

Chez les amphibiens, le stress oxydant a un rôle dans la senescence. L'activité enzymatique est différente selon l'âge avec un déclin des enzymes antioxydantes à des âges plus avancés. Dans le foie, les taux de SOD, catalase et GPx déclinent avec l'âge, ce qui suggère une capacité de détoxification moins efficace qui pourrait s'avérer délétère si les adultes sont soumis aux changements dans leur habitat (contamination environnementale). D'autres part, les dommages oxydatifs augmentent avec l'âge et les stressseurs environnementaux (Burraco et al., 2020). Dans un contexte de changement climatique, les organismes ont besoin de s'adapter aux fluctuations environnementales comme adapter leur stratégie de croissance. Mais cela peut avoir des conséquences sur la fitness. Des altérations métaboliques importantes sont induites par une croissance compensatoire suite à un stress environnemental pendant le développement larvaire (Burraco et al., 2017). Suite à un retard de développement induit par un stress thermique, les larves qui ont montré des retards d'éclosion ont montré une plus grande réponse compensatoire et se sont métamorphosés à une taille plus grande avec un développement moins long. Cependant, les différences de croissance ont altéré le statut redox et les niveaux de glucocorticoïdes (corticostérone) probablement liés à un métabolisme plus élevé.

Concernant le nicosulfuron, nous avons constaté que l'exposition à des concentrations faibles de cet herbicide persistant modifie le développement, la morphologie et le comportement. Plus particulièrement, l'absence d'effet du moment de l'exposition à différents stades de

développement (embryonnaire ou larvaire) sur les traits que nous avons étudiés suggère que des mécanismes similaires sont affectés pendant le développement embryonnaire ou larvaire. Il est clair que des études futures sont nécessaires afin d'identifier ces mécanismes. On sait que les herbicides sulfonylurés affectent l'activité et le comportement par la perturbation de l'activité normale de l'AChE (Bretaud et al., 2000 ; Saglio, Olsén, Bre, 2001), et il reste à vérifier si les mêmes mécanismes se produisent dans les embryons en développement (de Llamas et al., 1985).

Les dosages préliminaires de l'AChE chez les embryons en développement de *Bufo spinosus* seront effectués à l'automne 2021.

À notre connaissance, il s'agit de la première étude qui montre les effets du nicosulfuron sur les marqueurs biochimiques et les traits liés au développement chez une espèce aquatique non-cible. Étant donné que la fitness post-métamorphique est principalement expliquée par la qualité du développement larvaire chez les amphibiens (Bekhet et al., 2014 ; Boonekamp et al., 2018 ; Bredeweg et al., 2019), Enfin, nous soulignons que les effets sublétaux du nicosulfuron ont été négligés jusqu'à présent (mais voir Hackenberger et al., 2018) et nécessitent des investigations supplémentaires sur d'autres taxons non-cibles présents dans les paysages agricoles.

C) Le développement sous contrôle des hormones thyroïdiennes

La métamorphose chez les amphibiens est sous contrôle hormonal. Les hormones thyroïdiennes (TH) jouent un rôle important dans la régulation de la croissance et du développement et dans le maintien de l'homéostasie métabolique. La production d'hormones thyroïdiennes, responsable du remodelage des tissus commence peu après l'éclosion et le taux de thyroxine (T4) et tri-iodothyronine (T3) augmente au cours du développement larvaire pour atteindre un pic peu avant la métamorphose (Tableau 9)(Galton, 1992).

Tissu	Réponse	
	<i>Morphologique</i>	<i>Biochimique</i>
Cerveau	Re-structure, croissance des axons, renouvellement cellulaire	Division cellulaire, synthèse de protéines
Foie	Différentiation fonctionnelle, re-structuration	Induction d'albumine et d'enzymes du cycle de l'urée, différence de production d'hémoglobine
Œil	Repositionnement ; nouveaux neurones rétiniens ; modification du cristallin.	Changement de pigment visuel ; induction de la β -cristalline
Peau	Re-structuration ; kératinisation ; formation de glandes granuleuses	Induction du collagène de la kératine 63 kDa, de la magainine.
Membres, poumons	Morphogénèse <i>de novo</i> d'os, de peau, de muscle, de nerfs ect.	Prolifération cellulaire ; expression génétique
Queue, branchies	Remodelage majeur des tissus	Mort cellulaire programmée ; induction d'enzymes lytiques
Intestin, pancréas	Régression et élimination totale des tissus	Nouveaux constituants structurels et fonctionnels
Système immunitaire	Redistribution des populations de cellules immunitaires	Acquisition d'une nouvelle immunocompétence
Muscle	Croissance, différenciation, apoptose	Induction de la chaîne lourde de la myosine

Tableau 9 : Diversité des réponses morphologiques et biochimiques aux hormones thyroïdiennes pendant la métamorphose des amphibiens, adapté de Tata, 1998

Les signaux environnementaux (lumière, température, compétition, prédation) déterminent le relargage de ces hormones par l'axe hypothalamo-hypophysaire (**Figure 51**). Un grand nombre de produits chimiques synthétiques sont libérés dans l'environnement, et on sait que beaucoup d'entre eux interfèrent avec la fonction normale des hormones thyroïdiennes (TH) (Chai et al., 2017; Couderq et al., 2020). Par exemple, la métamorphose des têtards d'amphibiens étant un processus de développement déclenché et contrôlé par les TH peut se révéler utile comme outil de détection d'effet des polluants environnementaux soupçonnés de perturber les fonctions des TH (Thambirajah et al., 2019). On pense que la perturbation du TH est causée par divers mécanismes, notamment l'augmentation du métabolisme de la thyroxine (T4) par les uridine diphosphate glucuronyl transférases (UDPGT), le blocage de la signalisation du TH par les récepteurs du TH (TR) et l'induction de la transition de perméabilité de la membrane mitochondriale (MPT) (Thambirajah et al., 2019).

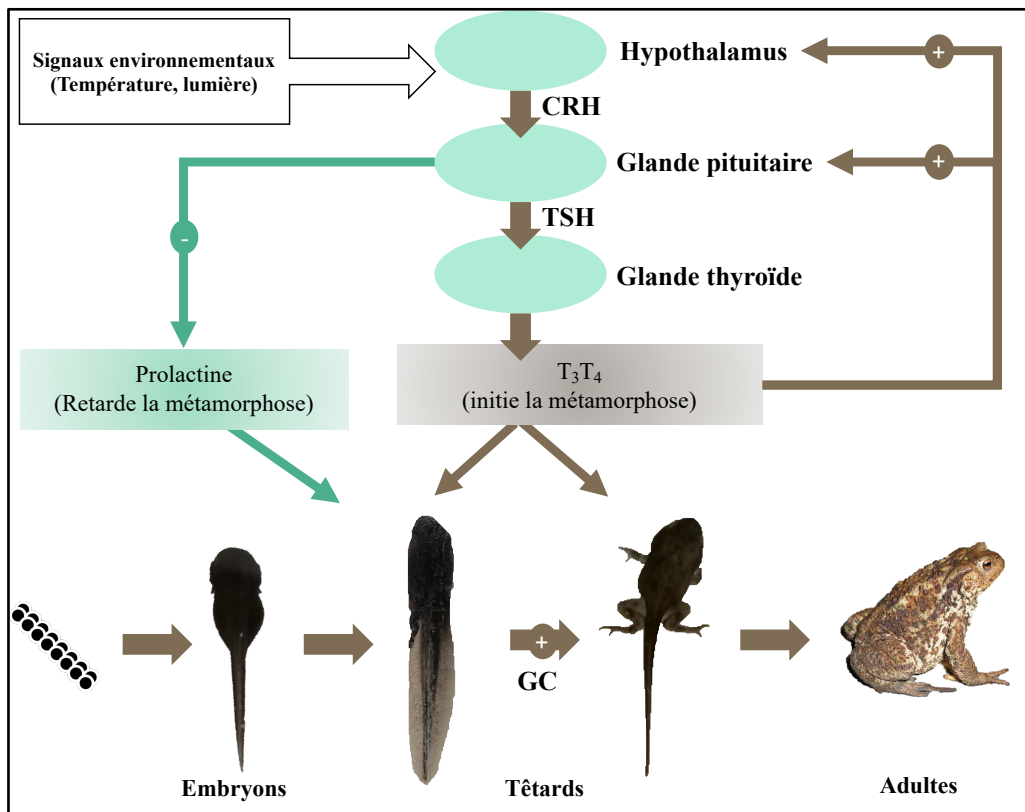


Figure 51 : Représentation schématique de la régulation hormonale de la métamorphose chez les amphibiens. En réponse à des signaux environnementaux, la glande activée pour produire les hormones thyroïdiennes T₄ (L-thyroxine) et T₃ (tri-iodothyronine) par les hormones hypothalamiques et hypophysaires TRH (thyrotrophin-releasing hormone), CRF (corticotrophin-releasing factor) et TSH (thyroid stimulating hormone). L'hormone thyroïdienne (TH) est obligatoirement requise pour initier et maintenir la métamorphose, son action étant potentialisée par les glucocorticoïde (GC) et retardée par la prolactine. Adaptée de Tata, 1998

Comme on a pu le voir, les contaminants étudiés dans cette thèse montrent des altérations aux niveaux enzymatiques et biochimiques se répercutant sur les traits de développement. On peut donc s'attendre à ce que des hormones sécrétées pendant le développement soit impactées par un xénobiotique.

Alors que la plupart des facteurs de stress environnementaux entraînent une augmentation de l'activité ou de la production de TH par l'activation des hormones de stress, il a également été démontré qu'un grand nombre de contaminants aquatiques inhibent l'action normale des TH chez les amphibiens, entraînant des modifications de la croissance, du développement et du métabolisme (voir Mann et al. 2009 ; Kashiwagi et al. 2009 ; Carr et Patiño 2011). Suite à nos expositions à l'AMPA et nicosulfuron sur les têtards de *Bufo spinosus*, on peut se demander si ces composés interfèrent avec la synthèse et la production de TH. Nous avons donc récolté des têtards post-éclosion pour détecter si les niveaux de T₃/T₄ étaient déjà perturbés par

l'exposition à la contamination. Si on détecte des signaux différents entre têtards traités et non traités dès l'éclosion, on peut s'attendre à ce que les différences observées pendant le stade larvaire en termes de morphologie et développement soient expliquées par cette perturbation de la balance hormonale. La mise en place et la calibration de la détection de ces hormones spécifiques a été mis en place en milieu d'année 2020 et devrait être effective vers le mois d'octobre 2021.

D) Coût de la contamination

Si notre hypothèse sur la mortalité sélective se vérifie, il est possible que l'utilisation à long terme du glyphosate ait agi comme une puissante pression sélective, expliquant ainsi pourquoi notre espèce peut persister dans les paysages agricoles (Guillot et al. 2016, **Annexe III**).

La mortalité sélective peut avoir des conséquences sur les embryons qui ont survécus et peut venir avec un coût en termes de réduction de compétence immunitaire, retard de croissance et une espérance de vie réduite (Bonier et al., 2009; Shalev et al., 2013). Si la mortalité pendant les premiers stades de vie est sélective, les variations phénotypiques qui en résultent peuvent avoir un effet important sur la survie future. Les pesticides sont faits pour rapidement affecter les espèces nuisibles et les adaptations génétiques comme la tolérance ou la résistance aux pesticides peuvent rapidement apparaître dans une population (Brausch & Smith, 2009; DiGiacopo & Hua, 2020; Jansen et al., 2011; Lopes et al., 2008). Les propriétés physicochimiques de l'AMPA lui permettent de rester dans les environnements non-cibles, il est donc attendu que seuls les individus les moins sensibles peuvent survivre dans les environnements contaminés par l'AMPA. Il est donc crucial d'évaluer les coûts de la résistance qui peuvent affecter la fitness et les interactions de la population (Athrey et al., 2007; Jansen et al., 2011).

En effet, les individus qui prospèrent dans des environnements contaminés peuvent montrer des baisses de fitness dans les zones sans contamination (DiGiacopo & Hua, 2020; Lenormand et al., 1999). Les coûts métaboliques associés aux mécanismes de détoxification, la sécrétion de protéines protégeant du stress oxydant et les schémas d'allocation d'énergie peuvent impacter la fitness si les mécanismes sont aussi exprimés dans des environnements sans contamination (Athrey et al., 2007). A l'inverse, des études ont suggéré que la résistance adaptative aux pesticides peuvent avoir des avantages de fitness plus tard dans la vie dans les environnements non contaminés (Arnaud & Haubruge, 2002). Par exemple, les populations de grenouilles des bois (*Lithobates sylvaticus*) de milieux agricoles ont une résistance accrue à

un insecticide largement utilisés sans induire de coûts de fitness et de performance (Cothran et al., 2013). En sachant que les populations de crapaud épineux persistent et se reproduisent en milieux agricoles (Adams et al., 2021; Guillot et al., 2016; Janin et al., 2012), les réponses évolutives suivant l'introduction de pesticides peuvent avoir modifié la tolérance à travers la variation génétique (Nusbaumer et al., 2021). Un axe intéressant à explorer serait de tester si la tolérance ou la résistance adaptative à l'AMPA ou au nicosulfuron peuvent avoir un effet sur les marqueurs de stress oxydatif et la dynamique des télomères pendant les premiers stades de développement.

Conclusion

Cette thèse a pour but de déterminer comment les conséquences indirectes (homogénéisation et contamination) de l'agriculture intensive impactent une espèce sentinelle de son environnement, le crapaud épineux. En prenant en compte tous les phases de développement de cette espèce, nous avons pu déterminer que les réponses en termes de morphologie, physiologie, comportement et développement sont directement impactés par ces facteurs. De plus, nous avons montré que des concentrations environnementales dites sublétales entraînaient des réponses concentration-effet différentes avec des réponses non-monotones pour le métabolite du glyphosate et des réponses linéaires classiques pour le nicosulfuron. L'exposition à ces contaminants altèrent les processus biologiques à plusieurs niveaux d'intégration : cellulaires (perturbation du statut oxydant), individuelles (perturbation du comportement, altération de trait de développement) et populationnel (baisse de la fitness). De plus, la perturbation du développement liée à la contamination pourrait expliquer les gammes de variations de phénotypes retrouvées entre les habitats agricoles ou forestiers chez les mâles adultes en période de reproduction. L'étude future sur l'exposition chronique des adultes de crapaud épineux devrait nous informer sur les effets directs de la contamination par les deux contaminants d'intérêts. L'exposition par le derme représentant une voie de contamination possible chez les amphibiens.

Par ailleurs, il est crucial de souligner que ces expérimentations se concentrent sur un stress simple mais qu'en milieux naturels les organismes sont soumis à des stress multiples. Ils peuvent se manifester par des combinaisons de facteurs biotiques ou abiotiques. Par exemple, le changement climatique pourrait entraîner des effets synergiques entre contamination et augmentation des températures. De plus, l'utilisation de produits phytosanitaires n'est pas limitée qu'à un seul produit mais à une multitude d'intrants chimiques qui se retrouvent dans les milieux non-cibles résultant en un effet cocktail menaçant les populations. Une voie de recherche prometteuse serait l'intégration de multiples facteurs dans les études d'impact des contaminants sur les espèces.

Pour finir, les résultats de cette thèse soulignent l'importance de prendre en compte des concentrations environnementales pertinentes pour mesurer les effets sur la biodiversité en comparaison aux doses létales fixant les normes gouvernementales.

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Article I

Matthias Renoirt, Frédéric Angelier, **Marion Cheron**, Paco Bustamante, Yves Cherel, François Brischoux

Stable isotopes of a terrestrial amphibian illustrate fertilizer-related nitrogen enrichment of food webs in agricultural habitats

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Short communication

Stable isotopes of a terrestrial amphibian illustrate fertilizer-related nitrogen enrichment of food webs in agricultural habitats

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ABSTRACT

To comprehensively assess the impacts of agricultural practices on biodiversity in complex landscapes mixing both agricultural habitats and remnants of other (presumably more favorable) types of habitats, a prerequisite is to evaluate to which extent agricultural habitats are actually used by a given species. Here, we tested whether the stable isotope method can help to discriminate habitat use of a wild vertebrate, the spined toad (*Bufo spinosus*). We expected habitat to influence their $\delta^{13}\text{C}$ values and the use of fertilizers to increase $\delta^{15}\text{N}$ values of individuals from agricultural landscapes. Based on 114 toads from seven sites characterized by contrasted habitats (agricultural, forest or mixed habitats), we found that toad blood $\delta^{15}\text{N}$ values were positively related to agricultural surface area, a result that was corroborated by diverging blood $\delta^{15}\text{N}$ values between habitat categories. Conversely, toad $\delta^{13}\text{C}$ values did not vary according to the habitat. Our results suggest that isotopic values (especially $\delta^{15}\text{N}$) could be a powerful tool to assess agricultural habitat use in terrestrial taxa. Further studies should usefully investigate whether individual $\delta^{15}\text{N}$ values can be used as a fingerprint of other constraints of agricultural habitats (e.g., contaminants) in agricultural landscapes.

1. Introduction

Anthropogenic activities are considered as the main factors responsible for the current loss of biodiversity (Chapin et al., 2000; Myers and Knoll, 2001; Brooks et al., 2002). Among these anthropogenic changes, modern agricultural practices have been shown to negatively influence biodiversity both directly and indirectly. The direct negative impacts of agriculture on flora and fauna are mainly linked to the destruction and simplification of habitat structures (Fahrig, 2003). Indirect effects are mainly mediated by the increasing reliance on chemical inputs that aim at improving crop productivity (Köhler and Triebkorn, 2013). For instance, the use of large amounts of fertilizers can eventually lead to disruptions of ecosystem functioning (Huang et al., 2017). In addition, the toxic effects of pesticides on non-target components have attracted considerable interest (Köhler and Triebkorn, 2013). Clearly, both direct and indirect effects are expected to affect the species inhabiting agricultural landscapes (McLaughlin and Mineau, 1995; Köhler and Triebkorn, 2013).

In some cases, it is relatively straightforward to assess the consequences of these habitat modifications on the ecology of animal species (e.g., when nesting trees or shrubs are lacking for birds, Mohring et al., 2021). Yet, in most cases, agricultural landscapes will be intersected with remnants of other types of habitats (e.g., small woods, hedgerows) that should allow the persistence of populations. In these cases, assessing the actual use of agricultural habitats versus remnant of native habitats is logistically complicated, especially when the species under focus is relatively mobile (i.e., most animal species). Nonetheless, in order to comprehensively assess the consequences of agricultural practices on biodiversity, it is necessary to evaluate to which extent such habitat is actually used by a given species (Street et al., 2016).

Stable isotopes can provide insights in this respect (Robinson, 2001; Rubenstein and Hobson, 2004; Perkins et al., 2014; Newton, 2016). The concept of the isotopic niche is based on the fact that an animal's chemical composition is influenced by what it consumes (Fry, 2006). Stable nitrogen isotope values ($\delta^{15}\text{N}$) are mostly used as a proxy of trophic position, but can be also a relevant proxy of consumers' foraging

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Table 1Summary of the sampling design (habitat categories, number of individuals) and of the corresponding toad blood mean (\pm standard deviation) of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values.

Sites	Categories	n	Agricultural surface area (ha)	$\delta^{15}\text{N}$ (mean \pm sd)	$\delta^{13}\text{C}$ (mean \pm sd)
1	Forest	16	0	0.49 \pm 0.54	-24.79 \pm 0.26
2	Forest	21	15	1.33 \pm 1.61	-23.93 \pm 0.53
3	Mixed	16	54	2.52 \pm 1.83	-23.62 \pm 0.38
4	Mixed	20	104	2.72 \pm 1.70	-24.41 \pm 0.59
5	Agricultural	19	260	3.43 \pm 1.64	-24.35 \pm 0.47
6	Agricultural	3	261	7.28 \pm 0.20	-23.10 \pm 0.76
7	Agricultural	19	286	4.77 \pm 1.86	-24.45 \pm 0.63

habitat (Kelly, 2000). In our context, because fertilizers widely used in agriculture show relatively high $\delta^{15}\text{N}$ (e.g., manure and compost [$\delta^{15}\text{N}$ up to 16.2‰], ammonium sulphate [$\delta^{15}\text{N}$ up to 6.6‰] and ammonium nitrate [$\delta^{15}\text{N}$ up to 2.2‰], Bateman and Kelly, 2007), it is expected that trophic chains influenced by fertilization will be enriched in $\delta^{15}\text{N}$ (Anderson and Cabana, 2005; Bateman and Kelly, 2007). As a consequence, individuals relying on agricultural areas to forage should display higher $\delta^{15}\text{N}$ values than individuals using other types of habitats. In contrast, stable carbon values ($\delta^{13}\text{C}$) vary little along the food chain, and often depend on foraging habitats ($\delta^{13}\text{C}$ source). Typically, $\delta^{13}\text{C}$ varies among specific primary producers (Farquhar et al., 1989), and we can use this parameter to examine differences in trophic support and thus presumably habitats.

In this study, we tested whether blood $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values can help to discriminate the foraging habitats of a wild vertebrate, the spined toad (*Bufo spinosus*). This species is particularly well suited to test these hypotheses for several reasons. First, this widespread amphibian can live in a variety of habitats and persist even in highly modified agricultural areas (Guillot et al., 2016, see also Salazar et al., 2016; Leeb et al., 2020). Second, spined toads forage for invertebrates and its prey spectrum has been shown to be highly conserved between habitats (Zamora-Camacho and Comas, 2017). Third, the terrestrial part of the life cycle occurs within 1 km from the breeding (sampling) sites, which allow a straightforward classification of the surrounding landscapes potentially used in the day-to-day life of individuals (Janin et al., 2011; Guillot et al., 2016). Finally, the remarkably long lifetime of erythrocytes of amphibians (Altland and Brace, 1962) indicates that stable isotopes from red blood cells actually reflect habitat use during the terrestrial life of our study species prior to breeding (see also Cloyed et al., 2015). In order to test whether isotopic values of spined toads can discriminate their foraging habitats, we assessed $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of red blood cells (herein blood) from 114 individuals from seven sites ranging from forested areas to highly agricultural sites.

2. Material and methods

2.1. Study species

In Western Europe, Spined toad (*Bufo spinosus*) is one of the most common species of amphibians. As most anuran species, spined toads have a biphasic life-cycle with an extensive use of terrestrial habitats during most of the year, and a short breeding season (\sim 1 month) in ponds. During breeding, male toads massively migrate towards ponds where they wait for females (Brischoux and Cheron, 2019) and a large number of males can be easily sampled at each pond.

2.2. Study sites and sampling

Sampling took place in February 2020 on seven breeding ponds situated in the south of the “Département des Deux-Sèvres” nearby the laboratory (46° 8'48.64"N; 0°25'30.86"W). Two sites were located in highly forested areas, three sites in agricultural landscapes, and the

remaining two sites at the interface between forested areas and agricultural areas (Appendix 1). Distances between different sites within a habitat type (e.g., \sim 18 km between the two forested sites; at least \sim 12 km between agricultural sites) were large enough to minimize spatial autocorrelation (Janin et al., 2011; Guillot et al., 2016). Such site selection allowed making simple habitat classifications (Table 1). Using QGIS.org 2.18.2, 2016 and satellite images (Google Earth), we drew a buffer around each pond (1000 m radius spanning the spatial scale traveled by toads during the breeding migration, Janin et al., 2011; Guillot et al., 2016) and we extracted the surface area of agricultural fields (Table 1).

We focused our sampling on the first breeding males arriving at each breeding sites. Captures occurred at night using a headlamp to locate individuals. Upon sighting, each toad (total N = 114, 3–21 different individuals per sampling sites, Table 1) was captured with a net, and a blood sample was collected (approx. 100 μ l) via cardiocentesis using a 1 ml syringe and a 30-G heparinized needle (Brischoux et al., 2018). All individuals were released at their location of capture after blood collection.

2.3. Stable isotope analyses

Whole blood was centrifuged, and red blood cells subsequently stored at -20 °C until analysis. Isotopic analyses were carried out on freeze-dried red blood cells at the LIENSs (La Rochelle, France). Aliquots of \sim 0.3 mg dry mass were analyzed with a continuous flow mass spectrometer (Thermo Scientific Delta V Advantage) coupled to an elemental analyzer (Thermo Scientific Flash EA 1112). Results are in δ notation relative to Vienna PeeDee Belemnite and atmospheric N_2 for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively. Internal laboratory standards (acetanilide) were used to check accuracy. Measurement errors were $<0.15\%$ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values.

2.4. Statistical analyses

All data were tested for homogeneity of variance, residues independence and normality with the Bartlett test, Dubin-Watson test and Shapiro-Wilks test, respectively. We also checked the residues normality using diagnostic plots. All statistical analyses were carried out with R. Studio v 1.2.1335 (R Core Team, 2019). We fitted linear mixed models (LMER, package lmerTest, Kuznetsova et al., 2015) to assess differences in blood $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values across agricultural surface area, with “sites” as a random factor. We analyzed these models with variances analysis (ANOVA). We also analyzed differences in blood $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values between categories (see habitat classification). We followed these analyses by post-hoc tests to performed pair-wise comparisons between categories using Tukey-Kramer tests for unbalanced sample sizes (implemented in the TukeyHSD function).

A site was characterized by very low sample size (N = 3, site #6, Table 1, Appendix 1). Excluding this site from our analyses yielded similar results and we present results with the whole dataset below.

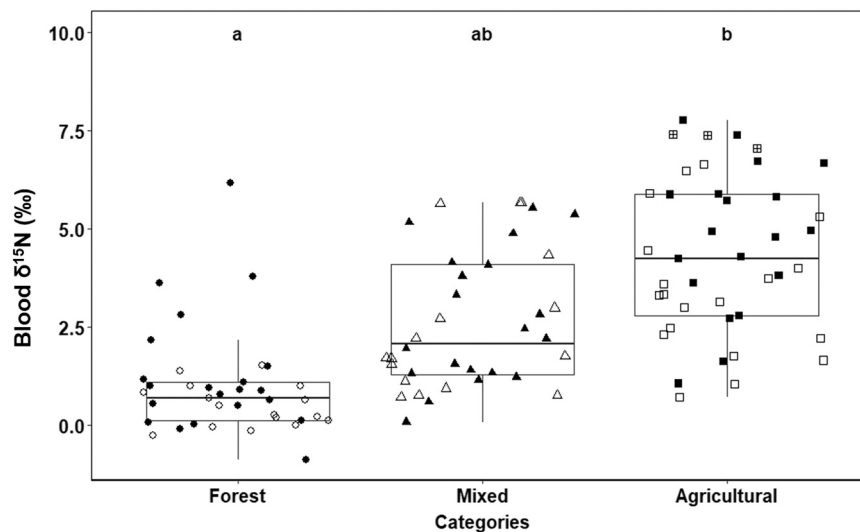


Fig. 1. Blood $\delta^{15}\text{N}$ values of toads from forest, mixed and agricultural habitats. The bottom and top of the boxes represent the first and third quartile, the line across the box represents the median, the whiskers represent the minimum and maximum values. The circles, triangles and squares represent individual data points: \circ Site 1, \bullet Site 2, \blacktriangle Site 3, \triangle Site 4, \blacksquare Site 5, \boxplus Site 6, \square Site 7. Different letters indicate significant differences.

3. Results

Blood $\delta^{15}\text{N}$ values were positively related to agricultural surface area ($F_{1,107} = 16.022$, $p < 0.001$). Blood $\delta^{15}\text{N}$ values were significantly different between habitat types ($F_{2,107} = 6.967$, $p < 0.001$, Fig. 1) with post-hoc tests showing that $\delta^{15}\text{N}$ of toads from forest habitats were significantly lower than $\delta^{15}\text{N}$ of toads from agricultural habitats ($p < 0.001$, Fig. 1), while individuals from mixed habitats were not different from the two other categories (both $p > 0.089$, Fig. 1).

Blood $\delta^{13}\text{C}$ values did not vary according to the agricultural surface area ($F_{1,107} = 0.120$, $p = 0.730$). Accordingly, $\delta^{13}\text{C}$ values were similar between habitat types ($F_{2,107} = 0.201$, $p = 0.81$).

4. Discussion

Overall, we found that habitat type influences isotopic values of toads. Blood $\delta^{15}\text{N}$ values were positively related to agricultural surface area. These results were corroborated by diverging toad $\delta^{15}\text{N}$ values between habitat categories with lower $\delta^{15}\text{N}$ values found in individuals from forest habitats and higher $\delta^{15}\text{N}$ values found in toads from agricultural areas. Conversely, blood $\delta^{13}\text{C}$ values of toads did not vary according to the habitat.

Toads that breed in ponds surrounded by agricultural environments, and thus likely living in such environments (Guillot et al., 2016; Salazar et al., 2016; Leeb et al., 2020), were characterized by higher blood $\delta^{15}\text{N}$ values than individuals breeding in ponds surrounded by forest. Two different hypotheses could explain such results. First, toads from agricultural landscapes may forage on different food items (higher in the trophic web) than individuals living in forest. This hypothesis seems unlikely as toad diet remains similar amongst habitats (Zamora-Camacho and Comas, 2017). In addition, the remarkably large variation of $\delta^{15}\text{N}$ values between habitat types (-0.9 to 7.9% , Fig. 1), spans approximately 2.1–2.7 theoretical trophic levels (DeNiro and Epstein, 1981). Although proportional abundances of invertebrates of different trophic levels may differ between habitat types (but see Zamora-Camacho and Comas, 2017), it can hardly explain the large variation of $\delta^{15}\text{N}$ - hence “trophic levels” - we found. Detailed analyses of the diet of toads and the $\delta^{15}\text{N}$ values of prey items in each habitat (see Perkins et al., 2014 for an example of food web stable isotopic table) are required to test this hypothesis. More likely, agricultural fertilization leads to very high amounts of ^{15}N -enriched fertilizers (e.g., manure, compost, ammonium sulphate and to a lesser extent ammonium nitrate,

Bateman and Kelly, 2007) deposited on arable land (Tamm, 2012). Relatively high $\delta^{15}\text{N}$ values of these fertilizers increases $\delta^{15}\text{N}$ baselines that propagate through the trophic webs in agricultural landscapes (Anderson and Cabana, 2005). Such process has already been highlighted in tadpoles developing in waters with high nitrate concentrations (Trakimas et al., 2011).

It is important to emphasize the large variations in $\delta^{15}\text{N}$ values between individuals from the same habitat type or study site (Table 1, Fig. 1). Such result seems to suggest different individual strategies of (micro-) habitat use to forage within a similar landscape (Miaud and Sanuy, 2005; Indermaur et al., 2009). This indicates that the influence of nitrogen fertilization on toad $\delta^{15}\text{N}$ values is spatially restricted. Such result suggests that, at least in our setting, $\delta^{15}\text{N}$ values can help to understand the use of micro-habitats, movement and/or dispersal and individual strategies in agricultural landscapes (Rickers et al., 2006; Dammhahn and Goodman, 2014). Future studies using both individual tracking (i.e. radio-tracking) and isotope analyses will be critical to test for this hypothesis.

In contrast to $\delta^{15}\text{N}$ values, our results showed that blood $\delta^{13}\text{C}$ values did not differ between toads from agricultural habitats and individuals living in forest. It is likely that the surface area occupied by corn fields (the main C4 plant expected to influence $\delta^{13}\text{C}$ values in agricultural habitats, Schwertl et al., 2005) is too low to significantly influence $\delta^{13}\text{C}$ values of individual toads using agricultural habitats: In our study area, corn crop represents only $\sim 4.5\%$ of agricultural surface area (Agreste, 2016). Future studies are required to investigate both the presence of toads and their potential prey in different crop types. Additionally the prey of toads could be sampled to assess their trophic level in each habitat to provide a better understanding of the habitat’s nutrient flow and its possible influence on toads’ use of various habitats (Perkins et al., 2014). Finally, future studies could incorporate other isotope markers such as sulphur or oxygen to further determine the nutrients in each habitat’s food web (Penna et al., 2020). Complementary approaches such as compound-specific isotopic analyses of amino acids may well prove useful to assess both fertilizer-related (e.g., phenylalanine) and trophic-related (e.g., glutamic acid) effects on $\delta^{15}\text{N}$ (McMahon and McCarthy, 2016).

To conclude, we suggest that $\delta^{15}\text{N}$ values could be a powerful tool to assess habitat use in a terrestrial meso-predator such as the spined toad. Whether such approach could be used on other taxa living in agricultural landscape deserve further investigations. Interestingly, $\delta^{15}\text{N}$ values, as an index of agricultural habitat use, may also help to reveal individual

susceptibility to disturbances linked to modern agricultural practices (i. e., pesticides). Futures studies should usefully investigate whether individual $\delta^{15}\text{N}$ values can be used as a fingerprint of concentrations of contaminants in agricultural landscapes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.agee.2021.107553](https://doi.org/10.1016/j.agee.2021.107553).

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Article II

Marion Cheron, Frédéric Angelier, Cécile Ribout, Charline Parenteau,
François Brischoux

Habitat influence morphological and physiological allometric scaling in a widespread amphibian species

In preparation for Science of Total Environment



The tailless batrachians of Europe. London :Printed for the Ray society,1897-98.

Habitat influence morphological and physiological allometric scaling in a widespread amphibian species

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Abstract

Landscape homogenization is one of the primary consequences of agricultural intensification, and the simplification of habitat structure, as well as increased use of agrochemicals are expected to influence the biodiversity that persist in agricultural landscapes. In order to comprehensively investigate the morphological and physiological correlates of living in agricultural habitats for wildlife, we used an integrative multi-component approach on a widespread terrestrial amphibian, the spined toad. We measured several morphological traits linked to morphology, mobility, antipredator ability, secondary sexual characters as well as markers of individual quality, metabolism and sexual activity in individuals from three contrasted habitat types (agricultural, forest or mixed). Interestingly, our results highlighted a strong contrast between simple comparisons of traits between habitat types (with almost no differences between habitats) and the strong habitat-mediated effects on allometric scaling of the same traits. We found that adult male toads originating from different habitats differed in the slopes and intercepts of allometric scaling of morphological features linked to locomotion or secondary sexual characteristics. In addition, the scaling of physiological and molecular indices of quality to body size and body condition also diverged between habitat types. Importantly, our study allows to identify critical avenues for future research including the effects of developmental (aquatic) habitats, the causes of the differences we found and the potential consequences in term of population dynamics. Answering these challenging questions will be critical if we are to comprehensively assess the consequences of anthropogenic activities on wildlife.

Key-words: Agrosystems, amphibian, allometry, testosterone, telomeres

Introduction

Since the beginning of the 20th century, anthropogenic pressures on the environment have constantly increased, and have modified ecosystem functioning (Kareiva et al., 2007; Moreno-Mateos et al., 2017). Strong changes in land-use priorities have changed the landscapes from natural or ancestral rural habitats to large scale agricultural and urban systems (Foley, 2005; Kareiva et al., 2007). For instance, in Europe, modern agricultural practices implemented post World War II have induced agricultural intensification (Tscharrntke et al., 2005). This intensification of agriculture was characterised by land consolidation, which has homogenised the landscapes, from mixed crop small fields and meadows bordered by hedges (hedgerows), to extended monoculture fields (Benton et al., 2003). In addition, modern agricultural practices are also characterized by the use of various agrochemicals which range from fertilizers to toxic pesticides (Poissant et al., 2008; Sharma et al., 2019). Overall, these changes linked to agricultural practices are now well recognized driving forces in the global biological diversity loss (Backhaus et al., 2012; Chopin et al., 2019; Kendall et al., 2016).

The impacts of habitat structure due to landscape homogenisation have been relatively well documented in some taxa (Chopin et al., 2019; Collins & Fahrig, 2017; Endenburg et al., 2019). Indeed, the spatial structuration of the habitat can influence the richness and composition of the plant community (shrubs, trees, and herbaceous strips), which in turn affect organisms from higher trophic levels (e.g., insects, birds, reptiles, mammals, Dominik et al., 2018; Fabian et al., 2013; Foster et al., 2019; Neumann et al., 2016). In animals, landscape homogenisation can negatively affect habitat use (Bjørneraas et al., 2012; Janin et al., 2012) or interspecific interactions (Robertson et al., 2013; Zarnetske et al., 2017) and ultimately species occurrence and population densities (Fahrig, 2007).

Importantly, despite this overall, worrying trend of lower species occurrence and/or population densities in agricultural landscapes, many species actually persist in such altered habitats (Collins & Fahrig, 2017). This suggests that these species can respond to the novel constraints linked to habitat structure through processes such as phenotypic plasticity, local adaptation, or a combination thereof (Edelaar et al., 2017; Lind & Johansson, 2011). For instance, morphological traits can respond to environmental pressures such as the physical structure of a habitat type (Grient & Rogers, 2019), and such process has been documented in insects (Gibb & Parr, 2013; Jiang et al., 2019), fishes (Foster et al., 2019; Franssen, 2011), or amphibians and reptiles (Bókony et al., 2019; Collar et al., 2010; Sparkman et al., 2018) in response to the physical attributes of agricultural habitats (Bókony et al., 2019; Guillot et al., 2016; Li et al., 2019). For example, homogenised habitats (as compared to dense hedgerows or forest habitats) can influence the attributes required to move, and thus to forage or to evade predation efficiently (Aviron et al., 2005; Trochet et al., 2019). In addition, lower availability of suitable micro-habitats to shelter (Lecq et al., 2017) may also influence susceptibility to predation and thus affect locomotor ability, anti-predator attributes, activity rhythms, and behaviour (Hansen et al., 2019; Vanhooydonck & Van Damme, 2003; Vasquez, 2002). Availability or types of food resources can also differ in agricultural habitats as compared to more natural landscape (Butet et al., 2010; Butler & Gillings, 2004; Červinka et al., 2013), and such variation has been shown to influence breeding phenology, reproduction traits and population dynamics in aerial insectivores (Paquette et al., 2013). Finally, diverging (micro-) climatic conditions (temperature, hygrometry) between open landscapes such as intensive agricultural habitats as compared to close forested habitats (J. Chen et al., 1993) have also been shown to influence critical traits such as reproductive phenology (Pincebourde et al., 2016), behaviour (Senior, 2020), thermal tolerance (Tougeron et al., 2016), or morphological attributes required to cope with constraining climate (e.g., surface area to

volume ratio, Ray, 2016). Overall, the simplification of the physical structure linked to intensive agriculture, and the resulting climatic consequences are likely to play a role on the ecology, behaviour and morphology of species that persist in such habitats.

Such plastic and/or adaptive responses of organisms that remain in agricultural habitats are not limited to the morphological (or behavioural) shifts mentioned above. In this respect, organismal physiology is also expected to vary between habitat types. Such variation of physiological responses can be linked to the parameters highlighted above. For instance, lower availability of suitable micro-habitats to shelter can influence susceptibility to predation and thus influence physiological processes linked to the stress response (Fardell et al., 2020; Mateo, 2007). Indeed, higher levels of glucocorticoids (the main stress hormones) have been shown to occur in species from agricultural habitats, as compared to their counterparts from other types of habitats (Bókony et al., 2021; Janin et al., 2011). Diverging climatic conditions can also influence several physiological processes (Qu & Wiens, 2020), including glucocorticoid levels (de Bruijn & Romero, 2018) which are also involved in energy allocation (Crespi et al., 2013; Emery Thompson, 2017; Kim, 2008; Sapolsky et al., 2000). Yet, physiological variations linked to agricultural habitats encompass a wider range of underlying mechanisms. Indeed, modern agriculture is characterised by the use of numerous agrochemicals, many of which are now classified as, or suspected to be endocrine disruptors (Hayes et al., 2006; Leemans et al., 2019). Although investigations on the endocrine consequences of agrochemicals have mainly been performed in laboratory conditions with model organisms (Hahn & Sadler, 2020; Segner & Baumann, 2016), there is now growing evidence that disruption of physiological systems linked to exposure to agrochemicals occur in wild organisms (Bókony et al., 2017; Hayes et al., 2006; Trudeau et al., 2020). Endocrine disruption of some physiological systems can bear strong consequences on individual fitness and thus population persistence (Hayes et al., 2006). For instance,

disruption of corticosterone (a major glucocorticoid involved in organismal allostasis, Bonier et al., 2009; Sapolsky et al., 2000) secretion can influence oxidative status and lead to cellular damages (Costantini et al., 2011). In turn, elevation of oxidative stress can induce damages to DNA as shown with increasing telomere attrition (Reichert & Stier, 2017) which can be used as a proxy of cellular senescence (Bize et al., 2009). Although telomere shortening occurs normally during cell division, this process can be magnified under stressful conditions (i.e., environmental contamination) especially when they are associated with increased oxidative stress (Reichert & Stier, 2017). Given the strong links that exist between organismal ecology, behaviour, morphology and the underlying physiological processes, it seems essential to incorporate such parameters to comprehensively investigate the consequences of living in agricultural habitats for wildlife.

To the best of our knowledge, very few studies have comprehensively and conjointly investigated the morphological and physiological correlates of living in agricultural habitats in wildlife (Chopin et al., 2019; Guillot et al., 2016). In this study, we investigated the impact of agricultural habitats on a widespread species, the spined toad, *Bufo spinosus*, using an integrative multi-component approach. This species is particularly well-suited for such investigations for several reasons. First, this species occurs in Western Europe where agricultural intensification has been relatively intense (Foley, 2005). Spined toad is a generalist species that can live in a variety of habitats and, importantly, has been shown to persist even in highly altered agricultural landscapes (Adams et al., 2021; Guillot et al., 2016). Such context allows to perform straightforward comparisons between habitats types. Second, as most amphibians, it displays a high level of phenotypic plasticity and thus allows to investigate the influence of habitat on phenotype (Bókony et al., 2019). Third, as most ectotherms, this species is characterized by an indeterminate growth (Duellman & Trueb, 1994), allowing to have access to a large range of body size and thus to investigate allometric

relationships. Fourth, this species is characterised by explosive breeding where large numbers of adult males gather at breeding sites (Janin et al., 2011), thereby allowing to obtain the relatively large sample sizes required to obtain robust results. For this reason, our investigations are restricted to male solely. Fifth, in adult individuals, most of the annual cycle occurs on land within ~1km from the breeding sites indicating that individuals are pertinent indicators of the potential effects of the surrounding habitats (Hartel & Demeter, 2015; Kovar et al., 2009).

Based on 150 male toads originating from 8 sites representing 3 habitat categories (forest, intensive farmland and mixed habitats), we measured several morphological traits linked to morphology (body size), individual quality (body mass, body condition), mobility (forearm and hindlimb length), antipredator ability (parotoid glands), secondary sexual characters (forearm width, nuptial pads). In addition, we included in our investigations molecular markers linked to individual quality (telomere length) and hormonal markers of metabolism and sexual activity during reproduction in this species (Brischoux et al., 2018). In order to comprehensively investigate the influence of habitat types on the morphology and physiology of wild toads, we adopted two complementary sets of analyses. First, we compared the morphological and physiological parameters under focus between habitat types in order to assess whether toads from agricultural areas differed from their forested counterparts (Guillot et al., 2016). Second, because of the wide range of body size available in adult individuals (see above), we investigated whether morphological and physiological scaling varied between habitat types. Indeed, allometric relationships between traits might differ in slope and/or intercept with differences in slopes indicating unequal morphological/physiological changes with increasing body size and differences in intercepts indicating variations in proportions of morphological/physiological traits. In this respect, we expected that morphological and physiological traits can covary with the body size (i.e.,

body mass, limbs length, parotoid glands size, secondary sexual characters, hormonal levels and telomere length) but also with the body condition (i.e., parotoid glands size, secondary sexual characters, hormonal levels and telomere length).

Materials and methods

Study species

Spined toad (*Bufo spinosus*) is one of the most common species of anurans in Western Europe. It is a generalist species which can occur in a diversity of habitats, including intensive agriculture (Guillot et al., 2016). In early spring, toads emerge from hibernation and migrate towards breeding sites. Males arrive early at breeding ponds where they remain throughout the entire reproductive period whereas females leave shortly after mating and egg-laying. As a consequence, male outnumber females and their abundance allow to access large sample sizes. Hence, our study is focused on adult males solely (Reading & Clarke, 1983). In order to avoid morphological bias linked to individual phenology (e.g., earlier individuals are usually smaller, (Brischoux & Cheron, 2019; Gittins et al., 1980; Semlitsch et al., 1993), we focused our sampling on the first males arriving at each study sites.

Study sites and classification

Sampling took place between early February and mid-March on 8 breeding sites in Deux-Sèvres (France). From aerial pictures of each study site, we drew buffers with a radius of 1000m, corresponding to the potential distance travelled by an individual to reach a breeding site (Kovar et al., 2009). We extracted surface area of forest cover and agricultural fields, and both variables were negatively correlated ($r=-0.96$ [-0.99: -0.80], $t(6)=-8.60$, $p<0.001$). The

surface area of forest cover allowed to create habitat categories: sites with forest cover > 90% were classified as “forest” (2 sites, Table 1), sites with forest cover < 15% were classified as “farmland” (4 sites, Table 1) and the remaining sites were classified as “mixed” (2 sites with forest cover ranging from 60% to 75%, Table 1). A previous study validated that using these habitat categories allowed to discriminate the isotopic signatures of toads, especially $\delta^{15}\text{N}$ which correlate with the use of fertilizers in agricultural areas (Renoirt et al., 2021). Importantly, ongoing investigations suggest that genetic structure and allelic diversity are similar between habitat types (unpublished data).

Capture and measurements

Field sessions took place at night using a headlamp to locate individuals. Upon sighting, each individual was captured. We collected blood (100 μl) through cardiocentesis with a 1ml syringe and a 30-G heparinized needle. Blood sample were collected in less than 5 minutes (mean \pm se, 2.58 \pm 1.08) in order to provide baseline hormonal levels (there was no effect of handling time on CORT or T levels (CORT: $F_{1,147} = 1.104$, $p = 0.359$; T: $F_{1,147} = 1.152$, $p = 0.299$). Toads were then weighted ($\pm 0.1\text{g}$), stored in individual boxes and brought back to the laboratory. At the laboratory, individuals were measured using a caliper ($\pm 0.01\text{mm}$) for snout-to-vent length (SVL), forearms length and width (left and right), tibias length (left and right), length of parotoid glands (left and right) following Watters et al. (2016). We took pictures of all individuals and used ImageJ (Schneider et al., 2012) to measure nuptial pad area of both sides (Orton et al., 2020) (Supplementary material Figure S1). A body condition index (BCI) was quantified from the residual scores of the linear regression between body size and body mass.

Individuals were marked with a small puncture of the toe web using a medical trocar in order to avoid multiple captures. All individuals were released at their site of origin within 24h.

Physiological assays

Blood was centrifuged, and the plasma and red-blood cells were separated and stored at -20°C until assays were conducted. Plasma concentrations of T and CORT were determined by radioimmunoassay at the CEBC (Brischoux et al. 2018) using $30\mu\text{L}$. The minimum detectable levels were 0.83 and 0.28 ng ml^{-1} , respectively, for T and CORT, and the intra- and inter-assay coefficients of variation were 13.16% and 17.56% and 10.66% and 11.77% , respectively, for T and CORT (that were run twice both within and between assays). Samples were assayed in duplicate, in 5 runs.

Telomere length was determined on red-blood cells by quantitative PCR (qPCR; BioRad CFX 96, Bio-Rad, USA) using an established protocol (McLennan et al., 2019), which was adapted for the Spined toad.

Statistical analysis

We were able to analyze data on 68 individuals from "farmland" habitats (N=4 sites), 38 individuals from "Mixed" habitats (N=2 sites) and 41 individuals from "Forested" habitat (N=2 sites).

All data were tested for homogeneity of variance and normality with the Barlett's test and the Shapiro-Wilks test, respectively. We used the mean value for the following morphometrics characters coming in pairs: Forearm length and width, hind leg length, parotoid glands length, nuptial pads of the same finger. We also checked the normality of the residuals using diagnostic plots. Overall, in habitat categories model, we kept site as a random factor because site identity influenced all our morphological and physiological parameters (all $p < 0.046$).

To test whether morphological and physiological scaling were different between site, we fitted linear models with $\log_{10}(\text{SVL})$ or BCI as covariate. To test whether allometry trajectories were influenced by habitat categories we fitted seven linear mixed-effect models (LMERs, package "lme4") to analyze differences in body mass, forearm length, tibia length, forearm width, parotoid gland length, nuptial pad area between the three categories. In all these seven models, we used $\log_{10}(\text{SVL})$ as covariate and tested the interaction between SVL and habitat categories. Moreover, to test whether physiology was influenced by habitat categories we fitted 3 linear mixed-effect models (LMERs, package "lme4") to analyze differences in corticosterone levels, testosterone levels and telomere length between the three categories. We did the same approach when using BCI as covariate instead of $\log_{10}(\text{SVL})$. When interaction was significant, we tested whether allometry slopes and intercept differed between habitat using *lstrends* (LSmean package).

All statistical analyses were conducted with R statistical software v.4.0.0 (R Core Team, 2020) and RStudio v 1.2.5042 (RStudio, Inc.).

Results

Simple comparisons between traits

All morphological and physiological traits are summarized in Table 2. We found that toads from the three habitats types were highly similar for most traits studied (Table 2), except for the length of the forearms that was significantly longer in toads from forest habitats as compared to individuals from mixed or farmland sites (respectively $p < 0.001$ and $p < 0.001$, Table 2).

Size-scaling across habitat categories

Allometric relationships between SVL and the other traits, as well as slopes and intercepts of these relationships are summarized in Table 3. As expected, most traits covaried positively with body size (except for hormonal levels and telomere length, Table 3, Fig. 1 & 2) but we found contrasts in the shape of these relationships between habitat types (Table 3, Fig 1 & 2).

Body mass was positively related to SVL with toads from mixed habitat showing lower slope compared to forest ($p = 0.001$) or farmland ($p = 0.012$) individuals (Fig. 1). The intercepts of the relationships between forearm length and SVL differed between habitat types, with toads from forest habitat showing higher estimates than farmland and mixed-habitats individuals ($p=0.011$ and $p=0.008$ respectively, Fig. 2). Forearm width was positively related to SVL (Table 3) with toads originating from farmland habitat having significantly higher slope than individuals from mixed habitat ($p = 0.014$, Fig. 1). Tibia length were positively related to SVL (Table 3) with toads originating from forested habitats having significantly higher slope than individuals from mixed habitats ($p = 0.05$, Fig. 1). Parotoid glands length was positively related to SVL (Table 3). Toads that originated from forested habitats had significantly higher slope than mixed habitat ($p = 0.029$, Fig. 1). In addition, toads from forest habitats differed in their intercept from individuals living in farmland ($p=0.045$, Fig. 2). Testosterone levels scaled negatively with SVL for forest individuals solely (Table 3), and the slope of this relationship differed significantly from toads from farmland habitats ($p = 0.02$, Fig 1).

Body-condition scaling across habitat categories

Allometric relationships between body condition and the other traits, as well as slopes and intercepts of these relationships are summarized in Table 4. Most of these traits covaried with body condition (Table 4, Fig. 3) but we found contrasts in the shape of these relationships between habitat types (Table 4, Fig 3).

Parotoid glands length was positively related to BCI (Table 4) with toads from mixed habitat showing lower slope compared to forest ($p = 0.031$) individuals (Fig. 3). Nuptial pad surface area of the second finger was marginally positively related to BCI with toads from farmland habitat showing lower slope compared to forest ($p = 0.068$) individuals (Fig. 3). Telomere length scaled either negatively (forest) or positively (farmland) with BCI (Table 4) and the slopes of these relationships were significantly different between forest individuals as compared to toads from farmland ($p < 0.001$, Fig. 3) or mixed-habitats ($p = 0.004$, Fig. 3).

Discussion

Overall, our study allowed to highlight morphological and physiological variations linked to habitat types (forest, farmland or mixed habitats) in a generalist, widespread amphibian species. Interestingly, our results highlighted a strong contrast between simple comparisons of traits between habitat types (with almost no differences between habitats) and the strong habitat-mediated effects on allometric scaling of the same traits. Our study model allowed to access a wide range of body size and body condition in sexually mature (reproducing) males from contrasting habitat types and such context has revealed a powerful opportunity to investigate variations of body architecture and physiological scaling across such habitat types.

In term of morphology, our results indicated different responses of slopes (indicating unequal changes with increasing body size) and intercepts (indicating variations in proportions of traits) between habitats. Differences in slopes of the allometric scaling of various traits to the body size of individuals highlighted a specific response of individuals

from mixed-habitat types as compared to individuals from either forest or farmland sites. In these individuals, allometric scaling was characterized by weaker slopes, a result that was convergent for most morphological traits under focus (body mass, length and width of forearms, length of hindlimbs, length of parotoid glands). The reasons underlying such patterns remain complicated to assess. Yet, it is plausible that the variety of habitats encountered during the day-to-day life of an individual can influence the ability of toads to optimally respond to diverging environmental constraints. For instance, it is possible that the breeding habitat (where eggs and tadpole develop) orients developmental responses of embryos and larvae to optimally perform in the same type of habitat later in life (Lind & Johansson, 2011). As a consequence, in highly heterogeneous habitat types (mixing forest and agricultural areas at a very small spatial scale), the probability for a post-metamorphic individual to disperse toward a contrasted habitat type is probably significant (e.g., an individual that has developed in an agricultural pond and that later live in forested habitat or vice versa, Kovar et al., 2009; Miaud & Sanuy, 2005). Accordingly, the quality of the terrestrial habitat surrounding the reproductive pond (to which toads are relatively faithful, Reading et al., 1991) can be critical for survival (Unglaub et al., 2018). This is especially true for terrestrial amphibians that display comparatively low agility and locomotor performance (Citadini et al., 2018) and in which avoidance of suboptimal micro-habitats might influence morphological traits divergence (Janin et al., 2012; Trochet et al., 2019). Clearly, future studies are required in order to assess whether developmental conditions can orient the phenotype of individuals to perform optimally in the same type habitat in species with a biphasic ecology (aquatic development *versus* terrestrial adulthood) such as spined toads.

Conversely, estimates of intercepts clearly indicated a similarity between individuals from mixed and farmland habitats as compared to toads from forest habitat. Such result is convergent with those highlighted by previous investigations (Guillot et al., 2016; Zamora-

Camacho & Comas, 2017) and which showed that toads from forest areas diverged from individuals from either intensive agricultural habitats or mixed traditional hedgerows habitats with individuals from these two later categories being similar in term of morphology (Guillot et al., 2016). Contrasts in intercept may suggest that allometric scaling differ through specific growth rate during the ontogeny under environmental constraints (Sanger et al., 2013). A variety of environmental constraints could explain this similarity between individuals originating from mixed and agricultural habitats (Chopin et al., 2019). Such relatively open habitats may influence prey availability, predation pressure and/or microclimatic conditions that are expected to influence traits related to locomotion (forearms and hindlimbs size), to foraging (body mass) and to antipredator attributes (parotoid glands) (Bókony et al., 2019; Guillot et al., 2016; Ingleby et al., 2016; Sumasgutner et al., 2019; Zamora-Camacho, 2018). The contrasted patterns of allometric scaling of morphology (i.e., slopes *versus* intercepts estimates) between habitat types clearly suggest complex responses linked to habitat structure that will require thorough investigations encompassing both aquatic and terrestrial habitats in order to assess relative contribution of developmental conditions versus post-metamorphic growth to comprehensively assess the influence of habitat structure on the morphology in such widespread, generalist species. In this respect, large-scale cross-fostering experiments *in natura* (e.g., translocation of post-metamorphic individuals) and/or common garden experiments in semi-controlled conditions may prove useful avenues of research in order to decipher the patterns we detected (Bachmann & Van Buskirk, 2021; Lind & Johansson, 2011; Van Buskirk & Arioli, 2005).

In contrast to the morphological responses highlighted above, physiological traits (including secondary sexual characters) provided more straightforward patterns with progressive changes in scaling according to expected differences linked to habitat types (mixed habitats having an intermediate position between forest and farmland). Such patterns were found for

two different but complementary indices of sexual activity. First, the relationship between testosterone levels and body size was negative in individuals from forest habitat solely. Second, body condition scaled positively with the surface area of nuptial pads (which allow males to clasp females during amplexus) but this relationship was steeper in individuals from forest habitats. Such results indicate that larger individuals from forest habitats secrete lower levels of testosterone than smaller ones, while no such relationship was found in individuals from other types of habitats. Three different hypotheses could explain this result. First, it is plausible that in agricultural habitats, increased mortality results in a different age structuration composed mainly of younger individuals (Zamora-Camacho & Comas, 2017). Moreover, in farmland habitat, body size is usually larger at younger age (Zamora-Camacho, 2018; Zamora-Camacho & Comas, 2017) which might be explained by a faster growth rate in farmland habitat and possibly a trade-off between lifespan and growth (Lee et al., 2013; Lemaître et al., 2015; Olsson & Shine, 2002; Rollo, 2002; Zamora-Camacho & Comas, 2017). Because testosterone levels decrease with age (Chen et al., 1994), differences in age structure between habitat types may underline the diverging relationships between testosterone levels and body size.

Second, because agricultural breeding sites are characterized by lower abundances of reproductive females (Renoirt et al. 2021b), competition for access to females may be more intense in these habitats, thereby influencing testosterone levels as a mediator of mating success in breeding ponds (Cayuela et al., 2017) where fiercer rivalry could induce more elevated testosterone levels (Burmeister & Wilczynski, 2001; Eikenaar et al., 2012; Hirschenhauser & Oliveira, 2006). This later hypothesis dovetails relatively well with the relationship found between body condition and nuptial pads surface area in individuals from forest habitats. Indeed, in such habitat, larger males have larger surface area of nuptial pads which allow to tightly clasp slippery females during amplexus, thereby decreasing the

risk of losing mating opportunities due to rivalry (Bowcock et al., 2009; Bruning et al., 2010; Orton et al., 2020). Indeed, in male anurans, nuptial pads size is positively related to reproductive success (Orton et al., 2020) and larger males can successfully remove smaller competitors already attached to a female during amplexus (Bruning et al., 2010; Davies & Halliday, 1977). In our context, larger nuptial pad surface area in larger individuals from forest habitats could help to secure mating opportunities (amplexus) and thus fitness while decreasing potential negative effects of elevated testosterone levels (McGlothlin et al., 2010; Wingfield et al., 2001)), a hormone that has been shown to reach remarkably high concentration in breeding males of our study species (Brischoux et al. 2018).

Alternatively, it is possible that living in sites subjected to agricultural activities (either farmland or mixed habitat types) may expose individuals to various agrochemicals, some of which are recognized as endocrine disruptors (e.g., Bókony et al., 2018; Hayes et al., 2006; Orton & Tyler, 2015). Such hypothesis may explain the contrast between habitats we found in the relationships that link body size to testosterone and may thus indicate that some compounds may disrupt such relationships in agricultural areas. Indeed, increased testosterone secretion as a response to environmental contamination has already been documented in toads (Hopkins et al., 1997). Such response may be detrimental to individuals as high levels of circulating testosterone have been shown to represent high energetic costs, reducing fat storage, increase mortality, altered behavior or suppress immune system (McGlothlin et al., 2010; Wingfield et al., 2001). If this hypothesis holds true, such mechanism may influence individual fitness (survival) and ultimately affect population persistence (McGlothlin et al., 2010; Wingfield et al., 2001). Clearly, future studies are required to test for these three competing hypotheses.

Finally, we found diverging relationships between telomere length and body condition between habitat types, these relationships being negative in forest individuals, positive in

toads from farmland and intermediate in individuals from mixed habitats. Telomere length is linked to senescence as they usually shorten with age (Bichet et al., 2020; Sánchez-Montes et al., 2020, but see Bize et al., 2009). If individuals in better body condition are also older individuals, the pattern we found in forest sites was expected (Rollings et al., 2017). Surprisingly, we found the reverse relationship in agricultural areas. Two different hypotheses can explain such result. First, this pattern could be a consequence of the diverging age structuration between habitat types as suggested above (Zamora-Camacho & Comas, 2017), with large but younger – hence, with longer telomeres - individuals in agricultural habitats. Second, and similarly to the hypothesis mentioned above, living in agricultural areas may expose individuals to various agrochemicals, some of which may disrupt the natural attrition of telomere length by activating telomerase (an enzyme that prevent telomere attrition, Blackburn, 1991, 2005; Hatakeyama et al., 2016). Such pattern has already been described in a different context with PFAS inducing a telomere elongation with age in bird species (Blévin et al., 2017; Sebastiano et al., 2020). Clearly, compounds in agricultural areas are different from those included in these studies, but the effect of different contaminants on telomere attrition dynamics has already been described in various contexts (Chatelain et al., 2020; Louzon et al., 2019). Because telomere dynamics predicts mortality risk (Wilbourn et al., 2018), the result we found clearly deserves further attention in order to decipher the mechanisms underlying contrasting telomere dynamics between habitat types, notably linked to environmental contamination by agrochemicals.

Conclusion

Overall, we found that habitat types influence the morphology and physiology of adult male toads. Interestingly, these differences occurred through subtle but significant variations of allometric scaling of the various morphological and physiological traits we investigated. In this respect, our study emphasize the need to encompass a wide range of body size as well as

a wide range of morphological and physiological traits if we are to comprehensively assess the influence of modern agricultural practices on wildlife. Importantly, our study allows to identify critical avenues for future research notably linked to the possible phenotypic matching induced by developmental habitat in species with a biphasic ecology mixing aquatic and terrestrial habitats. Additionally, the sources of the contrasts we highlighted (e.g., physical *versus* chemical structure of the environment, or a combination thereof) remain to be assessed. Finally, whether the differences we detected can have strong consequences on the population dynamics of toads living in agricultural *versus* forest habitats will require long-term mark-recapture studies. Clearly, answering these open questions is likely to be a challenging task but will be critical if we are to comprehensively assess the consequences of anthropogenic activities on wildlife.

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Ethics approval

All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities (COMETHEA ethic committee and Ministère de L'Enseignement Supérieur, de la Recherche et de L'innovation).

Competing interests

We declare we have no competing interests.

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Tables

Table 1: Categories discrimination according to tree cover percentages.

Site	Category	N	Tree cover (%)
Prissé	Modified	21	4.45
Javarzay	Modified	20	5.22
Ensigné	Modified	20	5.61
Préaut	Modified	7	13.14
Prioulet	Mixed	20	61.01
Zoo	Mixed	18	73.06
Labo	Forested	22	93.19
Aulnay	Forested	19	99.84

Table 2: Descriptive statistics and anova results of morphometrics measurement, hormonal levels and telomere length amongst habitats categories in *Bufo spinosus*.

	Categories			Statistical test
	Forested	Mixed	Modified	
Morphometry				
Snout-vent length (cm)	67.87 ± 0.67	70.89±0.65	69.83 ±0.60	F _(2,147) =0.982, p=0.439
Body mass (g)	45.93 ± 1.38	46.94 ±1.10	46.08 ± 1.10	F _(2,147) =0.019, p=0.981
Body condition index	1.42 ± 0.70	-0.85 ± 0.93	-0.45 ± 0.66	F _(2,147) =0.393, p=0.873
Mean forearm length (cm)	26.77 ± 0.42	23.04 ± 0.21	23.58 ± 0.28	F _(2,147) =0.416, p=0.023
Mean forearm width (cm)	8.70± 0.14	8.93 ± 0.15	8.53 ± 0.13	F _(2,147) =0.517, p=0.626
Mean hindlimb length (cm)	28.20 ± 0.27	28.11 ± 0.21	27.77 ± 0.23	F _(2,147) =0.139, p=0.873
Mean parotoid gland length (cm)	16.71 ± 0.25	16.32± 0.20	15.74 ± 0.17	F _(2,147) =2.897, p=0.1509
Mean nuptial pad area (cm ² , first finger)	0.19 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	F _(2,147) =2.129, p=0.216
Mean nuptial pad area (cm ² , second finger)	0.15 ± 0.00	0.13 ± 0.01	0.14 ± 0.01	F _(2,147) =0.723, p=0.534
Physiology				
Corticosterone level (ng.L ⁻¹)	87.19 ± 7.21	67.72 ± 5.01	68.69 ± 4.30	F _(2,147) =0.745, p=0.522
Testosterone levels (ng.L ⁻¹)	287.75 ± 21.94	259.44 ± 17.38	283.57 ± 12.47	F _(2,147) =0.224, p=0.809
Telomere length (T/S ratio)	0.93 ± 0.02	0.94± 0.02	0.94 ± 0.01	F _(2,147) =0.722, p=0.931

Table 3: Slope (b) and Intercept (a) estimates from OLS regressions of log10 morphometrics and physiological traits vs. log10 of Snout-vent length (SVL) \times 3 habitat categories in *bufo spinosus* male adults. Sample sizes (N) and confidence intervals (95% CI) of slopes are listed for each habitat categories. P-values are reported for the correlation between measured traits and SVL at each categories (*p*) as well as for a difference in slopes across habitat categories for each measured traits (*p slope*) and difference in intercept (*p intercept*) along with test statistics (test) and degrees of freedom (df).

Measured traits	Habitat categories	N	b (95 CI)	p	P slope (test, df)	a (95 CI)	p	P Intercept (test, df)
<i>Body mass (g)</i>	Forested	41	2.66(2.18-3.14)	<0.001	p=0.003 (6.15, 2)	1.68(1.64-1.72)	<0.001	p=0.543 (0.693, 2)
	Agricultural	71	2.38(2.02-2.73)	<0.001		1.66(1.63-1.68)	<0.001	
	Mixed	38	1.42(0.87-1.96)	<0.001		1.65(1.62-1.69)	<0.001	
<i>Mean forearm length (cm)</i>	Forested	41	0.68(0.31-1.05)	<0.001	p=0.65 (0.43, 2)	1.43(1.41-1.45)	<0.001	p=0.007 (16.92, 2)
	Agricultural	71	0.72(0.46-0.99)	<0.001		1.37(1.36-1.38)	<0.001	
<i>Mean forearm width (cm)</i>	Mixed	38	0.49(0.07-0.91)	p=0.02	p=0.02 (4.08, 2)	1.36(1.34-1.38)	p=0.001	p=0.159 (2.81, 2)
	Forested	41	1.04(0.62-1.45)	p<0.001		0.95(0.93-0.96)	p<0.001	
<i>Mean hindlimb length (cm)</i>	Agricultural	71	1.30(1.02-1.58)	p<0.001	p=0.04 (3.33, 2)	0.93(0.92-0.94)	p<0.001	p=0.248 (1.88, 2)
	Forested	41	0.82(0.64-1.00)	p<0.001		0.94(0.93-0.96)	p<0.001	
<i>Mean parotoid gland length (cm)</i>	Mixed	38	0.48(0.01-0.95)	p=0.05	p=0.04 (3.38, 2)	1.46(1.45-1.47)	p<0.001	p=0.063 (5.21, 2)
	Forested	41	0.77(0.53-1.01)	p<0.001		1.44(1.43-1.46)	p<0.001	
<i>Mean nuptial pad area *</i>	Mixed	38	0.35(-0.04-0.73)	p=0.08	p=0.52 (0.66, 2)	1.23(1.21-1.25)	p<0.001	p=0.22 (2.07, 2)
	Forested	40	1.39(0.63-2.15)	p<0.001		-0.71(-0.80--0.62)	p<0.001	
	Agricultural	71	1.00(0.44-1.56)	p<0.001		-0.77(-0.83--0.70)	p<0.001	
<i>Mean nuptial pad area**</i>	Mixed	38	1.54(0.68-2.39)	p<0.001	p=0.10 (2.33, 2)	-0.85(-0.94--0.76)	p<0.001	p=0.43 (1.01, 2)
	Forested	40	1.35(0.69-2.00)	p<0.001		-0.87(-0.91--0.82)	p<0.001	
	Agricultural	71	0.50(0.02-0.98)	p=0.04		-0.91(-0.98--0.85)	p<0.001	
<i>Corticosterone level</i>	Mixed	38	1.12(0.38-1.85)	p<0.001	p=0.87 (0.14, 2)	1.87(1.71-2.03)	p<0.001	p=0.74 (0.32, 2)
	Forested	41	0.54(-2.08-3.15)	p=0.69		1.79(1.68-1.91)	p<0.001	
	Agricultural	69	-0.29(-2.20-1.62)	p=0.77		1.78(1.62-1.95)	p<0.001	
<i>Testosterone levels</i>	Mixed	38	0.31(-2.66-3.27)	p=0.84	p=0.02 (4.00, 2)	2.38(2.28-2.48)	p<0.001	p=0.852 (0.32, 2)
	Forested	41	-2.73(-4.93--0.53)	p=0.02		2.41(2.34-2.49)	p<0.001	
	Agricultural	69	1.17(-0.41-2.74)	p=0.15		2.38(2.27-2.48)	p<0.001	
<i>Telomere length (T/S ratio)</i>	Mixed	38	-1.04(-3.53-1.46)	p=0.43	p=0.13 (2.11, 2)	-0.04(-0.07--0.01)	p=0.04	p=0.91 (0.09, 2)
	Forested	41	-0.51(-1.11-0.08)	p=0.09		-0.03(-0.05--0.01)	p=0.05	
	Agricultural	66	0.26(-0.18-0.69)	p=0.25		-0.03(-0.06-0.00)	p=0.10	

b = slope estimate, a = intercept estimate

* (cm², first finger); ** (cm², second finger)

Table 4 : Slope (b) and Intercept (a) estimates from OLS regressions of log10 morphometrics and physiological traits vs. log10 of body condition index (BCI) × 3 habitat categories in *bufo spinosus* male adults. Sample sizes (N) and confidence intervals (95% CI) of slopes are listed for each habitat categories. P-values are reported for the correlation between measured traits and SVL at each categories (*p*) as well as for a difference in slopes across habitat categories for each measured traits (*p slope*) and difference in intercept (*p intercept*) along with test statistics (test) and degrees of freedom (df).

Measured traits	Habitat categories	N	b (95 CI)	p	P slope (test, df)	a (95 CI)	p	P Intercept (test, df)
<i>Mean forearm width (cm)</i>	Forested	41	0.01(0.00-0.01)	p<0.001	p=0.32 (1.13, 2)	0.93(0.90-0.96)	p<0.001	p=0.58 (0.61, 2)
	Agricultural	71	0.00(0.00-0.00)	p<0.001		0.93(0.90-0.95)	p<0.001	
	Mixed	38	0.00(0.00-0.01)	p<0.001		0.95(0.92-0.99)	p<0.001	
<i>Mean parotoid gland length (cm)</i>	Forested	41	0.00(0.00-0.00)	p<0.001	p=0.04 (3.31, 2)	1.21(1.20-1.23)	p<0.001	p=0.19 (2.53, 2)
	Agricultural	71	0.00(0.00-0.01)	p=0.001		1.20(1.19-1.21)	p<0.001	
	Mixed	38	0.00(0.00-0.00)	p=0.58		1.21(1.20-1.23)	p<0.001	
<i>Mean nuptial pad area*</i>	Forested	40	0.00(0.00-0.01)	p=0.08	p=0.78 (0.25, 2)	-0.73(-0.80--0.66)	p<0.001	p=0.21 (2.15, 2)
	Agricultural	71	0.00(0.00-0.01)	p=0.04		-0.77(-0.82--0.72)	p<0.001	
	Mixed	38	0.00(0.00-0.01)	p=0.24		-0.83(-0.90--0.76)	p<0.001	
<i>Mean nuptial pad area**</i>	Forested	40	0.01(0.00-0.01)	p<0.001	p=0.07 (2.64, 2)	-0.86(-0.91--0.81)	p<0.001	p=0.54 (0.69, 2)
	Agricultural	71	0.00(0.00-0.01)	p=0.03		-0.87(-0.91--0.83)	p<0.001	
	Mixed	38	0.00(0.00-0.01)	p=0.03		-0.90(-0.95--0.84)	p<0.001	
<i>Corticosterone level</i>	Forested	41	-0.02(-0.03-0.00)	p=0.05	p=0.18 (1.75, 2)	1.89(1.73-2.06)	p<0.001	p=0.71 (0.36, 2)
	Agricultural	69	0.00(-0.01-0.01)	p=0.76		1.79(1.67-1.91)	p<0.001	
	Mixed	38	0.00(-0.01-0.02)	p=0.63		1.79(1.62-1.95)	p<0.001	
<i>Testosterone levels</i>	Forested	41	-0.01(-0.02-0.01)	p=0.41	p=0.40 (0.92, 2)	2.42(2.29-2.55)	p<0.001	p=0.86 (0.15, 2)
	Agricultural	69	0.01(0.00-0.01)	p=0.26		2.41(2.32-2.51)	p<0.001	
	Mixed	38	0.01(-0.01-0.02)	p=0.37		2.37(2.25-2.50)	p<0.001	
<i>Telomere length (T/S ratio)</i>	Forested	41	-0.01(-0.01-0.00)	p<0.001	p<0.01 (7.70, 2)	-0.03(-0.06-0.00)	p=0.12	p=0.99 (0.01, 2)
	Agricultural	66	0.00(0.00-0.01)	p=0.02		-0.03(-0.05--0.01)	p=0.04	
	Mixed	36	0.00(0.00-0.01)	p=0.13		-0.03(-0.06-0.00)	p=0.10	

b = slope estimate, a = intercept estimate

* (cm², first finger); ** (cm², second finger)

Caption to figures

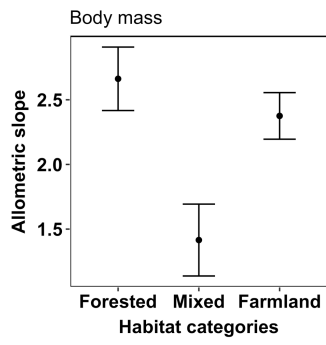
Figure 1: Allometric (SVL) slopes of morphometrics measurements in Spined toad (*Bufo spinosus*) across habitat categories. **A/** Body Mass allometry (g), **B/** Forearm length allometry (mm), **C/** Tibia length allometry (mm), **D/** Nuptial pad area for the second finger (mm²), **E/** Forearm width allometry (mm), **F/** Parotoids gland length allometry (cm) , **G/** Corticosterone levels (ng.ml⁻¹), **H/** Testosterone levels (ng.ml⁻¹), **I/** Telomere length (T/S ratio). All measurements were log₁₀. Measurements were performed on reproductive male adults. Asterisks represent significant statistical tests.

Figure 2: Intercept estimates (SVL) of morphometrics measurements in Spined toad (*Bufo spinosus*) across habitat categories. **A/** Body Mass allometry (g), **B/** Forearm length allometry (mm), **C/** Tibia length allometry (mm), **D/** Nuptial pad area for the second finger (mm²), **E/** Forearm width allometry (mm), **F/** Parotoids gland length allometry (cm) , **G/** Corticosterone levels (ng.ml⁻¹), **H/** Testosterone levels (ng.ml⁻¹), **I/** Telomere length (T/S ratio). All measurements were log₁₀. Measurements were performed on reproductive male adults. Asterisks represent significant statistical tests.

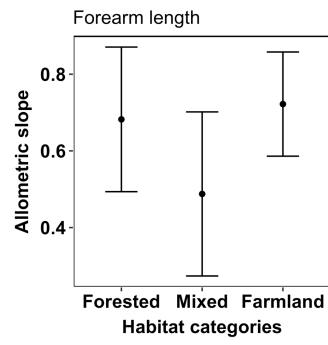
Figure 3: Allometric (BCI) slopes of morphometrics measurements in Spined toad (*Bufo spinosus*) across habitat categories. **A/** Forearm width allometry (mm), **B/** Parotoids gland length allometry (cm), **C/** Nuptial pad area for the second finger (mm²), **D/** Corticosterone levels (ng.ml⁻¹), **E/** Testosterone levels (ng.ml⁻¹), **F/** Telomere length (T/S ratio). All measurements were log₁₀. Measurements were performed on reproductive male adults. Asterisks represent significant statistical tests.

Figure 1:

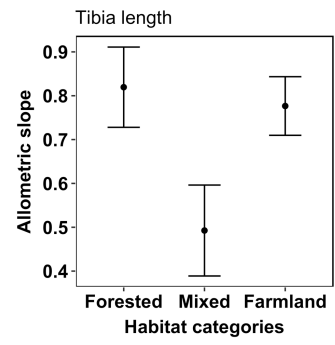
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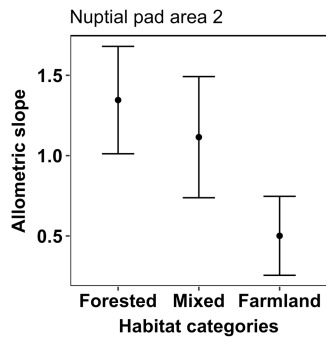
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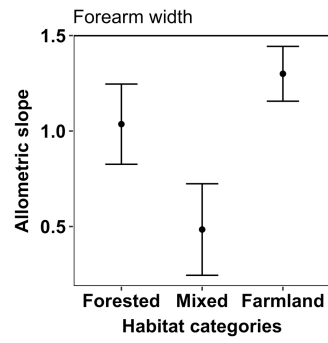
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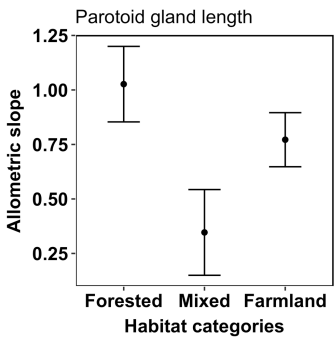
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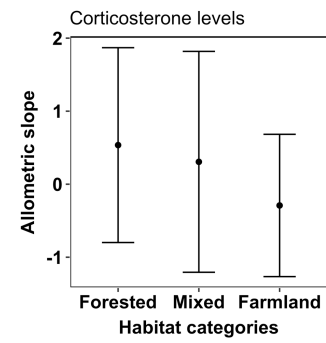
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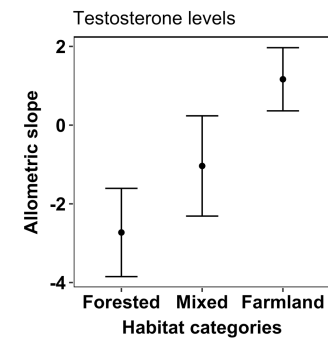
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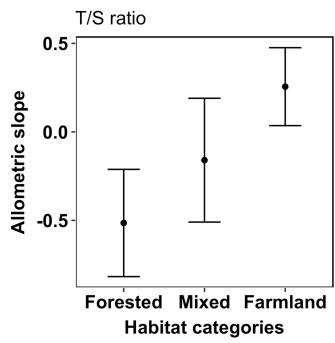


Figure 2:

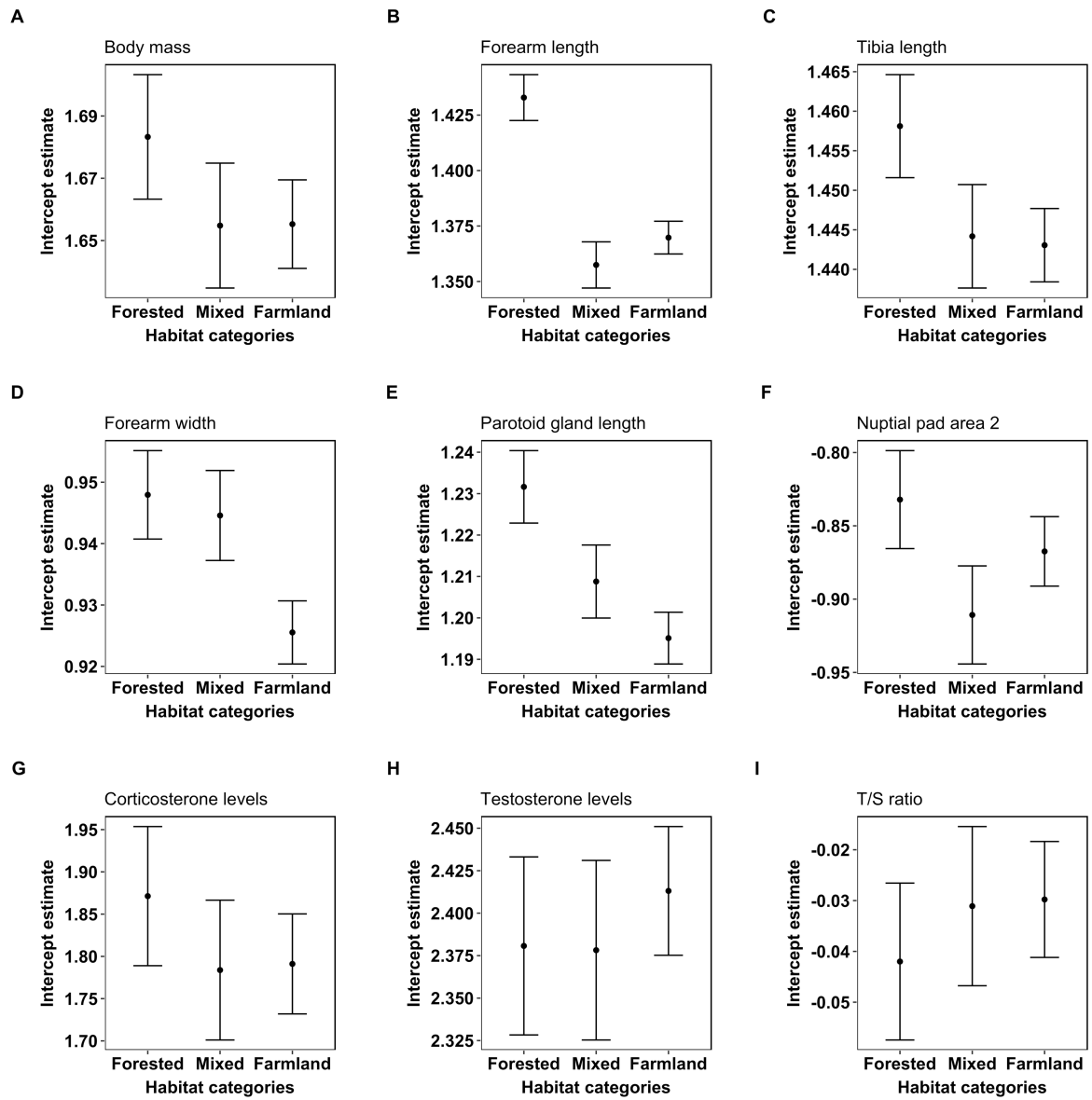
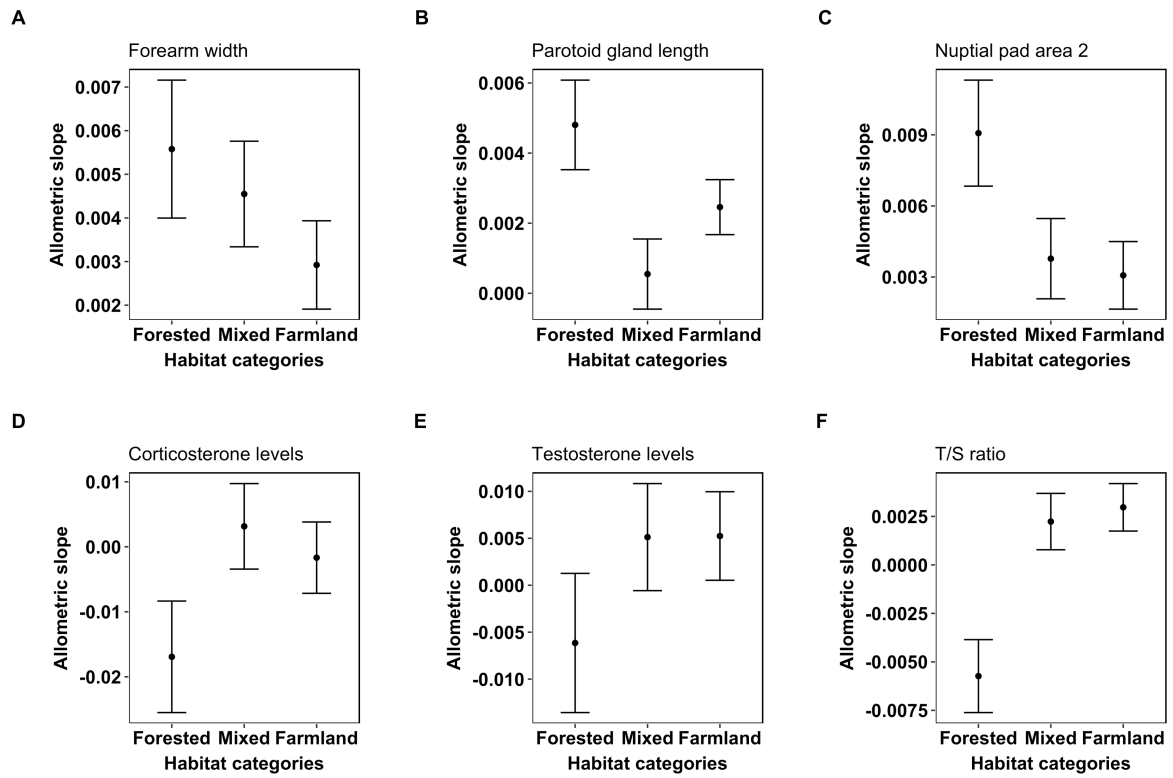


Figure 3:

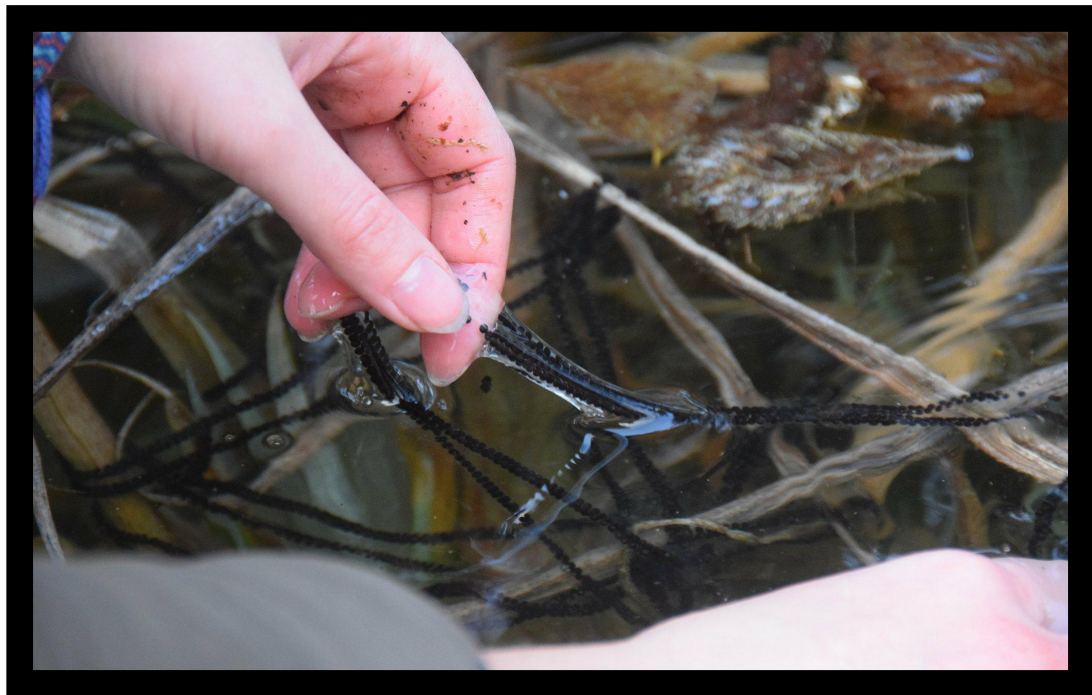


Article III

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Clutch quality is related to embryonic development duration, hatchling body size and telomere length in the spined toad (*Bufo spinosus*)

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Clutch quality is related to embryonic development duration, hatchling body size and telomere length in the spined toad (*Bufo spinosus*)

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Reproductive success is often related to parental quality, a parameter expressed through various traits, such as site selection, mate selection and energetic investment in the eggs or progeny. Owing to the complex interactions between environmental and parental characteristics occurring at various stages of the reproductive event, it is often complicated to tease apart the relative contributions of these different factors to reproductive success. Study systems where these complex interactions are simplified (e.g. absence of parental care) can help us to understand how metrics of parental quality (e.g. gamete and egg quality) influence reproductive success. Using such a study system in a common garden experiment, we investigated the relationships between clutch hatching success (a proxy of clutch quality) and offspring quality in an amphibian species lacking post-oviposition parental care. We found a relationship between clutch quality and embryonic development duration and hatchling phenotype. We found that hatchling telomere length was linked to hatching success. These results suggest that clutch quality is linked to early life traits in larval amphibians and that deciphering the influence of parental traits on the patterns we detected is a promising avenue of research.

ADDITIONAL KEYWORDS: amphibian – clutch quality – early life – embryonic development – hatching success – reproductive success.

INTRODUCTION

Reproductive success is mediated, in part, by parental quality, expressed through complex interactions among ecology, physiology and behaviour during a reproductive event. The influence of parental quality on reproductive performance can occur at early stages of reproduction, such as during mate selection (Amos *et al.*, 2001), breeding site selection (Smiseth *et al.*, 2012), gamete production and energetic investment (Ratikainen *et al.*, 2018). Other determinants of reproductive success take place later during the reproductive event and are often mediated through the expression of parental care to the progeny (Trillmich, 2010; Smiseth *et al.*, 2012) or the quality of the reproductive environment (Trevail *et al.*, 2019). Owing to the complex interactions between environmental and parental characteristics occurring at various stages of the reproductive event, it is often

complicated to tease apart the relative contributions of these different factors to reproductive success (Ridley, 2007; Fox *et al.*, 2018).

In species that do not exhibit parental care, the influence that parents have on reproduction is often reduced to a few simple components of reproductive investment, namely the quality of the gamete, the energetic investment in the eggs (Ratikainen *et al.*, 2018) and the selection of optimal oviposition sites (Smiseth *et al.*, 2012). In situations where breeding site selection can be excluded as a parental effect (e.g. communal nesting), studies can focus simply on how metrics of parental quality (the quality of gametes and eggs; Ratikainen *et al.*, 2018) influence reproductive success.

Amphibians represent a taxonomic group particularly suitable for investigations of the relationships between parental investment in reproduction (egg quality) and indicators of offspring quality (hatchling phenotype). In amphibians lacking parental care, many species lay their eggs communally (i.e. in the same breeding pond),

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thereby largely eliminating the potential influence of oviposition site selection as a parental effect (Doody *et al.*, 2009; Refsnider & Janzen, 2010). In this study, we explore the relationships between clutch hatching success and offspring quality in an amphibian species that breeds communally and lacks post-oviposition parental care, the spined toad (*Bufo spinosus*).

Hatching success in toad species is known to be highly variable (Häkkinen *et al.*, 2001; Rowe *et al.*, 2001; Bishop *et al.*, 2010; Bókonyi *et al.*, 2018). Although environmental conditions can influence hatching success (Chivers *et al.*, 2001; Bishop *et al.*, 2010; Delia *et al.*, 2019), studies have shown that maternal transfer (i.e. glucocorticoid hormones: Saino *et al.*, 2005; contaminants: Unrine *et al.*, 2006, 2007; Bergeron *et al.*, 2010; Metts *et al.*, 2013) can also affect hatching success. This effect suggests that hatching success is under strong parental influence and might be used as a proxy of clutch quality.

In this study, we investigated the relationship between clutch quality (assessed as hatching success) and offspring quality in clutches originating from the same breeding site. Our laboratory setting (common garden in controlled conditions) allowed us to assess hatching success independently from natural factors affecting embryonic development (Chivers *et al.*, 2001; Gomez-Mestre *et al.*, 2013; Egea-Serrano *et al.*, 2014). Offspring quality was assessed by using two complementary sets of markers that are known to be linked to fitness. First, we used two traits of offspring, namely embryonic development duration and hatchling body size, because these traits can be used as markers of offspring quality in amphibians (Van Buskirk, 2002; Delia *et al.*, 2019). Second, we assessed the telomere length of hatchlings, because this marker has been suggested as a promising molecular tool to evaluate individual quality (Angelier *et al.*, 2019; Eastwood *et al.*, 2019; Bichet *et al.*, 2020).

Telomeres are non-coding, repetitive short sequences of DNA situated at the ends of eukaryotic chromosomes. Telomere attrition can lead to programmed cell death (Campisi & d'Adda di Fagagna, 2007). Telomere length can vary across environmental conditions (Blévin *et al.*, 2016; Angelier *et al.*, 2018; Chatelain *et al.*, 2020), ontogenetic stages (Burraco *et al.*, 2020) and species (Whittemore *et al.*, 2019). Thus, telomeres can provide insight into phenotypic and physiological responses to environmental conditions during early life stages (Heidinger *et al.*, 2012; Herborn *et al.*, 2014). As in most vertebrates, telomeres shorten through adult life in amphibians (Sánchez-Montes *et al.*, 2020), and the developmental period is thought to be a critical period for hatchling telomere length (Foote *et al.*, 2011; Eastwood *et al.*, 2019; Burraco *et al.*, 2020; Stier *et al.*, 2020). Importantly, hatchling telomere length is also linked to fitness parameters, such as

longevity, in multiple vertebrate species (Heidinger *et al.*, 2012; Boonekamp *et al.*, 2014; Wilbourn *et al.*, 2018), emphasizing the relevance of this measurement to assess hatchling quality. Accordingly, we predicted that offspring from lower-quality clutches (i.e. with greater embryonic mortality) would have reduced body sizes and telomere lengths.

MATERIAL AND METHODS

ETHICAL APPROVAL

All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities (COMETHEA ethic committee and Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation) under permit #APAFIS#13477-2018032614077834 v7.

EGG SAMPLING AND DEVELOPMENT

Spined toad (*B. spinosus*) egg strings ($N = 10$) were collected by hand during a single day in January 2018 in a pond near the Centre d'Etudes Biologique de Chizé (46°8'48.624; 0°25'32.661W, France), brought back to the laboratory and kept in controlled conditions (see below). Eggs were collected immediately after laying (direct observations) in order to avoid development in the natural environment. A piece of each string containing 30 eggs was selected randomly and kept for our experiment, and the remaining eggs (i.e. 3000–5000) were released at their site of origin.

The 30 eggs from each clutch were placed in separate glass tanks containing 2 L of dechlorinated tap water (changed once a week) and monitored until hatching (total $N = 300$ eggs). We determined the stage of development according to Gosner (1960). Hatching occurred at Gosner stage 22 after 16.09 ± 0.04 days (see Results). All experiments took place in a thermally controlled room, with air and water temperature set at 17 °C and with a natural day–night photoperiod (9 h–15 h).

MEASUREMENTS

We monitored egg segments and counted all the individuals that hatched (Gosner stage 22), which we considered a metric of hatching success. Undeveloped embryos were counted individually. Our observations showed that undeveloped embryos had stopped their development at early stages, usually within the first few hours, and that this number of undeveloped embryos remained steady during the remaining developmental period. Development duration was assessed as the time elapsed between the date of egg collection and the date

of hatching. We did not observe variation in embryonic development duration within clutch. We took photographs of all live hatchlings using a camera (Panasonic Lumix DC-TZ55) in order to assess the length of individuals ($N = 285$). Morphological measurements were performed with the software IMAGEJ (Schneider *et al.*, 2012).

RELATIVE TELOMERE LENGTH

Immediately after hatching, we selected two hatchlings randomly from the embryos that hatched in each clutch, for telomere analysis ($N = 20$ hatchlings from ten clutches). The remaining hatchlings were released in their pond of origin. Hatchlings were euthanized, and we used the whole individual for the assay. We deliberately kept small sample sizes for this subset of analyses for ethical reasons.

Telomere length was determined by quantitative PCR (qPCR; BioRad CFX 96; Bio-Rad, USA) using an established protocol (McLennan *et al.*, 2019), which was adapted for the spined toad. Whole hatchlings were digested with proteinase K, and DNA was extracted using the Nucleospin Tissue Kit (Macherey-Nagel), following the manufacturer's instructions. The DNA concentration and purity were assessed with a Nanodrop ND1000 spectrophotometer (Thermo Scientific). Universal telomere primers were used, and the control single-copy gene recombination activating gene 1 (*RAG1*) was selected and amplified using specific primers designed for the spined toad using the sequence alignment methodology: RAG1-F 5'-GGTCTCTGATAGCCGAAA-3' and RAG1-R 5'-CATCATAACCTGTACCCCGGA-3'. This single-copy gene has previously been used successfully in multiple species (birds: Sebastiano *et al.*, 2020; fish: Petitjean *et al.*, 2020; reptiles: McLennan *et al.*, 2019; Dupoué *et al.*, 2020), including amphibians (Canestrelli *et al.*, 2021). All qPCRs were performed on three plates for each gene (*RAG1* and telomere) using 7.5 ng of DNA per reaction. The telomere and single-copy gene primers were used at concentrations of 800 and 300 nM, respectively. To generate a six-point standard curve (from 50.0 to 1.5 ng) for controlling the amplification efficiency of the reactions, serial dilutions of DNA from a pooled sample of ten tadpoles were included on the plate (in triplicates). For both telomere and *RAG1* amplification, the melt curves displayed single sharp curves, validating amplification specificity of these primers. A reference toad sample was run in triplicate in all plates to account for inter-plate variation. All samples were run in duplicates and distributed randomly across the PCR plates. The cycle threshold (*Ct*) values determined for duplicates were averaged, and samples with a *Ct* SD > 0.2 between duplicates were repeated. Amplification efficiencies reached 87.48 ± 5.92 (mean \pm SE, %) for *RAG1* and

92.60 ± 6.00 for the telomere, and all R^2 values were very high (> 0.99). The relative telomere length (expressed as the *T/S* ratio) was calculated as the number of telomere copies (*T*) relative to the single-copy gene (*S*; *RAG1*), according to Cawthon (2002). Inter-plate variations for telomere and *RAG1* were 2.07 and 1.89%, respectively. Inter-plate variation for the *T/S* ratio was 5.62% ($N = 3$).

STATISTICAL ANALYSES

All statistical analyses were conducted with R.STUDIO v.1.2.5042 (R Core Team, 2020).

All data were tested for homogeneity of variance and normality using the Barlett's test, the Shapiro–Wilks test and diagnostics plots of residuals. All clutches were collected on the same date; hence, we did not include laying date as a predictor.

First, on the whole dataset, to test whether there were correlations between hatching success and development duration and hatchling size, we fitted two linear models (lm, 'stats' package). We used hatching success as the fixed effect in our models. Hatching success was expressed as the proportion of eggs that hatched from the total number of eggs. In these models, we did not include 'clutch identity' as a random effect because each clutch was represented by a single hatching success.

Second, on the subset of individuals for which we measured telomere length, we investigated whether there was a correlation between hatching success, embryonic development duration and hatchling size, and relative telomere length. When hatching success was used as a fixed effect in our models, we did not include 'clutch identity' as a random effect for the same reason as above, and we fitted linear models (lm, 'stats' package). When embryonic development duration or hatchling size was used as a fixed effect in our models, we added 'clutch identity' as a random effect and thus used a linear mixed model (LMER, 'lmerTest' package). Further information on model outputs is available in the Supporting Information (Table S1).

RESULTS

The Supporting Information (Table S2) provides the whole dataset.

Hatching success was variable among clutches, ranging from 86 to 100% (coefficient of variation = 5%) and was positively related to embryonic development duration of the eggs that hatched ($F_{1,283} = 432.9, P < 0.0001, R^2 = 0.60$; Fig. 1). Hatchling size was different among clutches ($F_{9,275} = 61.08, P < 0.0001, R^2 = 0.67$) and positively related to hatching success ($F_{1,283} = 95.96, P < 0.0001,$

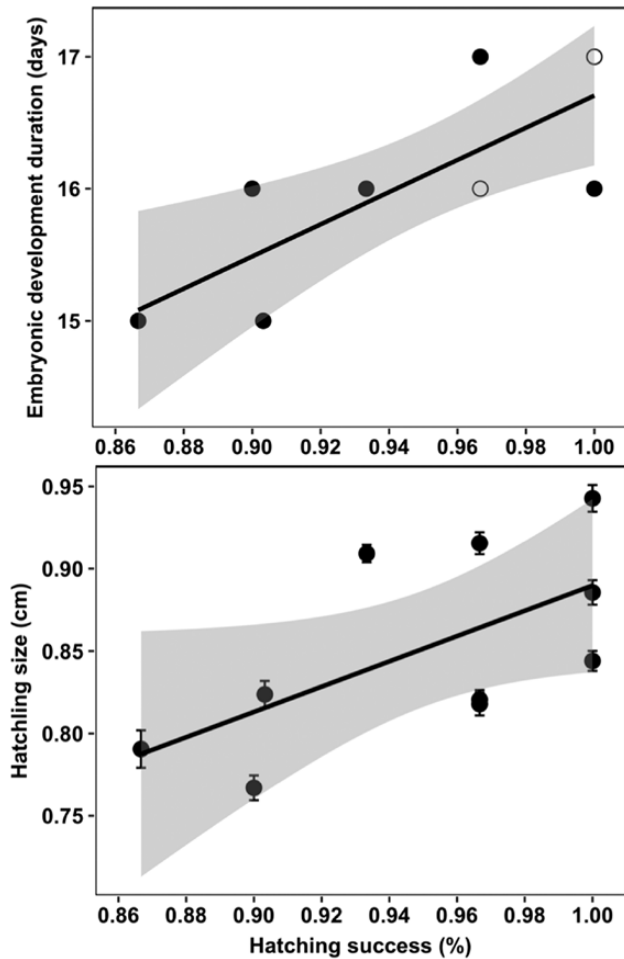


Figure 1. Upper panel, development duration relative to hatching success. Filled circles represent all the individuals within one clutch. Given that two clutches displayed similar hatching success and development duration, we represented them with open circles; hence, the six filled circles represent six clutches and the two open circles represent four clutches. Lower panel, hatchling size (mean \pm SE) relative to hatching success. Grey shading indicates 95% confidence intervals.

$R^2 = 0.25$; Fig. 1). Hatchling size was positively correlated with development duration ($F_{1,283} = 44.44$, $P < 0.0001$).

Among individuals for which telomere length was assessed, telomere length was positively related to hatching success ($F_{1,18} = 5.71$, $P = 0.03$, $R^2 = 0.20$; Fig. 2) but not related to embryonic development duration ($F_{1,9} = 3.26$, $P = 0.11$). Hatchling size was not correlated with telomere length ($F_{1,9} = 2.25$, $P = 0.164$). On this subset of individuals and like the whole dataset analysed above, hatchling size was different among clutches ($F_{1,9} = 10.78$, $P < 0.001$) and positively related to hatching success ($F_{1,18} = 9.33$, $P = 0.007$, $R^2 = 0.30$; Fig. 2).

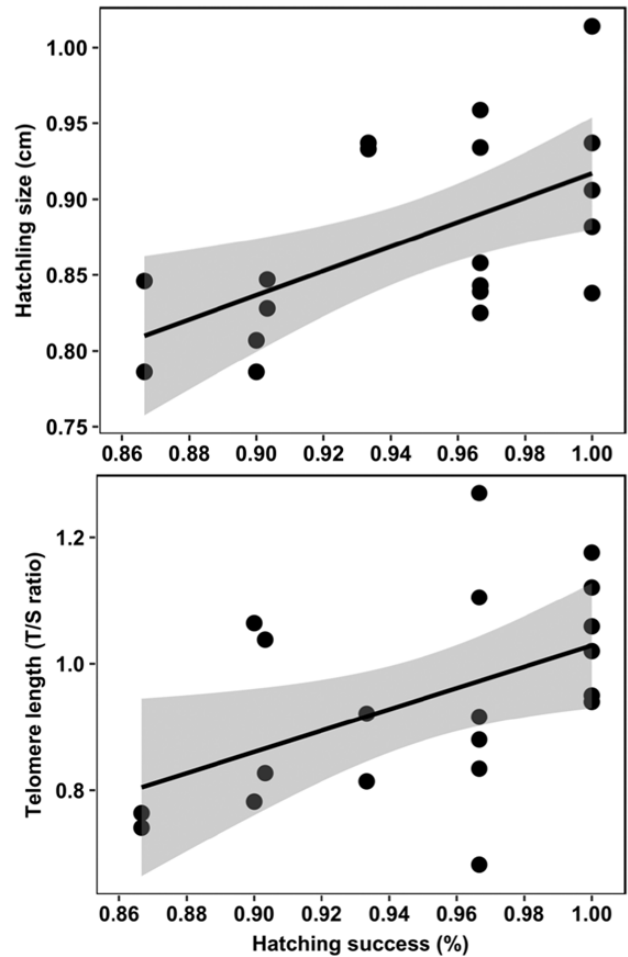


Figure 2. Hatchling size (upper panel) and relative telomere length (lower panel) relative to hatching success for the individuals for which telomere length was assessed. Grey shading indicates 95% confidence intervals.

DISCUSSION

Overall, we found that hatching success was related to the duration of embryonic development and the size of hatchlings. Among the group of individuals for which telomere length was assessed, we found that telomere length was also linked to hatching success. These results suggest that clutch quality has a strong relationship with early life traits of larval amphibians. Using hatching success as a metric for clutch quality has the advantage that it is relatively straightforward to measure.

We found that clutches with lower hatching success were characterized by shorter embryonic development durations for the individuals that hatched. Such a relationship could be the result of an intrinsic characteristic of the clutch, whereby some clutches develop more rapidly than others (Magrath, 1990; Nager *et al.*, 2000). In turn, rapid embryonic

development might lead to an accumulation of cellular damage by overproduction of reactive oxygen species, resulting in lower hatching success (Lee *et al.*, 2013). Alternatively, such a relationship could be an adaptive response of embryos to environmental cues. Embryos can respond to chemical cues secreted by neighbouring dead embryos and accelerate their development in order to evade detrimental environmental conditions (Lee *et al.*, 2013; Delia *et al.*, 2019). Hatching plasticity is known to occur in amphibians and has been hypothesized as an adaptive response to predation risks (Sih & Moore, 1993; Warkentin, 1995; Lee *et al.*, 2013; Touchon *et al.*, 2013). No damage to the embryos occurred in our experimental system, and we suggest that dead and decaying embryos that might be used as a substrate for potential pathogens (e.g. bacteria and/or fungi) might secrete cues indicating a possible susceptibility to the spread of pathogens to neighbouring live embryos. Future studies are required to disentangle these hypotheses and to assess whether amphibian embryos can respond to predation risk and/or susceptibility to pathogens during embryonic development.

We also found that embryos that took less time to develop hatched as smaller hatchlings. This result was expected given the well-known negative relationship between the duration of embryonic development and size at hatching across ectothermic vertebrates (Gillooly & Dodson, 2000). For instance, ectotherms are known to develop faster and mature at smaller body size when exposed to warmer temperature (Zuo *et al.*, 2012). Thermal conditions were identical in our experimental study, and we suggest that the size at hatching is simply a consequence of embryonic development duration. Whether the duration of embryonic development is linked to maternal investment in eggs remains to be tested (Martin & Schwabl, 2008; Chen *et al.*, 2013).

In support of these results, we found that telomere length was linked to hatching success. Clutches with lower hatching success produced hatchlings with shorter telomeres. It is known that the rate of cell replication during embryonic development is negatively correlated with the size of telomeres at hatching (Jennings *et al.*, 2000; Foote *et al.*, 2011; Stier *et al.*, 2020). Given that shorter telomeres during early life are linked to lower fitness later in life (e.g. longevity: Heidinger *et al.*, 2012; Boonekamp *et al.*, 2014; Wilbourn *et al.*, 2018; Stier *et al.*, 2020), our results suggest that offspring from lower-quality clutches might experience overall relatively poorer performances later in life. Although we failed to detect the expected relationship between telomere length and body size (Scott *et al.*, 2006; Ringsby *et al.*, 2015), we believe that the relatively low number of hatchlings included in our telomere analysis might have obscured

such a pattern (but see Monteforte *et al.*, 2020), and future studies are required to test this hypothesis.

Whether smaller body sizes and shorter telomeres have long-term consequences for developing tadpoles remains to be assessed. Future studies are required to test whether shorter telomeres influence tadpole development until metamorphosis (Cabrera-Guzmán *et al.*, 2013; Burraco *et al.*, 2017; Semlitsch *et al.*, 2000) or, indeed, until adulthood (Sánchez-Montes *et al.*, 2020). Given the correlates of shorter telomere size on life-history traits (e.g. shorter life expectancy, lower quality and senescence: Heidinger *et al.*, 2012; Wilbourn *et al.*, 2018; Angelier *et al.*, 2019; Eastwood *et al.*, 2019; Bichet *et al.*, 2020), we believe that our study system might provide insights to assess the trade-offs between early life development and individual quality later in life. For instance, although fast development might allow embryos and tadpoles rapidly to evade the high levels of competition and predation occurring in breeding ponds (Burraco *et al.*, 2020), it might have consequences later in life on crucial attributes, such as individual quality and life expectancy (Burraco *et al.*, 2020).

Futures studies should investigate the effect of clutch size on hatching success and offspring quality. We did not record clutch size in our study, but given the well-known trade-off between clutch size and egg size (Gould *et al.*, 2020), we can hypothesize that clutch size has a direct effect on hatching success (Brown & Shine, 2009). In addition, multiple paternity is known to occur in explosive breeding amphibians (Laurila & Seppä, 1998; Liebgold *et al.*, 2006), and deciphering whether variation in hatching success is linked to a paternal genetic contribution is required. Finally, future studies should explore the implications that the positive relationship between hatching success and telomere length can have for ectothermic species, such as amphibians (e.g. interactions between clutch quality and suboptimal environments). Overall, deciphering the influence of parental traits (e.g. quality and fecundity) on the patterns we detected is a promising avenue of research.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Linear model coefficient and estimates.

Table S2. Whole dataset.

Article IV

Marion Cheron, François Brischoux

Aminomethylphosphonic acid alters amphibian embryonic development at environmental concentrations

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Aminomethylphosphonic acid alters amphibian embryonic development at environmental concentrations

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ABSTRACT

Despite intense societal and scientific debates regarding glyphosate toxicity, it remains the most widely used herbicide. The primary metabolite of glyphosate, AMPA (aminomethylphosphonic acid), is the main contaminant detected in surface waters worldwide, both because of the extensive use of glyphosate and because of other widespread sources of AMPA (i.e., industrial detergents). Studies on potential effects of glyphosate using environmentally relevant concentrations of AMPA on non-target wildlife species are lacking. We experimentally tested the effects of AMPA on embryonic development in a common European toad at concentrations spanning the range found in natural water bodies (from 0.07 to 3.57 $\mu\text{g l}^{-1}$). Our experimental concentrations of AMPA were 100–6000 times lower than official Predicted-No-Effect-Concentrations. We found that these low-level concentrations of AMPA decreased embryonic survival, increased development duration and influenced hatchling morphology. Response patterns were more complex than classical linear concentration-response relationships, as concentration responses were nonmonotonic, with greater effects at low-concentrations of AMPA than at high levels. Based on our results we recommend that investigators focus not only on effects of “parent compounds,” but also their metabolites at environmentally relevant concentrations in order to comprehensively assess impacts of anthropogenic contaminants on the environment.

1. Introduction

Anthropogenic activities have been responsible for the creation of a multitude of synthetic chemicals that have been released into the environment (Kendall et al., 2016). Many synthetic chemicals are now (in)famous for their high levels of toxicity for organisms (Tsui and Chu, 2003; Kendall et al., 2016; Giaquinto et al., 2017). Persistent organic pollutants (POPs) can be highly toxic and are widespread, threatening ecosystems worldwide (Ashraf, 2017). Environmental contamination by POPs and other chemicals is now recognized to play a major role in the current loss of biodiversity, along with climatic influences and habitat modifications (Kendall et al., 2016).

Among the numerous sources of environmental contamination, modern agricultural practices play a significant role because of the widespread use of agrochemicals, which span from fertilizers (that can lead to organic matter enrichment and eutrophication; Khan and Mohammad, 2014) to pesticides (i.e., herbicides, insecticides and fungicides; Prakash et al., 1997). Although pesticides are designed to alter specific components of ecosystems (e.g., weeds, insect pests, fungi) that

can be deleterious to crop productivity, they can also affect non-target species either because of their toxicity, or because they can be transported to non-target environments (Hasenbein et al., 2017; Relyea and Hoverman, 2006; Isidori et al., 2005). Pesticides tend to accumulate in aquatic habitats due to the transfer of agrochemicals from contaminated agricultural fields into wetlands by soil erosion during rain events (Solomon and Thompson, 2003). In addition, many agrochemicals exhibit properties (e.g., solubility, polarity) which promote transfer to and persistence in aquatic ecosystems (Mackay and Boethling, 2000; Giesy et al., 2000).

Although traditional toxicity studies are critical to assess median lethal doses (LD50) and associated deleterious effects, they usually fail to detect more subtle sub-lethal effects at very low concentrations of agrochemicals (i.e., levels below Predicted-No-Effect-Concentrations [PNEC]), especially on non-target (non-model) organisms. Sub-lethal effects may influence multiple traits (e.g., physiological effects due to endocrine disruption; Hayes et al., 2010), and they may also impact the persistence of natural populations. In addition to not examining sub-lethal effects, many toxicity studies focus solely on parent

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compounds (i.e., the listed active substances), but ignore the potential effects of their metabolites. Yet, many metabolites can be found in natural environments more frequently and at higher concentrations than their parent compounds (Grandcoin et al., 2017; Matozzo et al., 2019).

This is the case for the most widely used non-selective herbicide — glyphosate — and its primary metabolite AMPA, which are the main pesticides detected in surface waters in North America and Europe (Grandcoin et al., 2017; Coupe et al., 2012; Bonansea et al., 2017). The great abundance of AMPA in surface waters is presumably linked to the worldwide use of glyphosate (Grandcoin et al., 2017), but also because AMPA is an organic phosphonate derived from water treatment facilities, textile industries, and industrial or household detergents (Grandcoin et al., 2017). Despite the global presence of AMPA in surface waters, surprisingly few studies have investigated its potential effects on non-target organisms (Giesy et al., 2000), and even fewer studies have focused on actual environmental concentrations of AMPA (Matozzo et al., 2018, 2019).

Amphibians are a particularly well-suited non-target taxon to investigate the potential effects of AMPA at low, realistic environmental concentrations. First, amphibians are often very susceptible to changes in water quality because they have highly permeable skin, which allows for the exchange of gases, water and ions, but may also facilitate the diffusion of harmful substances (Quaranta et al., 2009). Second, in many species, larvae filter water or graze on submerged surfaces to feed. As a consequence, larvae can directly ingest sediment, which can accumulate pesticides even if rates of contaminant inputs are low (Hayes et al., 2010). Third, many amphibians have biphasic life-cycle, with terrestrial adults breeding in aquatic sites (ponds) where eggs and larvae develop. Early developmental (embryonic and larval) phases bear strong and long-lasting influences on life-history traits such as body size, growth, survival, and reproduction (Kashiwagi et al., 2009; Arrighi et al., 2013). As a consequence, disturbances to aquatic habitats during early development may have lifelong effects on an individual phenotypic variation. For instance, the development and metamorphosis of larvae are hormonally dependent, and amphibian larvae are particularly sensitive to xenobiotics, which can disrupt hormonal pathways (Kashiwagi et al., 2009; Chai et al., 2017; Crump et al., 2002). Accordingly, amphibians have been particularly studied to investigate the consequences of environmental contaminants (Mann et al., 2009; Hayes et al., 2006).

In this study, we experimentally investigated the effects of AMPA through environmentally relevant concentrations (i.e., actual concentrations found in surface waters, 100 to 6000 times lower than PNEC) on embryonic development in a common European amphibian, the Spined toad (*Bufo spinosus*). This non-model species is particularly well-suited to investigate the effects of AMPA for several reasons. This species can live in a variety of habitats and even persist in highly modified agricultural areas (Guillot et al., 2016), suggesting that individuals can be exposed to agrochemicals and their metabolites at multiple life stages. In some instances, eggs, tadpoles, juveniles, and adults of *B. spinosus* are found in and around ponds located in agricultural areas (Bókonyi et al., 2018). In addition, eggs may be intensely exposed to AMPA, as breeding/oviposition time often coincides with the timing of herbicide (glyphosate) application in agroecosystems (Lenhardt et al., 2015; Berger et al., 2013).

2. Material and methods

2.1. Eggs collection

Spined toad (*Bufo spinosus*) eggs strings (number of egg string = 15) were collected from 29/01/2018 to 28/02/2018 in two ponds near the Centre d'Etudes Biologique de Chizé (46°09'N, 0°24'W) and returned to the laboratory. The two ponds were monitored twice a day to insure that the eggs were collected immediately after oviposition to minimize embryonic development in the natural environment. A subset of 120 eggs on each clutch was collected for our experiment, while the

remaining eggs (i.e., 3000–5000) were released at their oviposition site.

2.2. Treatment concentrations and chemical solutions

Environmental concentrations found in aquatic environments in France range from 0.1 $\mu\text{g l}^{-1}$ to 6.6 $\mu\text{g l}^{-1}$ (data from Water Agencies, “Agence de l'eau Loire Bretagne” and “Agence de l'eau Adour-Garonne”) and are similar to concentrations found in aquatic environments in Northern America and Europe (Grandcoin et al., 2017; Coupe et al., 2012; Bonansea et al., 2017). Because our goal was to mimic the range of concentrations found in the wild, we produced three treatments spanning from low to relatively high concentrations (Table 1). Stock solutions (0.1 g/L) of AMPA were obtained by dissolving commercial crystalline powder (Aminomethylphosphonic acid, 99% purity, ACROS ORGANICS™) in dechlorinated tap water. These stock solutions were further diluted with dechlorinated tap water to reach each of the treatment (Table 1). We produced 3 additional water samples for each treatment in order to perform analytical verifications of the concentrations. These analytical verifications of the actual concentrations were performed by an independent accredited analytical laboratory (QUALYSE, Champdeniers-Saint-Denis, France). Overall, these verifications showed that actual concentrations were similar to concentrations found in aquatic environments (see above) and that the differences between “low”, “medium” and “high” treatments were significant (Table 1).

For clarity, we will refer to the treatments as Low, Medium and High hereafter.

2.3. Experimental design

Eggs were subjected to different concentrations of AMPA (Low, Medium and High) and a control during the whole embryonic development until hatching. To maintain relatively constant exposure levels throughout the experiment but to avoid excessive mechanical disturbances to developing eggs, water was changed once a week according to the half-life of AMPA ranging from 7 to 14 days in water (Battaglin et al., 2014). Egg jelly was maintained throughout the experiment.

Each subset of each clutch (containing ~120 eggs) was further separated into segments containing ~30 eggs (ie. 120/4 treatments) that were randomly placed in different glass-molded tanks containing 2 L of water at one of our experimental treatments (i.e., Control, Low, Medium or High) and monitored until hatching (total N = 4 treatments * 15 clutches representing 60 tanks containing 30 eggs each).

2.4. Measurements

We determined the stage of development using Gosner stages (Gosner, 1960). Embryos were checked twice a day. Hatching occurred at Gosner stage 22 (hereafter GS 22) after 16.10 ± 0.02 days.

At GS 22, hatching success, mortality and deformation rates were assessed as follows: undeveloped eggs and embryos that did not hatch were counted, and hatchlings (GS 22) were qualified as normal, dead or deformed. All live and undeformed tadpoles were put in a Petri dish with water from their own tank and photographed above graph paper in order to measure their length (body; tail and total length, N = 1639). Morphological measurements were performed with the software ImageJ (Schneider et al., 2012). All measurements were performed by the same

Table 1
Concentrations ($\mu\text{g.l}^{-1}$) measured in the experimental tanks (Mean \pm SD).

Treatment	AMPA concentrations
Control	0
Low	0.07 \pm 0.01
Medium	0.32 \pm 0.052
High	3.57 \pm 0.153

person throughout the experiment.

All experiments took place in a thermally controlled room with the temperature set at 17 °C (both air and water) and under natural 12:12 h day/night photoperiod.

2.5. Statistical analyses

All data were tested for homogeneity of variance and normality with Barlett's and Shapiro-Wilks tests, respectively. We also checked normality of the residuals using diagnostics plots. The only variable that slightly diverged from normality was development duration. Yet, because the F-statistic is extremely robust to violation of the normality assumption when sample sizes are equivalent among groups and degrees of freedom are large (both conditions were met in our analyses), we also used parametric test to analyze development duration.

Clutch identity significantly influenced all response variables (all $P < 0.001$) and was kept as a random factor in all our analyses. Conversely, the sampling site never influenced our results, and it was excluded from our final analyses.

We did not use tank as the replication unit in our analytical design because each tank represented a single treatment (either control, low, medium or high) and contained only the eggs of one clutch. Balanced sample sizes across clutches and treatments (e.g., each clutch was represented by 30 eggs in each treatment) and the use of clutch identity as a random factor allow to avoid significant pseudoreplication issues.

All statistical analyses were conducted with R.Studio v1.2.1335 (R Core Team, 2019).

First, we used Generalized Linear Models (GLMERs, (package *lme4*)) with a log-link function for variables following binomial distribution to test whether there were correlations between treatment and embryonic mortality. Second, we fitted two mixed-effect linear models (LMERs, package *nlme* (Pinheiro et al, 2017)) to analyze differences in development duration and total length with "Clutch" as a random factor to control for the non-independence of individuals coming from the same clutches. Lastly, we checked for linear relationship between covariate (body length) and response variable (body proportion) using visual inspection of scatter plot, which allowed us to conduct analysis of covariance (ANCOVA) with total length as the covariate.

We performed effect size tests with statistical power analysis to assess the magnitude of the difference between treatments (Cohen, 1977). The magnitude of differences (at $P < 0.05$) between two groups is considered significant when Cohen's δ is close to or above 0.2.

3. Results

AMPA influenced embryonic mortality ($\chi^2 = 20.894$, $P = 0.0001$, Fig. 1). Relative to control, embryonic mortality was higher for the lowest and intermediate AMPA concentrations (all $P < 0.04$) but similar for the highest AMPA concentration ($P = 0.99$, Fig. 1).

Similarly, AMPA influenced the duration of embryonic development ($F_{3,1615} = 13.33$, $P < 0.001$, Fig. 2). Relative to control, all treated embryos took longer to develop into tadpoles (Fig. 2). Among treatments, the highest AMPA concentration took less time to hatch than the lower and intermediate concentrations (respectively $P < 0.001$ and $P = 0.004$).

Among hatchlings, the number of deformed tadpoles (overall: $6.07 \pm 0.57\%$), or the number of tadpoles that died upon hatching (overall $0.18 \pm 0.10\%$) were similar between treatments (ANOVA, all $P > 0.10$).

The morphology of viable hatchlings (i.e., not deformed) was also influenced by AMPA concentrations ($F_{3,1615} = 11.22$, $P < 0.001$, Fig. 3). Relative to control ones, tadpoles exposed to the lowest AMPA concentration were larger (TukeyHSD, $P < 0.001$) while tadpoles exposed to higher AMPA concentrations were marginally smaller (TukeyHSD, $P = 0.063$, Fig. 3). Importantly, AMPA modified body proportions (Fig. 3). Although body lengths were similar between treatments (ANCOVA with the total length as a covariate, $F_{3,1615} = 0.64$, $P = 0.59$, Fig. 3), tail

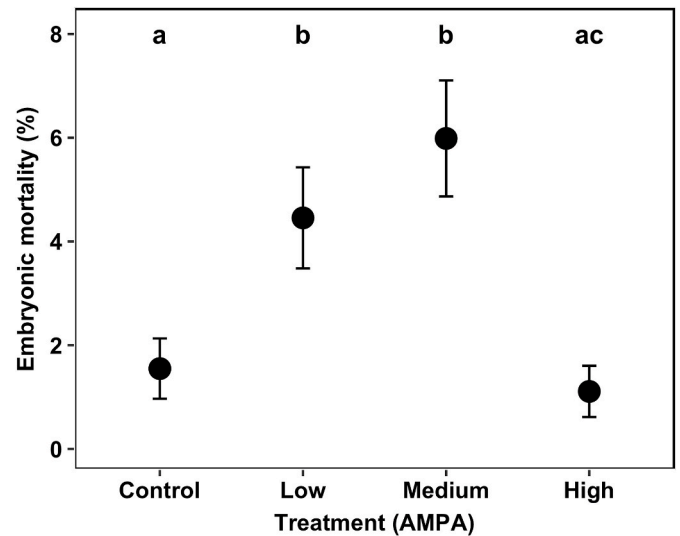


Fig. 1. Embryonic mortality relative to AMPA concentrations. Lowest and intermediate concentrations yielded significantly higher mortality. Letters indicate significant differences (Tukey HSD, $p < 0.05$).

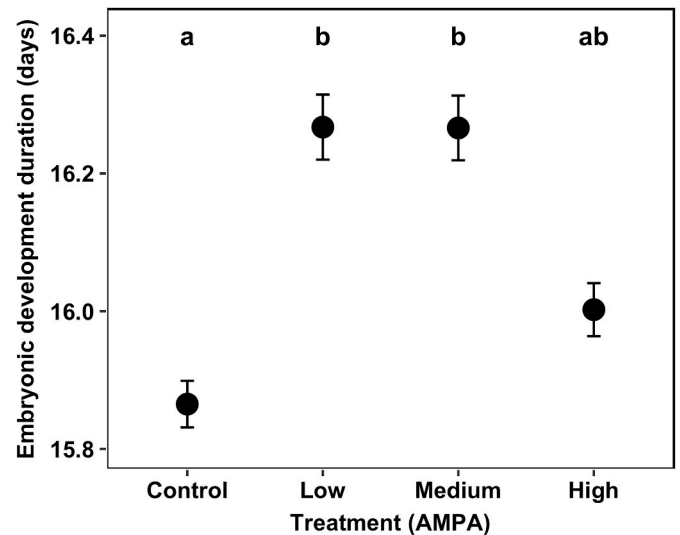


Fig. 2. Embryonic development duration relative to AMPA concentrations. All AMPA concentrations yielded delayed hatching. Letters indicate significant differences with controls (Tukey HSD, $p < 0.05$).

lengths were different (ANCOVA with the total length as a covariate, $F_{3,1615} = 3.2241$, $P = 0.02$, Fig. 3). Relative to the Low treatment, tadpoles exposed to the highest AMPA concentration had shorter relative tail length (TukeyHSD, $P = 0.01$).

Tests of effect sizes supported these results (Appendix 1).

4. Discussion

Based on a very large sample size (i.e., >2000 eggs), our results show that chronic exposure to low-level, environmentally relevant concentrations of AMPA, a primary contaminant detected in surface waters (Grandcoïn et al., 2017; Coupe et al., 2012; Bonansea et al., 2017) alters the quality of embryonic development in Spined toads. More specifically, our results show that AMPA influences embryonic mortality, development duration and body architecture in a complex way. Interestingly, the response patterns of all measured traits diverged from classical linear concentration-response relationships.

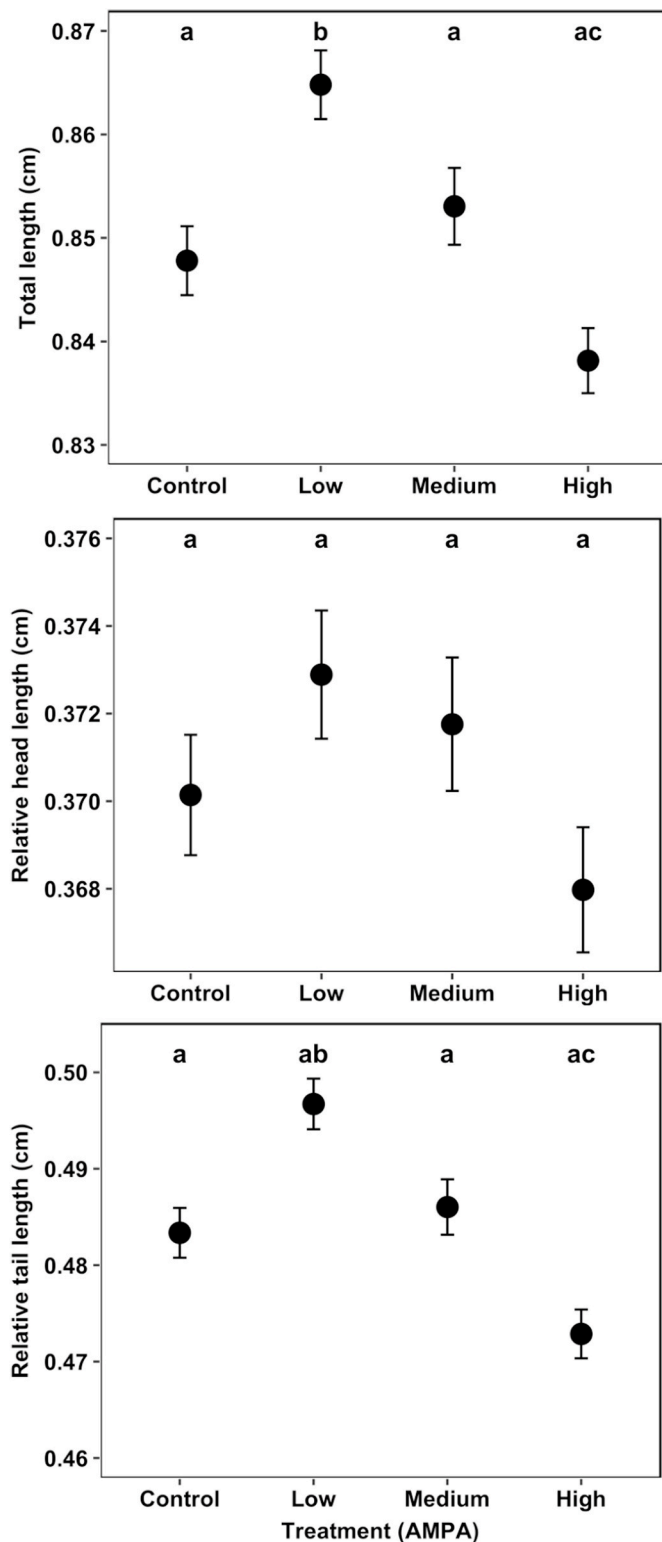


Fig. 3. Hatchlings morphology at Gosner stage 22 (upper panel: total length, middle panel: head length, lower panel: tail length) relative to AMPA concentrations. Lowest AMPA concentration yielded longer tadpoles (with longer tails) while highest AMPA concentration yielded shorter tadpoles (with shorter tails). Letters indicate significant differences with controls (Tukey HSD, $p < 0.05$).

Embryonic mortality was 2–3 times higher in eggs that developed with the lowest and the intermediate AMPA concentrations. Focusing solely on the differences among control embryos and the embryos raised at the two lower AMPA concentrations, the pattern we found could suggest a direct lethal effect of AMPA on embryos, presumably through increased toxicity driven by increased AMPA concentration (Broomhall and Shine, 2003; García-Muñoz et al., 2010; Baier et al., 2016a,b). However, the results from the highest AMPA concentration do not support the hypothesis of increasing AMPA concentrations causing increased mortality. Indeed, embryos that were exposed to the highest AMPA concentration developed and survived as well as those in the control treatment. A non-linear survivorship pattern is clearly more complex than classical linear concentration-response relationships and future studies are required to assess whether this pattern is maintained with other increasing test concentrations (see also below). Development duration was also significantly influenced by a chronic exposure to AMPA. Indeed, chronic exposure to AMPA seems to delay hatching as development duration was longer in all groups exposed to AMPA, irrespective of the concentration. The two lower AMPA concentrations yielded similar development durations, while the highest concentration of AMPA produced development duration that were significantly shorter than that of the two lower AMPA concentrations, although longer than that of control embryos. Again, this pattern seems more complex than classical concentration-responses and future studies should usefully investigate the outcome of similar approaches with higher AMPA concentrations. We found that chronic exposure to AMPA influences not only the size of tadpoles upon hatching, but also their body proportions. Embryos submitted to the lowest AMPA concentration produced longer tadpoles, with similar body length than other groups but longer tails. Conversely, embryos submitted to the highest AMPA concentration produced shorter tadpoles, with shorter tails. Similarly to the previous metrics of embryonic development we studied, these response patterns of morphology contrast from classical concentration-responses, and seem to point to a concentration-dependent effect of AMPA on body architecture. Such response clearly deserves future investigations in order to clarify the mechanisms through which exposition to AMPA during embryonic development influences overall morphology.

Whether the alteration of embryonic development we highlighted bears consequences for developing tadpoles in natural ponds remains complicated to directly assess with our experimental design. Increased embryonic mortality could decrease the reproductive success of toads that lay their eggs in a contaminated pond (Hayes et al., 2006; Richter et al., 2003). Delayed hatching may potentially increase the vulnerability of immobile embryos to predators (Zamudio et al., 2016). In addition, delayed embryonic development may also induce cascading effects on the subsequent larval (tadpole) development duration, a potentially deleterious consequence if spawning takes place in ephemeral water bodies. Finally, reduced body size may decrease mobility and thus foraging ability while increasing susceptibility to predation (Broomhall and Shine, 2003; Hoff and Wassersug., 2000). Although taken together all of these elements tend to suggest ecological consequences for embryos (and may be tadpoles) developing in water contaminated by environmental concentrations of AMPA, these hypotheses need to be taken with caution and will require thorough testing, especially as post-embryonic survival may be more important to population persistence than egg survival (Vonesh and De la Cruz., 2002). In addition, it is important to highlight the fact that the absolute magnitude of the effects we found appears relatively small. Indeed, although embryonic mortality was marked by a two- or three-fold increase in some of our experimental concentrations, development duration increased by ~2.5% and body size was either increased by ~2.1% or decreased by ~1.2%. Although we cannot entirely rule out that the ecological significance of these effects may be trivial they could equally induce consequences on fitness-related traits such as parent reproductive success (embryonic mortality) and offspring survival (development duration, tadpole morphology) and thus may ultimately affect

population persistence (Hayes et al., 2010; Orton and Tyler, 2015). Such results are clearly important to take into account at a time when amphibians are dramatically declining (Hayes et al., 2010; Berger et al., 2013; Grant et al., 2016., Wake, 2012). Importantly, our results contrast with those found with the parent compound on similar species (Baier et al., 2016a,b) suggesting that AMPA can be at least as toxic as glyphosate or its formulation containing surfactants (Giesy et al., 2000; Relyea, 2009; Moore et al., 2012). Yet, we clearly lack studies regarding its toxicity at ecologically relevant concentrations in non-target species (Matozzo et al., 2018, 2019).

Perhaps more importantly than the magnitude of the effects *per se*, we emphasize the complex patterns of concentration-response curves we found. Indeed these patterns contrast with other studies that have focused on much higher test concentrations (FAO, 2013), and may indicate low-concentration effects and nonmonotonic concentration responses (Vandenberg et al., 2012). Such nonmonotonic responses were not expected, and thus we lack the power to thoroughly test for this hypothesis because verifying nonmonotonicity requires at least two concentrations on either side of the inflection point (OECD, 2014). Yet, it is important to stress that low-concentration effects and nonmonotonic responses seems to be strongly linked to endocrine disruption (Vandenberg et al., 2012), a possible underlying mechanism to the complex patterns we found for all metrics studied. If such hypothesis is true, deciphering the targets of this putative endocrine disruption will be critical to comprehensively assess the impact of glyphosate's primary metabolite. In addition, this hypothesis would add to the recently growing evidences that, for anthropogenic contaminants, nonmonotonic response curves at low concentrations are much more widespread than previously suspected (Hill et al., 2018). Yet, nonmonotonicity, although fundamental for regulatory decision-making, occurs at minute concentrations that are usually disregarded by regulatory toxicology (i.e., in our case 6000 times lower than PNEC). Clearly, decision-making needs to go beyond the use of high-dose studies to infer official No Effect Concentrations (Hayes et al., 2010; Hill et al., 2018). As a consequence,

our study pleads for the investigation of parent compounds but, perhaps more importantly, also their metabolites at environmentally relevant concentrations in order to comprehensively assess impacts of anthropogenic contaminants on the environment.

More generally, our results emphasize the potential effects of AMPA on human and environmental health (e.g., Matozzo et al., 2018, 2019; Martinez and Al-Ahmad, 2019). Indeed, the putative nonmonotonic responses we detected, and thus the underlying potential endocrine disruption (Vandenberg et al., 2012) can be of critical importance for both humans and wildlife. This seems especially important given that our tested concentrations are very similar to concentrations found in aquatic environments in Northern America and Europe. Future studies are required to assess whether other study models can be affected by environmental concentrations of AMPA.

Authors' contribution

M.Cheron and F.Brischox designed and conducted the experiment, analyzed the data and wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix 1. Effect size of pair-wise comparisons using Cohen's δ statistics. C stands for control. The magnitude of differences (at $p < 0.05$) is considered significant for Cohen's δ that are reaching values close to or above 0.2 and are indicated in bold. Cohen's δ is not indicated for non-significant differences ($p > 0.05$)

Pair-wise comparisons	Mortality	Development duration	Morphology
C - Low	0.170	0.314	0.253
C - Medium	0.234	0.230	–
C - High	–	0.164	0.147
Low - Medium	–	–	0.168
Medium - High	0.187	0.144	0.217
Low - High	0.249	0.160	0.409

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2020.109944>.

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Article V

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Aminomethylphosphonic acid (AMPA) alters oxidative status during embryonic development in an amphibian species

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Aminomethylphosphonic acid (AMPA) alters oxidative status during embryonic development in an amphibian species

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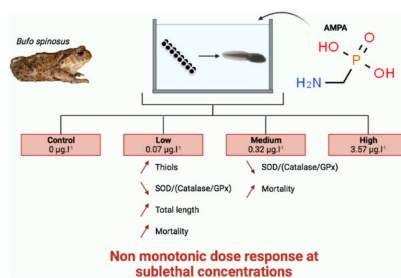
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HIGHLIGHTS

- Spined toad hatchlings were exposed to three AMPA concentrations.
- AMPA affects oxidative status markers and embryonic development.
- Low concentrations induced non-monotonic doses responses.
- Telomere length was not affected by AMPA treatment.

GRAPHICAL ABSTRACT



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ABSTRACT

Glyphosate's primary metabolite (aminomethylphosphonic acid, AMPA) is known to alter embryonic development at environmentally relevant concentrations in amphibians. However, we have limited understanding of the physiological mechanisms through which AMPA affects organisms. In this study, we tested whether alteration of the oxidative status is one mechanism through which AMPA affects organism performance. To this end, we analysed several oxidative status markers in hatchling tadpoles that were exposed to sublethal concentrations of AMPA during embryonic development (~16 days). We compared the influence of environmentally relevant concentrations of AMPA (from 0.07 to 3.57 $\mu\text{g l}^{-1}$) on the relation between developmental traits (i.e., embryonic development duration, embryonic mortality and hatchling size) and oxidative status markers known to alter homeostasis when unbalanced (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), thiols and ratios thereof). We included measures of telomere length as an indicator of physiological state. We found that AMPA concentrations induce non-monotonic effects on some oxidative status markers with hatchlings displaying elevated antioxidant responses (elevated thiols and unbalanced SOD/(GPx + CAT) ratio). The lack of effect of AMPA on the relation between developmental traits, oxidative status and telomere length suggests that selective mortality of embryos susceptible to oxidative stress may have occurred prior to hatching in individuals less resistant to AMPA which display lower hatching success. Future studies are required to disentangle whether oxidative unbalance is a cause or a consequence of AMPA exposition. This study highlights the need to investigate effects of the metabolites of contaminants at environmental concentrations to comprehensively assess impacts of anthropogenic contamination on wildlife.

Abbreviations: AMPA, aminomethylphosphonic acid; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; SODGPx, ratio between SOD and GPx; SODCAT, ratio between SOD and CAT + GPx; GS, Gosner stage.

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1. Introduction

Among the numerous sources of environmental contamination (e.g., fossil fuel combustion, industrial activities, (Kendall et al., 2016), modern agriculture is well-known for the massive release of toxic substances in the environments (Jayaraj et al., 2016; Solomon and Thompson, 2003). Indeed, pesticides are specifically designed to alter the functioning of diverse organisms (weeds, fungi, and insects) which are detrimental to crop productivity (Sharma et al., 2019). Although traditional toxicology studies have convincingly demonstrated the potential toxicity of such compounds on non-target organisms (Crump et al., 2002; de Brito Rodrigues et al., 2019; Hasenbein et al., 2017), they are not sufficient to assess their impact on wild animals for several reasons. First, they often rely on relatively high, environmentally irrelevant concentrations of agrochemicals in order to assess mortality thresholds (i.e., LD50, Relyea and Edwards, 2010). As a consequence, reliance on LD50 limits our ability to identify more subtle, sublethal effects occurring at lower concentrations (Beiras, 2018). However, such sublethal effects have been shown to occur at extremely low concentrations and to be responsible for the disruption of several organismal traits that can affect population persistence (Cauble and Wagner, 2005; Cheron and Brischoux, 2020; Lenkowski et al., 2010).

Importantly, field monitoring often reveals that environmental contamination occurs at concentrations that are well below LD50 (Awkerman and Raimondo, 2018; Egea-Serrano et al., 2012). Second, most toxicology studies use laboratory models which can differ from their wild counterparts in their response to environmental contamination (Hahn and Sadler, 2020; Patisaul et al., 2018). Indeed, laboratory conditions simplify the complexity that organisms face in natural environments and long-term selection of laboratory lineages are likely to influence the responses of these species to environmental constraints (Hahn and Sadler, 2020; Segner and Baumann, 2016). Overall, one of the prominent current challenges is to assess the detrimental sublethal effects of contaminants at environmentally relevant concentrations on non-model organism's representative of exposure in the wild. This is particularly relevant for pesticides, because their breakdown after spread in agricultural fields can expose organisms to concentrations much lower than those originally used or tested in laboratory experiments (Bonansea et al., 2017; Wang et al., 2019).

Glyphosate remains one of the most widely used non-selective herbicide (Clapp, 2021; Helander et al., 2012). Accordingly, the effects of glyphosate on wildlife, including investigations of commercial formulations and/or of different surfactants have been relatively thoroughly studied (Berger et al., 2018; Bókonyi et al., 2017; Cauble and Wagner, 2005; de Brito Rodrigues et al., 2019; Giaquinto et al., 2017; Gill et al., 2018; Guilherme et al., 2010; Herek et al., 2020; Lenkowski et al., 2010; Matozzo et al., 2020; Muñoz et al., 2021; Solomon and Thompson, 2003). However, its primary metabolite, aminomethylphosphonic acid (AMPA) is arguably the most common compound found in surface and groundwater worldwide (Bonansea et al., 2017; Medalie et al., 2020; Okada et al., 2020; Silva et al., 2018). The greater occurrence and abundance of AMPA in natural environments is likely linked to the extensive use of glyphosate and also because AMPA is an organic phosphonate derived from detergents (Grandcoin et al., 2017). In North American wetland, AMPA occurred at 97% ranging from 0.25 to 10 $\mu\text{g l}^{-1}$ (Okada et al., 2020). Environmental concentrations found in aquatic environments in France range from 0.1 $\mu\text{g l}^{-1}$ to 6.6 $\mu\text{g l}^{-1}$ (data from Water Agencies, "Agence de l'eau Loire Bretagne" and "Agence de l'eau Adour-Garonne"). Despite its greater environmental occurrence and abundance than glyphosate, studies on the effects of AMPA at environmental concentrations on wildlife are comparatively fewer (Cheron and Brischoux, 2020; de Brito Rodrigues et al., 2019; Domínguez et al., 2016; Guilherme et al., 2014; Martinez and Al-Ahmad, 2019; Matozzo et al., 2018, 2019). Importantly, these studies have revealed sublethal effects on wild species, such as genotoxic effect (Battaglin et al., 2014; Medalie et al., 2020; Okada et al., 2020; Silva et al., 2018) and cellular

and biochemical alterations (Matozzo et al., 2018, 2019). AMPA has been shown to alter embryonic development in amphibians, increasing embryonic mortality and altering hatchling phenotype through processes occurring at low concentrations and suggesting nonmonotonicity (Cheron and Brischoux, 2020).

Our goal was to identify potential physiological mechanisms that may be responsible for the alteration of embryonic development due to environmentally relevant concentrations of AMPA (Cheron and Brischoux, 2020). In this respect, we focused our investigations on oxidative stress, as such cellular biochemical process is known to reflect how environmental stressors affect organismal physiology (Costantini, 2014). The metabolic activity of cells is responsible for a continuous generation of reactive chemical species (e.g. reactive oxygen species ROS), which cause oxidation of numerous organic substrates (e.g. lipids, DNA). Organisms are equipped with a complex array of molecular defence mechanisms, generally referred to as antioxidants. The cellular oxidative status is, therefore, determined by a complex network of molecules, including oxidative damages to organic substrates, levels of both enzymatic and non-enzymatic antioxidants, or ROS production (Costantini, 2019). Any changes in these molecular components of cellular oxidative status provide evidence for oxidative imbalance. There is growing consensus that oxidative status alteration is one relevant molecular mechanism that links environmental conditions (including contaminant exposure) to fitness, through effects on growth, fertility, reproduction and senescence (Alonso-Alvarez et al., 2004; Beaulieu and Costantini, 2014; Costantini et al., 2014; Stier et al., 2012). In addition to markers of oxidative status, we included in our investigations telomere length that may reveal complementary effects of AMPA on DNA damage during amphibian embryonic development (Burraco et al., 2017; Chatelain et al., 2020; Cheron et al., 2021). Indeed, telomeres are repetitive nucleotide sequences located in each chromosome to ensure stability and viability, and telomere length is increasingly suggested to be an integrative biomarker of organismal, cellular, and molecular damages. Although telomere shortening occurs normally in cell division, this process can be magnified under stressful conditions especially when they are associated with oxidative stress (Reichert and Stier, 2017). Accordingly, stress-induced telomere attrition may be linked to an intense cellular oxidative stress (Chatelain et al., 2020) because guanine (the main nucleotide of telomeres) is particularly sensitive to oxidation (Bjelland, 2003). Finally, telomere length might be seen as a proxy of individual quality although such relationship is not well established in ectotherms species (Burraco et al., 2020a; Olsson et al., 2018).

Specifically, we hypothesized that environmentally relevant concentrations of AMPA, which have been shown to induce altered embryonic mortality, development duration and hatchling phenotype (Cheron and Brischoux, 2020), will induce significant changes in oxidative status. We expected that exposition to AMPA concentrations will induce increase in antioxidant enzymes such as SOD, GPx and CAT and a depletion of thiols levels. Moreover, we expected non monotonic relationships between oxidative markers and AMPA concentrations given that some studies showed nonlinear or hormetic effects of AMPA or glyphosate on organisms (Brito et al., 2018; Cheron and Brischoux, 2020; Domínguez et al., 2016). If exposure to AMPA leads to increased oxidative stress, we also predicted that exposure to AMPA should be associated with shorter telomeres.

2. Materials and methods

2.1. Study species

Spined toad (*Bufo spinosus*) is a widespread amphibian species that persists in agricultural landscapes (Gagné and Fahrig, 2007; Guillot et al., 2016). Breeding pond (46°090 N, 0°240 W, France) was monitored every day during the reproductive period. All eggs strings (N = 10) were collected during a single day in January 2018 immediately after

laying in order to avoid development in the natural environment. A subset of each string containing 120 eggs was randomly selected and kept for our experiment (see below), while the remaining eggs (i.e., 3000–5000) were released at their site of origin.

2.2. Treatment concentrations and chemical solutions

Environmental concentrations found in aquatic environments in France range from $0.1 \mu\text{g l}^{-1}$ to $6.6 \mu\text{g l}^{-1}$ (data from Water Agencies, “Agence de l’eau Loire Bretagne” and “Agence de l’eau Adour-Garonne”) and are similar to concentrations found in aquatic environments in Northern America and Europe (Bonansea et al., 2017; Coupe et al., 2012; Grandcoin et al., 2017). Because our goal was to mimic the range of concentrations found in the wild, we aimed at three nominal concentrations spanning from low to relatively high concentrations ($0.1 \mu\text{g l}^{-1}$; $0.30 \mu\text{g l}^{-1}$ and $3.00 \mu\text{g l}^{-1}$, Table 1). Stock solutions (0.1 g/L) of AMPA were obtained by dissolving commercial crystalline powder (aminomethylphosphonic acid, 99% purity, ACROS ORGANICS™) in dechlorinated tap water. These stock solutions were further diluted with dechlorinated tap water to reach the selected concentration for each treatment group (Table 1). We produced additional water samples for each treatment in order to perform analytical verifications of the concentrations. These analytical verifications of the actual concentrations were performed by an independent accredited analytical laboratory (QUALYSE, Champdeniers-Saint-Denis, France). Determination of AMPA in water samples was assessed using liquid chromatography-mass spectrometry (LC/MS/MS) with 9-fluorenylmethyl chloroformate (FMOC-Cl) used as derivatization agent. The limit of quantification was $0.1 \mu\text{g l}^{-1}$ for the determination of AMPA in water samples. Overall, these verifications showed that actual concentrations were similar to concentrations found in aquatic environments (see above) and that the differences between “low” ($0.07 \pm 0.01 \mu\text{g l}^{-1}$), “medium” ($0.32 \pm 0.052 \mu\text{g l}^{-1}$) and “high” ($3.57 \pm 0.153 \mu\text{g l}^{-1}$) treatments were significant (Table 1).

For clarity, we will refer to the treatments as Low, Medium and High hereafter.

2.3. Experimental design

Each subset of each clutch (containing 120 eggs) was further separated into segments containing 30 eggs (i.e., 120/4 treatments) that were randomly placed in different glass-molded aquariums containing 2 L of water and one of our experimental treatments (i.e., Control, Low, Medium or High) and monitored until hatching (total $N = 4$ treatments * 10 clutches representing 60 tanks containing 30 eggs each).

Eggs were subjected to different concentrations of AMPA (Low, Medium and High) and a control during the whole embryonic development until hatching. To maintain relatively constant exposure levels throughout the experiment but to avoid excessive mechanical disturbances to developing eggs, water was changed once a week according to the half-life of AMPA in water (Battaglin et al., 2014). Our procedures did not alter egg jelly matrix surrounding the developing embryos throughout the experiment. We determined the stage of development according to the developmental table of Gosner (1960). Eggs were exposed from laying to hatching and hatching occurred at Gosner stage 22 (hereafter GS 22) after 16.10 ± 0.16 days (see Table 2). All

Table 1
Concentrations ($\mu\text{g.l}^{-1}$) measured in the experimental tanks (Mean \pm SD).

Treatment	Actual AMPA concentrations
Control	0.00 ± 0.00
Low	0.07 ± 0.01
Medium	0.32 ± 0.052
High	3.57 ± 0.153

experiments took place in a thermal-controlled room with air and water temperature set at $17 \text{ }^\circ\text{C}$ and with a natural day/night photoperiod (9 h/15 h). We monitored egg segments and counted all the individuals that hatched (GS22) which we considered a metric of hatching success. Development duration was assessed as the time elapsed between the date of egg collection (egg laying) and the date of hatching. We took pictures of all live hatchlings using a camera (Panasonic Lumix DC-TZ55) in order to assess the length of individuals ($N = 80$). Morphological measurements were performed with the software ImageJ (Schneider et al., 2012).

Upon hatching, for each clutch and within each treatment, we randomly collected 2 hatchlings that were pooled to assess oxidative status (because a single individual did not yield enough tissue for these assays) and 2 hatchlings that were used for telomere analyses. Hence, we measured clutch levels of oxidative status parameters.

2.4. Oxidative status markers

We randomly selected 80 hatchlings (20/experimental group), including two hatchlings that hatched from each single clutch. We pooled together these two hatchlings because a smaller portion did not provide enough tissue for the analyses. Thus, we had 10 pools per experimental group (i.e. 40 samples in total). We homogenized hatchlings in Dulbecco’s Phosphate Buffered Saline (Sigma-Aldrich, France) supplemented with 1 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich, France) as an inhibitor of proteases using a TissueLyser II (Qiagen) at 30 Hz for 4 min. Afterwards, we centrifuged tubes for 10 min at $4 \text{ }^\circ\text{C}$ to obtain clean supernatants to be used for the assays. We measured the concentration of thiols using the -SHp test (Diacron International, Grosseto, Italy); the activity of the antioxidant enzyme superoxide dismutase (SOD) using the Ransod assay (RANDOX Laboratories, France); the activity of the antioxidant enzyme glutathione peroxidase (GPx) using the Ransel assay (RANDOX Laboratories, France); the activity of CAT using the OxiSelect CAT Activity Assay (Euromedex, France). We ran all analyses in duplicate and an average value of each duplicate was used for the statistical analyses (coefficients of variation $<10\%$). We normalized values of markers to the amount of

Table 2

Mean values of oxidative status markers and developmental-related traits amongst treatment groups of aminomethylphosphonic acid (AMPA).

	Treatment			
	Control (n = 10)	Low (n = 10)	Medium (n = 10)	High (n = 10)
Oxidative stress markers				
SOD*	3.822 ± 0.447	3.139 ± 0.360	2.835 ± 0.386	3.277 ± 0.509
Thiols*	0.075 ± 0.002	0.082 ± 0.002	0.072 ± 0.002	0.070 ± 0.002
CAT*	13.813 ± 1.436	16.943 ± 2.347	18.728 ± 2.612	11.212 ± 2.702
GPx*	0.110 ± 0.013	0.106 ± 0.009	0.114 ± 0.008	0.118 ± 0.012
SOD/(GPx + CAT)	0.307 ± 0.048	0.204 ± 0.039	0.175 ± 0.035	0.352 ± 0.067
SOD/GPx	39.274 ± 6.419	30.239 ± 2.896	$25.1453.355$	28.077 ± 3.514
Developmental-related traits				
Total length (cm)	0.842 ± 0.016	0.896 ± 0.016	0.864 ± 0.020	0.837 ± 0.013
Hatching success	0.997 ± 0.002	0.963 ± 0.020	0.953 ± 0.032	0.997 ± 0.003
Telomere length (T/S ratio)	0.951 ± 0.041	0.942 ± 0.020	0.928 ± 0.042	0.972 ± 0.044
Development duration (days)	16.10 ± 0.233	16.30 ± 0.213	16.30 ± 0.163	16.45 ± 0.221

Values are Mean \pm SE.

SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: CAT.

*: (units/mg protein).

proteins as quantified using the Bradford protein assay with albumin as reference standard (Sigma-Aldrich, France). All assays were run according to manufacturer's instructions and have been validated in amphibian species (Liparoto et al., 2020).

In addition to markers alone we chose to show balanced or unbalanced ratio of markers activities following de Haan et al. studies (1992, 1996) (de Haan et al., 1992, 1996). An unbalanced ratio of superoxide dismutase and glutathione peroxidase (SOD/GPx) can lead to a proliferation of H₂O₂ leading to accelerated cellular senescence and altered morphology (de Haan et al., 1996). Moreover, since both GPx and catalase convert H₂O₂ into H₂O, an unbalanced ratio of superoxide dismutase on GPx and catalase activities (SOD/(GPx + CAT)) can lead to a proliferation of deleterious pro-oxidants (de Haan et al., 1992).

2.5. Relative telomere length

Similarly to sampling for oxidative stress markers, we randomly selected two hatchlings among the embryos that hatched of each clutch for telomere analysis (N = 20 hatchlings from 10 clutches). Although, smaller portions of individuals (e.g., head, tail, muscle) can be used for telomere assays (Burraco et al., 2017; Sánchez-Montes et al., 2020), hatchling spined toads were too small to provide enough tissue for these analyses and we used whole individuals (Cheron et al., 2021). We used quantitative PCR (qPCR; BioRad CFX 96, Bio-Rad, USA) to determine telomere length using an established protocol (McLennan et al., 2019), which was adapted for spined toad (Cheron et al., 2021). Whole hatchlings were digested with proteinase K and DNA was extracted using the Nucleospin Tissue Kit (Macherey-Nagel), following the manufacturer's instructions. DNA concentration and purity were assessed with a Nanodrop ND1000 spectrophotometer (Thermo Scientific). Universal telomere primers were used, and the control single-copy gene Recombination Activating Gene 1 (RAG-1) was selected and amplified using specific primers designed for the spined toad: RAG1-F 5'-GGGCTCTGATAGCCGAAA-3' and RAG1-R 5'-CATCA-TAACCTGTACCCCGGA-3'. This single-copy gene has previously been successfully used in multiple species (birds (Sebastiano et al., 2020); fish (Dupoué et al., 2020; McLennan et al., 2019; Petitjean et al., 2020)) including amphibians (Canestrelli et al., 2020). All qPCRs were performed on three plates for each gene (RAG-1 and telomere) using 7.5 ng of DNA per reaction. The telomere and single-copy gene primers were respectively used at a concentration of 800 nM and 300 nM. To generate a six-point standard curve (from 50.0 ng to 1.5 ng) for controlling the amplifying efficiency of the reactions, serial dilutions of DNA from a pooled sampled of 10 tadpoles were included on the plate (in triplicates). For both telomere and RAG-1 amplification, the melt curves displayed single sharp curves, validating amplification specificity of these primers. A reference toad sample was run in triplicate in all plates to account for inter-plate variation. All samples were run in duplicates and were randomly distributed across the PCR plates. Amplification efficiencies reached Mean ± SE: RAG-1, 87.48 ± 5.92; TEL, 92.60 ± 6.00 and all R² were always very high (>0.99). The relative telomere length (expressed as T/S ratio) was calculated as the telomere copy number (T) relative to single-copy gene (S; RAG1), according to Cawthon (2002). Intra-plate variation for telomere and RAG1 were 2.07% and 2.53% respectively. Intra-plate variation for the T/S ratio was 3.46%. Inter-plate variations for telomere and RAG-1 were 2.07% and 1.89%, respectively. Inter-plate variation for the T/S ratio was 5.62%.

2.6. Statistical analysis

All statistical analyses were conducted with R statistical software v.4.0.0 (R Core Team, 2020) and RStudio v 1.2.5042 (RStudio, Inc.).

All data were tested for homogeneity of variance and normality with the Bartlett's test and the Shapiro-Wilks test, respectively. We also checked the normality of the residuals using diagnostic plots. We checked which distribution fitted the best the model using Cullen & Frey

plot (bootstrap at 500, "fittedplus" package (Delignette-Muller and Dutang, 2015)). We computed z-scores in all predictor variables (scaled to mean of 0 and standard deviation of 1) to avoid multicollinearity between treatment when we tested interaction or relation with developmental-related traits but also to avoid large differences in scale in the model. We checked for multicollinearity using the variance inflation factor (VIF, non-multicollinearity when below 2. "car" package). Clutch identity was used as random effect in all our models.

Each clutch was assigned with the value of average telomere length ratio of the two tadpoles sampled. Because we did not measure oxidative status markers and telomere length on the same individual instead, we pooled individuals of the same family or used average values for telomere length, correlations between the two parameters were at clutch levels.

We used η^2 to measure the magnitude of the main effect (package "effect size").

η^2 : <0.01 considered negligible, between 0.01 and 0.06 considered small, between 0.06 and 0.14 considered medium and >0.14 considered large effect.

First to assess whether our AMPA treatment had an effect on oxidative status markers we fitted six linear mixed-effect models (LMERs, package *lme4* (Bates et al., 2015), with "Treatment" as fixed effect and "SOD", "GPx", "CAT", "thiols", "SOD/GPX" "SOD/(GPx + CAT)" as dependent variables. Four individuals displayed proportionally elevated CAT (tested with "rosmertest" and "EnvStats" packages) and were excluded from the analyses. We calculated 95% confidence intervals, p value and coefficient for each levels of fixed effect factor from 1000 bootstraps iterations ("parameters", packages, Appendix A; table S1).). When $p < 0.05$, the magnitude of the main effect was measured using η^2 (package "effectsize").

Second, to test whether developmental-related traits differed between AMPA treatment groups, we fitted three linear mixed-effect models ("lmer" function, "lme4" package (Bates et al., 2015)) with "Treatment" as fixed effect and "embryonic development duration", "total length" and "telomere length" as dependent variables. Because hatching success followed binomial distribution, we fitted one generalized linear mixed models ("glmer", "lme4" package (Bates et al., 2015)) with "hatching success" as dependent variable.

Finally, to test if there was a relation between developmental-related traits and oxidative status and if this relation differed between treatment, we fitted LMER models with development-related trait ("embryonic development duration", "total length" and "telomere length") as dependent variables and z_scored oxidative status markers ("SOD", "GPx", "CAT", "thiols", "SOD/GPX" "SOD/(GPx + CAT)") as fixed effect in interaction with "Treatment". We ran GLMER for binomial data ("hatching success"). To compare the trends and regression slopes we used "emmeans" function in "emmeans" package. Due to high correlation between oxidative status markers (SOD, GPx, CAT) and ratios, we performed statistical models separately (Appendix A; table S2).

3. Results

All oxidative status and developmental-related traits at each AMPA concentrations are summarized in Table 2.

3.1. Effect of AMPA treatment on oxidative status markers

CAT, SOD and GPx were not influenced by AMPA treatment (respectively: $F_{3,25} = 2.05$, $p = 0.125$; $F_{3,27} = 0.92$ $p = 0.439$; $F_{3,27} = 0.24$, $p = 0.869$, Table 3). When including four outlier values for CAT, AMPA did not influence enzymatic activities ($F_{3,27} = 0.70$, $p = 0.560$). AMPA treatment influenced concentration of thiols ($F_{3,26} = 3.66$, $p = 0.021$, $\eta^2 = 0.21$, Table 3). Hatchlings from low AMPA group displayed higher thiols than all other treatment groups (all $p < 0.028$, Tukey HSD, Fig. 1).

AMPA treatment significantly influenced SOD/(GPx + CAT) ratio

($F_{3,25} = 3.93$, $p = 0.017$, $\eta^2 = 0.27$, Table 3). When including four outlier values for CAT, we found a marginal effect of AMPA in SOD/(GPx + CAT) ratio ($F_{3,27} = 2.92$, $p = 0.052$). Ratios at Low and Medium concentrations were significantly lower compared to High concentration (respectively: $-0.16[-0.30, -0.01]$, $p = 0.029$; $-0.21[-0.35, -0.06]$, $p = 0.006$, Fig. 1). In Medium treatment, SOD/(GPx + CAT) ratio decreased compared to Control treatment ($-0.13[-0.24, -0.02]$, $p = 0.026$).

Finally, treatment did not affect SOD/GPx ratio ($F_{3,36} = 2.02$, $p = 0.127$, Table 3) nor telomere length ($F_{3,36} = 0.51$, $p = 0.673$).

3.2. Effect of AMPA treatment on developmental-related traits

Hatching success varied among treatments ($\chi^2_3 = 25.168$, $p < 0.001$); Low and Medium treatment yielded lower hatching success than Control or High treatment of AMPA (all $p > 0.003$).

AMPA treatment did not influence embryonic development duration ($F_{3,67} = 1.15$, $p = 0.33$). However, total length of hatchlings differed between treatments ($F_{3,67} = 5.57$, $p = 0.002$). Hatchlings were larger at Low treatment compared to individuals from Control and High treatments (respectively, $p = 0.004$, $p = 0.002$).

3.3. Relationships among oxidative status markers and developmental-related traits

We did not find any relationships among any oxidative status marker and telomere length (all interaction $p > 0.26$). Total length of hatchlings was not related to any oxidative status markers ($p > 0.25$). Relation between total length of hatchlings and oxidative status markers in interaction with treatment was never significant (all $p > 0.201$). We did not find any relation between oxidative status markers and embryonic development duration across or among groups (all $p > 0.186$).

Finally, although we found a significant interaction between SOD/GPx and SOD/(CAT + GPx) ratios, and hatching success, after close examination of the plots and removals of outliers, such interactions were no longer supported.

4. Discussion

Our study shows that the primary metabolite of glyphosate – AMPA –

Table 3

Comparisons of outputs of linear mixed models (LMER, “lmerTest” package) of oxidative stress markers amongst treatment of aminomethylphosphonic acid (AMPA). Clutch identity was used as random factor in all our models.

Variable	Effect	df	F-value	p-value	Clutch identity	
					Variance	Residuals variance
SOD*	Intercept	1,9	79.21	< 0.001	0.365	1.401
	Treatment	3,36	0.92	0.439		
Thiols*	Intercept	1,9	727.65	< 0.001	<0.001	<0.001
	Treatment	3,36	3.66	0.02		
CAT*	Intercept	1,9	37.38	< 0.001	4.11	44.35
	Treatment	3,36	2.05	0.125		
GPx*	Intercept	1,9	103.99	< 0.001	<0.001	<0.001
	Treatment	3,36	0.24	0.869		
SOD/(GPx + CAT)	Intercept	1,9	44.85	< 0.001	0.0012	0.019
	Treatment	3,36	3.93	0.017		
SOD/GPx	Intercept	1,9	84.28	< 0.001	23.08	157.44
	Treatment	3,36	2.03	0.127		

SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: CAT. Values in bold are considered significant $P < 0.05$.

alters oxidative status, but not telomere length, during embryonic development in an amphibian species that occurs in agricultural landscapes. Interestingly, these physiological effects occurred at environmentally relevant-concentrations representative of actual concentrations of this contaminant in the wild. Furthermore, our results suggest non-monotonicity for some markers of oxidative status such as thiols and SOD/(CAT + GPx) ratio as shown for developmental-related traits in a related study (Cheron and Brischoux, 2020). SOD/(CAT + GPx) ratio is likely driven by CAT levels considering the trend for CAT to increase at Low and Medium level while GPx remained steady.

Exposure to low concentrations of AMPA influenced the levels of thiols but did not influence CAT levels, although both markers showed similar trends. For these markers, levels were higher at the lowest or the intermediate AMPA concentrations tested which suggest low concentration responses and non-monotonicity (Hill et al., 2018; Shi et al., 2016; Vandenberg et al., 2012). Interestingly, although we did not detect any influence of AMPA concentrations on SOD and GPx, we found that relevant ratios of these antioxidant enzymes (i.e., SOD/(GPx + CAT)) were influenced by AMPA treatments. Moreover, since both GPx and CAT convert H_2O_2 into H_2O , an unbalanced ratio of superoxide dismutase on GPx and CAT activities (SOD/(GPx + CAT)) can lead to a proliferation of deleterious pro-oxidants (de Haan et al., 1992). SOD/(GPx + CAT) ratios showed low concentration responses and non-monotonicity, with the lowest values obtained for the lower and intermediate AMPA concentrations. These results suggest a possible low-concentration stimulatory effect of AMPA with upregulation of both CAT and thiols. One important feature of the regulation of oxidative status involves, first the dismutation by superoxide dismutase (SOD) of O_2^- into H_2O_2 and then the conversion of H_2O_2 to H_2O catalysed by CAT and/or GPx. However, when H_2O_2 is produced at high concentrations, CAT is more efficient in the conversion of H_2O_2 in H_2O than GPx, which might explain why we observed upregulation of CAT but not of GPx (Halliwell and Gutteridge, 2015). It is important to stress that antioxidants play an important role in protecting early development and, accordingly, their levels are known to increase during embryonic development in amphibians (Dandapat et al., 2003; Anguiano et al., 2001). Moreover, during early development, CAT levels are high while GPx levels are low which show the important role of CAT in removing toxic hydrogen peroxide (Aceto et al., 1994), and GPx activities tend to gradually increase during growth (Pašková et al., 2011).

To our knowledge, only few studies investigated relevant concentration of AMPA on biochemical parameters (Guilherme et al., 2014; Matozzo et al., 2018, 2019) and most studies have investigated its parent compound, glyphosate (de Brito Rodrigues et al., 2019). Guilherme et al. (2014) found DNA and chromosomal damage when *Anguilla Anguilla* were exposed for 1 or 3 days at sublethal concentration (11.8 and 23.6 $\mu g l^{-1}$). In marine invertebrates, AMPA exposure altered oxidative status and enzymatic activities in *Mytilus galloprovincialis* when exposed for 7, 14 or 21 days to 1, 10 and 100 $\mu g l^{-1}$ (Matozzo et al., 2018, 2019). In tadpole of another amphibian species (*Pelobates cultripes*), exposure to sublethal concentration of glyphosate induced altered antioxidant activity by decreasing glutathione reductase activity but did not affect SOD nor CAT levels (Burraco and Gomez-Mestre, 2016). In *Oncorhynchus mykiss*, glyphosate-based formulations altered energy metabolism (Le Du-Carrée et al., 2021) when exposed to 1 $\mu g l^{-1}$ and in *Anguilla Anguilla* increased CAT levels in gills when exposed for 1 or 3 days at 58 and 116 $\mu g l^{-1}$ (Guilherme et al., 2012). Focusing on glyphosate's primary metabolite, our study reinforces the potential of this widely used herbicide and of its metabolite AMPA (the main contaminants detected in surface waters worldwide, Battaglin et al., 2014; Medalie et al., 2020; Okada et al., 2020; Silva et al., 2018) to alter oxidative status in wildlife.

Interestingly, given the lower hatching success in Low and Medium groups, selective mortality of embryos susceptible to oxidative stress may have occurred in these two groups prior to hatching. According to this hypothesis, surviving embryos may be the individuals that are more resistant to both AMPA and AMPA-mediated oxidative stress. Such

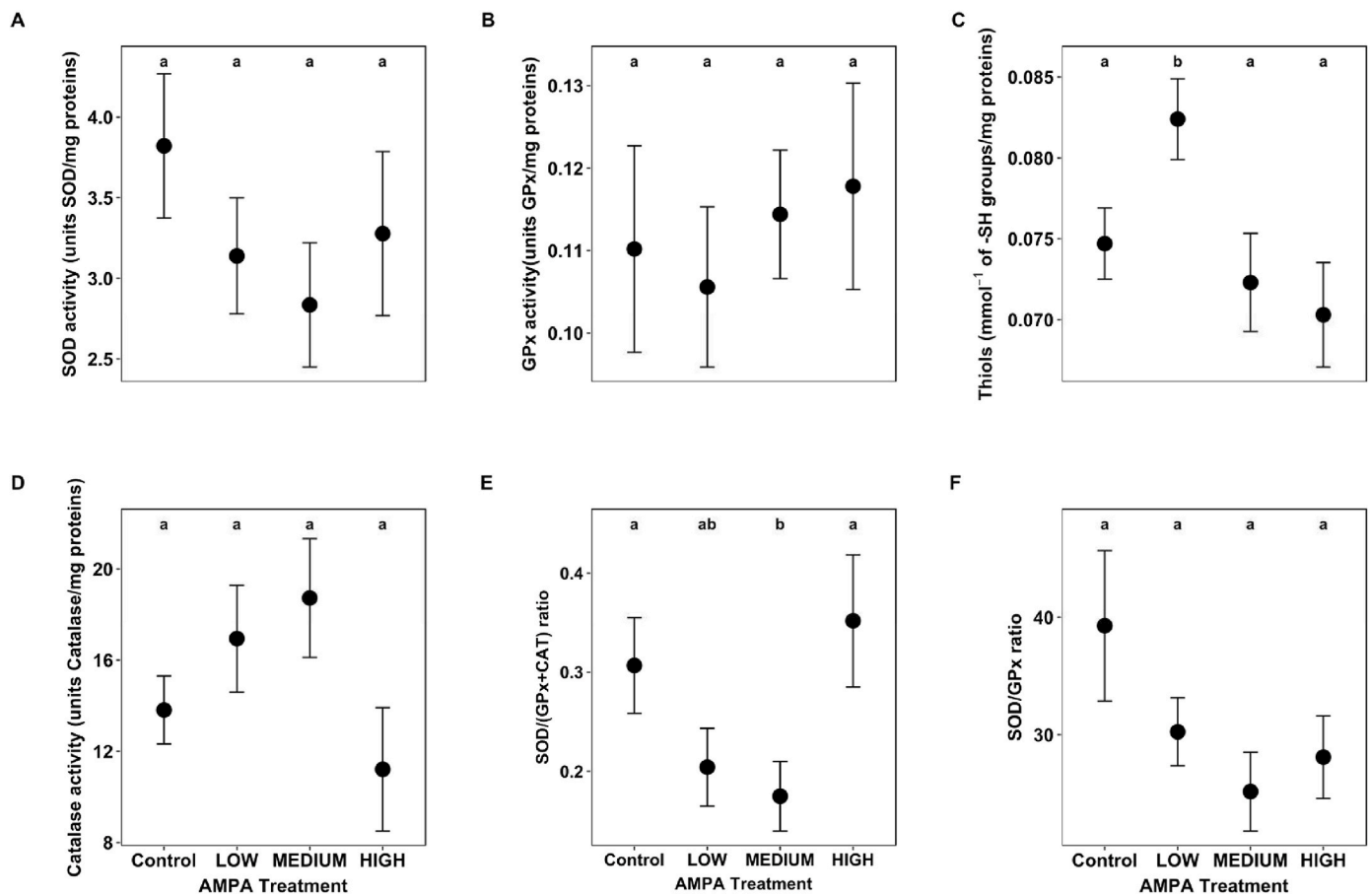


Fig. 1. A/Superoxide dismutase activity (units SOD/mg proteins), B/Glutathione peroxidase activity (units GPx/mg proteins) C/Thiols (mmol⁻¹ of -SH groups/mg proteins) D/CAT activity (units CAT/mg proteins) E/SOD/(GPx + CAT) ratio F/SOD/GPx ratio relative to AMPA treatments in *Bufo spinosus* tadpoles. Data represent mean ± SE. Letters indicate significant differences.

hypothesis dovetails with the lack of relationship between developmental traits and oxidative status markers. Indeed, embryonic development can be constrained by oxidative stress through oxidation of the thiol group of proteins (Goto, 1992), and accelerated development might enhance metabolism and induced cellular oxidative stress (Buraco et al., 2020b; Smith et al., 2016).

This process might also explain why we did not detect any correlation between telomere length and oxidative status parameters at clutch level. Telomere dynamics are influenced by environmental conditions and telomere length generally shortens under environmental stress (Chatelain et al., 2020). We expected that a reduction in telomere length because prior work showed that exposure to environmentally relevant concentrations of AMPA may damage DNA (de Brito Rodrigues et al., 2019; Guilherme et al., 2014). In our context, telomere length may be influenced by increased production of pro-oxidants or decreased of antioxidant defences (Chatelain et al., 2020; Reichert and Stier, 2017). However, if surviving embryos were more able to cope with oxidative stress, damages to their DNA may be reduced (Coluzzi et al., 2014). Since the proliferation of H₂O₂ induces cellular senescence (de Haan et al., 1996) or telomere attrition in human primary fibroblasts (Coluzzi et al., 2014), selective mortality might select coping individuals with preserved telomere length. Early development constitutes a phase where oxidative stress levels are high because of the presumed high metabolic needs. However, our results on AMPA exposure suggest an alteration of the trade-off between oxidative stress and developmental traits, thus the potential for AMPA to affect expression of life-history traits.

5. Conclusions

Our results provide rare evidence for physiological effects of environmentally-relevant AMPA concentrations on amphibian embryos (Cheron and Brischoux, 2020). Interestingly, the integration of markers of oxidative status in our investigations allows to suggest potential physiological mechanisms responsible for the effects on developmental related traits detected in a previous study (Cheron and Brischoux, 2020). Our results also strengthen the idea that AMPA may also induce non-monotonic effects. These effects might indicate stimulation of the antioxidant machinery to buffer any pro-oxidant effect of AMPA, but also selected embryos that were more resistant to oxidative stress, two hypotheses which are not mutually exclusive. Further experiments using non-lethal concentration of AMPA are required in order to disentangle whether AMPA acts directly on the antioxidant machinery of tadpoles or if surviving individuals have higher ability to cope with oxidative stress. If our hypothesis on selective mortality holds true, it is possible that the long-term use of glyphosate may have acted as a potent selective pressure, thereby explaining why our study species can persist in agricultural landscapes (Guillot et al., 2016, but see Renoirt et al., 2021). In light of our findings, we urge for regulatory toxicology to investigate effects of parent compounds but, more importantly, also of their metabolites at environmentally relevant concentrations on non-model species in order to comprehensively assess impacts of anthropogenic contaminants on the environment.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

We declare we have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2021.131882>.

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Ethics approval

All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities (COMETHEA ethic committee and Ministère de L'Enseignement Supérieur, de la Recherche et de L'innovation) under permit #APAFIS#13477-2018032614077834 v7.

Authors' contributions

F.B. & M.C. conceived and designed the study, M.C. performed the experiment and collected the data, F.A., C.R. & M.C. performed telomeres assays, D.C performed oxidative stress markers assays, M.C, D.C., F.A & F.B. interpreted the data, M.C. & F.B. designed the figures and wrote the initial draft, M.C, D.C., F.A & F.B. revised the paper. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Author statement

Brischoux François & Cheron Marion: **Conceptualization**; Brischoux François, Cheron Marion, Costantini David, Angelier Frederic: **Data curation**; Brischoux François: **Funding acquisition**; Brischoux François, Cheron Marion, Costantini David, Angelier Frederic: **Investigation**; Brischoux François, Cheron Marion: **Methodology**; Brischoux François: **Supervision**; Brischoux François & Cheron Marion: **Writing - original draft**; Brischoux François, Cheron Marion, Costantini David, Angelier Frederic, Ribout Cecile: **Writing - review & editing**.

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Article VI

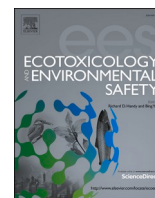
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Nicosulfuron, a sulfonylurea herbicide, alters embryonic development and oxidative status of hatchlings at environmental concentrations in a widespread amphibian.

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Nicosulfuron, a sulfonylurea herbicide, alters embryonic development and oxidative status of hatchlings at environmental concentrations in an amphibian species

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ABSTRACT

The widespread use of agrochemicals for controlling pests and diseases of crops is recognized as a main threat to biodiversity. Sulfonylurea herbicides are being increasingly used and display low levels of degradation in water which suggest that they might affect non-target organisms. In a common garden experiment, eggs of a widespread amphibian (*Bufo spinosus*) were exposed to sublethal environmentally relevant concentrations of a widely used sulfonylurea herbicide, nicosulfuron, during the whole embryonic development. We assessed development-related traits (i.e., development duration, hatching success, hatchling size and occurrence of malformation) as well as antioxidant markers in response to contamination (i.e., SOD, GPx, catalase, thiols and relevant ratios thereof). We found that sublethal concentrations of nicosulfuron increased embryonic development duration, increased hatchling size and tended to increase malformations. Embryos exposed to nicosulfuron displayed decreased thiols and increased catalase activity suggesting alteration of oxidative status. We did not find any effect of nicosulfuron on SOD and GPx levels. Interestingly, higher catalase activity was linked to higher proportion of malformed individuals, suggesting that exposure to nicosulfuron induced teratogenic effects. Our results suggest that alteration of antioxidant levels might be one physiological mechanism through which nicosulfuron might cause detrimental effects on amphibian embryos. Sublethal effects of pesticides at environmentally relevant concentrations have been overlooked and require further investigations, especially in non-target taxa occurring in agricultural landscapes.

1. Introduction

Environmental contamination is one of the major threats to biodiversity since the beginning of the century (Backhaus et al., 2012; Ceballos et al., 2015). The effects of contaminants on wildlife range from consequences at the individual scale (i.e., genotoxicity, physiological alterations, Beasley, 2020; Kendall et al., 2016; Lushchak, 2016; Relyea, 2009) to large scale populational effects (Hamilton et al., 2016; McCarthy, 2018; Sievers et al., 2019). The majority of environmental contamination comes from agricultural practices through the release of agrochemical compounds (e.g. pesticides) in the wild (Prakash et al., 2018). Effects of pesticides on wildlife are well studied but often focus on compounds that have been on the market for long time and used massively (i.e., legacy pesticides such as glyphosate, neonicotinoids,

atrazine or DDT, Annett et al., 2014; Lenkowski et al., 2010; Pandey and Mohanty, 2015).

Herbicides have been used to control weeds since decades (e.g., glyphosate, Prakash et al., 2018) and new molecules are regularly developed and used, among which sulfonylurea herbicides that are applied during the spring in a large variety of crops including wheat, oat, soybean or maize (Eizenberg et al., 2003; Kearney, 1988). These herbicides are increasingly being introduced into the environment through direct application and runoff from agricultural activities (Battaglin et al., 2009, 2000; Cessna et al., 2015; de Lafontaine et al., 2014). Among sulfonylurea herbicides, nicosulfuron is characterized by elevated DT50 values (75days) and slow photodegradation in water (Fenoll et al., 2012). This relative stability in aquatic ecosystems suggests low levels of abiotic and microbial degradations (Cessna et al., 2015). As a

Abbreviations: SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase, GS: Gosner stage.

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consequence, the persistence of nicosulfuron in aquatic ecosystems further suggests that environmentally relevant concentrations may affect non target organisms. For instance, nicosulfuron is frequently detected in surface waters (e.g., rivers and ponds) and wetland sediments in northern America (Battaglin et al., 2009, 2000; de Lafontaine et al., 2014; Degenhardt et al., 2009) and Europe (data from Agences de l'Eau). Although detected concentrations are usually low (i.e., between $0.01 \mu\text{g.l}^{-1}$ and $3.2 \mu\text{g.l}^{-1}$, Battaglin et al., 2009, de Lafontaine et al., 2014; as compared to a no observed effect concentration [NOEC] of 5.2mg.l^{-1} assessed on *Daphnia magna*, EFSA, 2007), nicosulfuron causes increased oxidative stress and increased AChE activity in earthworms (Hackenberger et al., 2018) as well as erratic behaviour and increased AChE in goldfish (Bretaud et al., 2000; Saglio et al., 2001) at environmentally relevant concentrations. Conversely, it has been suggested that nicosulfuron has small effects in some mammal, bird or amphibian laboratory species (EFSA, 2007). Yet, these conflicting results are based on a limited number of studies and species. It remains thus essential to assess the effects of environmentally relevant concentrations of nicosulfuron on non-target organisms, and to identify the physiological mechanisms mediating such effects.

Amphibians are particularly well-suited to assess the effects of agrochemicals on non-target organisms (Beasley, 2020; Mann et al., 2009). Many amphibian species have complex (biphasic) life cycles with terrestrial adults breeding in aquatic sites (ponds) where eggs and larvae develop. Breeding and post metamorphosis migrations often coincide with agrochemical application in agricultural lands, which makes amphibians particularly vulnerable to pesticides (Berger et al., 2013). Their highly permeable skin, which allows for the exchange of gases, water and ions, may also facilitate the diffusion of harmful substances, such as agrochemicals, especially during their aquatic larval stage (Quaranta et al., 2009). Many species of amphibians are particularly affected by environmental contamination (Beasley, 2020), and they have been highlighted as relevant model organisms to investigate the consequences of environmental contaminants (Hayes et al., 2006; Mann et al., 2009). The use of oxidative status as a biomarker of contaminant exposure represents a powerful tool for assessing the quality of an aquatic environment. Antioxidant enzyme activity, the amount of thiol groups and markers of oxidative damage (ROS damage) to biomolecules (lipids, vitamin, DNA) are often used to monitor the presence of pro-oxidant substances in aquatic environments (Trevisan et al., 2013). For instance, in *Bufo spinosus* embryos, exposure to a metabolite of glyphosate induced perturbation in antioxidant levels at environmental concentrations (Cheron et al., 2022). Similarly, in *Pelobates cultripes* tadpoles, exposure to sublethal concentrations of glyphosate decreased glutathione reductase activity but did not affect SOD nor CAT levels (Burraco and Gomez-Mestre, 2016).

In this study, we experimentally investigated for the first time the effects of environmentally relevant concentrations of nicosulfuron on embryonic development and physiology of the spined toad (*Bufo spinosus*), a widespread amphibian species known to persist in agricultural areas (Guillot et al., 2016) and presumably exposed to contaminants (Storrs Méndez et al., 2009; Van Meter et al., 2015). We examined the influence of exposure to nicosulfuron on metrics of embryonic development (embryonic survival, development duration and hatching morphology, (Cheron et al., 2021a) in conjunction with markers of oxidative status that can be influenced by nicosulfuron (e.g., Hackenberger et al., 2018). We tested low but environmentally relevant concentrations (i.e. $\sim 0.1 \mu\text{g.l}^{-1}$ and $\sim 0.8 \mu\text{g.l}^{-1}$, data from water agencies) and we expected that exposure to nicosulfuron should alter embryonic development (survival, duration and morphology) and affects oxidative status.

2. Materials and methods

2.1. Study species

We collected egg strings ($N = 20$) of spined toad between the end of February and the beginning of March in a pond near the present study site. Eggs were collected immediately after laying and brought back to the laboratory. A subset of each string containing 90 eggs was randomly selected and kept for our experiment, while the remaining eggs (i.e., 3000–5000) were released at their site of origin. The 90 eggs from each clutch were separated into segments containing ~ 30 eggs (ie. 90/3 treatments, see below) and placed in separate glass tanks containing 2 L of dechlorinated tap water (changed once a week). We monitored the eggs until hatching (total $N = 2400$ eggs). We determined the stage of development using Gosner (1960). Hatching occurred at Gosner stage 22 (hereafter GS 22) after 12.97 ± 0.04 days (see results). All experiments took place in a thermally controlled room with air and water temperature set at 17°C . Photoperiod was controlled in a 12:12 h cycle using LEDs placed across the room.

2.2. Treatment concentrations and chemical solutions

In surface waters close to our study site, mean nicosulfuron concentrations range from $0.005 \mu\text{g.l}^{-1}$ to $0.920 \mu\text{g.l}^{-1}$ (mean: $0.043 \pm 0.076 \mu\text{g.l}^{-1}$) and maximum values range from $0.005 \mu\text{g.l}^{-1}$ to $10.72 \mu\text{g.l}^{-1}$ (mean of maximum concentrations: $0.142 \pm 0.076 \mu\text{g.l}^{-1}$, data from Agence de l'Eau Loire-Bretagne).

In order to mimic the range of concentrations found in the wild, we produced three experimental concentrations of nicosulfuron: control treatment ($0 \mu\text{g.l}^{-1}$), low concentration ($0.15 \mu\text{g.l}^{-1}$) and high concentration ($0.83 \mu\text{g.l}^{-1}$, Table 1). We obtained stock solutions (0.1g.l^{-1}) of nicosulfuron by dissolving commercial crystalline powder (PESTANAL, Sigma-Aldrich) in dechlorinated tap water. These stock solutions were further diluted with dechlorinated tap water to reach the concentration required for each treatment (Table 1). Analytical verifications of the concentration were performed in triplicate by an independent accredited analytical laboratory (QUALYSE, Champdeniers-Saint-Denis, France). Determination of nicosulfuron in water samples was assessed using liquid chromatography-mass spectrometry (LC/MS/MS) by direct injection with a limit of quantification of $0.1 \mu\text{g.l}^{-1}$. Overall, these verifications showed that actual concentrations were similar to concentrations found in aquatic environments and that the differences between “low” and “high” treatments were significant (Table 1). For clarity, we will refer to the treatments as Low and High hereafter.

2.3. Experimental design

Eggs were exposed to two concentrations of nicosulfuron (Low and High) and a control during the whole embryonic development until hatching. To maintain relatively constant exposure levels throughout the experiment but to avoid excessive mechanical disturbances to developing eggs, water was changed once a week even though the half-life of nicosulfuron can range from 15 to 75 days (EFSA, 2007; Cessna et al., 2015). Egg jelly was maintained throughout the experiment.

2.4. Measurements

We monitored egg segments and counted all the individuals that

Table 1
Concentrations ($\mu\text{g.l}^{-1}$) measured in the experimental tanks (Mean \pm SD).

Treatment	Nicosulfuron concentrations
Control	0.00 ± 0.00
Low	0.15 ± 0.05
High	0.83 ± 0.04

hatched (GS22) which we considered a metric of hatching success. Undeveloped embryos and embryos that did not achieve total development were individually counted. Development duration was assessed as the time elapsed between the date of egg collection (egg laying) and the date of hatching (Cheron et al., 2021a). Development duration did not differ within egg segment of each clutch. Malformed tadpoles (axial malformation, oedema, scoliosis of the tail, Cooke, 1981; Wagner et al., 2014) were also individually counted.

We took pictures of all live and undeformed hatchlings using a camera in order to assess the standardized length (body and tail, according to Watters et al., 2016) of individuals (N = 1252). Morphological measurements were performed with the software ImageJ (Schneider et al., 2012).

2.5. Oxidative status markers

We randomly selected 60 hatchlings (20/experimental group), including two hatchlings per clutch. Because smaller portion did not provide enough tissue, we pooled together these two hatchlings for the analysis. We gave us 10 pools per experimental group (i.e. 30 samples in total). We homogenized hatchlings in Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, France) supplemented with 1 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich, France) as an inhibitor of proteases using a TissueLyser II (Qiagen) at 30 Hz for 4 min. Afterwards, we centrifuged tubes for 10 min at 4 °C to obtain clean supernatants to be used for the assays. We measured the concentration of thiols using the -SHp test (Diacron International, Grosseto, Italy); the activity of the antioxidant enzyme superoxide dismutase (SOD) using the Ransod assay (RANDOX Laboratories, France); the activity of the antioxidant enzyme glutathione peroxidase (GPx) using the Ransel assay (RANDOX Laboratories, France); the activity of catalase using the OxiSelect Catalase Activity Assay (Euromedex, France). We ran all analyses in duplicate and the CV of measurements was always < 10%. We standardized values of markers by the amount of proteins as quantified using the Bradford protein assay with albumin as reference standard (Sigma-Aldrich, France). All assays were run according to manufacturer's instructions.

Balanced or unbalanced ratio of markers activities are known to alter cells integrity (de Haan et al., 1996, 1992). Hence, in addition to markers alone we chose to display ratio of superoxide dismutase and glutathione peroxidase (SOD/GPx) and ratio ratio of superoxide dismutase on GPx and catalase activities (SOD/(GPx+CAT)). Unbalanced ratio of (SOD/(GPx+CAT)) can lead to a proliferation of deleterious radicals (de Haan et al., 1992) and unbalanced ratio of (SOD/GPx) can lead to a proliferation of H₂O₂ heading to accelerated cellular senescence and altered morphology (de Haan et al., 1996).

2.6. Statistical analysis

All statistical analyses were conducted with R statistical software v.4.0.0 (R Core Team, 2020) and RStudio v 1.2.5042 (RStudio, Inc.).

All residuals were tested for homogeneity of variance and normality with the Barlett's test and the Shapiro-Wilks test, respectively. We also checked the normality of the residuals using diagnostic plots. We checked which distribution fitted the best the model using Cullen & Frey plot (bootstrap at 5000, "fitdist" package). We scaled to mean of 0 and standard deviation of 1 (z_score of oxidative status markers and developmental-related traits) to avoid multicollinearity between treatment when we tested interaction or relation with developmental-related traits. We checked for multicollinearity using the variance inflation factor (VIF, non-multicollinearity when below 2. "car" package). We kept clutch identity as a random factor to avoid significant pseudoreplication issues. We did not use tank as the replication unit in our analytical design because each tank represented a single treatment (either control, low or high) and contained only the eggs of one clutch.

Conversely, sampling date never influenced our results, and it was excluded from our final analyses. Sample sizes were balanced across

clutches and treatments (e.g., each clutch was represented by 30 eggs in each treatment).

We performed effect size tests with statistical power analysis to assess the magnitude of the difference between treatments (Table S1, Cohen, 2013). When $p < 0.05$, the magnitude of the main effect was measured using η^2 and the magnitude between two groups was measured using Hedge's g (package "effectsize", Supplementary materials table S1). DABEST package was used to provide Gardner-Altman estimation plot as well as between- and within-group effect sizes as well as bias-corrected, bootstrapped, 95% confidence intervals around these estimates (Supplementary material, Fig. S1, S2). All confidence intervals were produced via bootstrap with 5 000 resamples. Effect sizes were considered to be small when $g = 0.2$ (1% of the variance), intermediate when $g = 0.5$ (9% of the variance), or large when $g = 0.8$ (25% of the variance) as suggested by Cohen (2013).

First, on the whole dataset, to test whether nicosulfuron treatment affected developmental traits, we fitted two linear mixed models (LMERs, package lme4) with "Treatment" as fixed and "Total length of hatchling" and "Embryonic development duration" as dependent variables. Then, we fitted two generalized linear models (GLMERs, (package lme4)) with a log-link function for variables following binomial distribution (hatching success and occurrence of malformed tadpoles).

Second, to assess whether our nicosulfuron treatment affected oxidative status we fitted six linear mixed-effect models (LMERs, package lme4 (Bates et al., 2015)) with "Treatment" as fixed effect and "SOD", "GPx", "catalase", thiols, "SOD/GPx" "SOD/GPxCAT" as dependent variables. We used "emmeans" package for pair-wise comparison.

Finally, on the subset of the individuals for which we assessed oxidative status markers to test whether there is a relation between developmental-related traits and oxidative status markers and whether such relation differed between treatments, we fitted LMER models with developmental-related traits as dependent variable and z_scored oxidative status markers in interaction with treatment in fixed effect. We fitted GLMER for binomial data with "hatching success", "malformed" as dependent variable and z_scored oxidative status markers in interaction with treatment as explanatory variables. To compare the trends and regression slopes we used "emtrends" function in "emmeans" package. P -value ≥ 0.05 were considered not significant.

3. Results

All data on embryonic traits and oxidative status markers are summarized in Table 2.

Table 2

Mean values of markers of oxidative status and developmental-related traits amongst treatments of nicosulfuron.

	Treatment		
	Control	Low	High
Developmental-related traits			
Total length (cm)	0.77 ± 0.00	0.77 ± 0.00	0.78 ± 0.00
Hatching success	0.80 ± 0.02	0.77 ± 0.02	0.80 ± 0.02
Malformation	0.07 ± 0.01	0.06 ± 0.01	0.10 ± 0.01
Development duration (days)	12.94 ± 0.06	12.94 ± 0.04	13.03 ± 0.04
Oxidative status markers			
SOD ^a	6.35 ± 0.65	6.76 ± 0.56	6.40 ± 0.52
Thiols	0.14 ± 0.01	0.12 ± 0.01	0.12 ± 0.00
Catalase ^a	18.82 ± 2.07	24.33 ± 3.55	28.43 ± 3.50
GPx ^a	0.09 ± 0.01	0.11 ± 0.02	0.11 ± 0.01
SOD/(GPx+CAT)	0.37 ± 0.05	0.32 ± 0.04	0.26 ± 0.03
SOD/GPx	97.60 ± 15.31	99.04 ± 29.44	73.57 ± 12.29

Values are Mean±SE

SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: Catalase

^a (units/mg protein)

3.1. Effect of nicosulfuron treatment on embryonic traits

On the whole dataset, we found that nicosulfuron significantly influenced total length of hatchlings ($F_{3,1234} = 5.51$, $p = 0.004$, Fig. 1). Post hoc tests showed that hatchlings from High treatment were longer than individuals from the Low treatment ($p = 0.003$, $g = -0.22$). Nicosulfuron slowed down embryonic development duration ($F_{3,1234} = 19.185$, $p < 0.001$, Fig. 1); post hoc tests showed that High groups differed from Control groups ($p = 0.001$, $g = -0.08$) and Low groups ($p = 0.001$, $g = -0.12$). We did not find any effect of nicosulfuron on hatching success ($p = 0.13$, Fig. 1) and a marginal effect on the occurrence of malformed individuals ($p = 0.06$, Fig. 1).

3.2. Effect of nicosulfuron treatment on oxidative status

Neither SOD, GPx nor SOD/GPx and SOD/(GPx+CAT) ratios were affected by nicosulfuron (all $p > 0.214$, Fig. 2). Nicosulfuron influenced concentration of thiols ($F_{2,28} = 5.14$, $p = 0.017$, Fig. 2). Hatchlings from Control group displayed higher thiols than all other treatment groups (all $p < 0.007$, all $g > 0.94$, Fig. 1). Catalase activity was marginally

influenced by nicosulfuron treatment ($F_{2,28} = 3.41$, $p = 0.055$, Fig. 2), a result that was supported by effect size estimates ($\eta^2 = 0.15$). (Table 3).

3.3. Relationships between embryonic traits and oxidative status

In the subset of individuals in which we quantified oxidative status, total length of hatchlings was influenced by the treatment ($F_{2,20} = 3.721$, $p = 0.044$, same pattern than above) but not by oxidative status markers (all $p > 0.085$) except for a marginal relation with thiols levels ($F_{2,24} = 3.262$, $p = 0.055$), or their interaction (all $p > 0.089$) except for a marginal relation between development related traits, GPx and treatment ($F_{2,20} = 3.262$, $p = 0.059$).

Embryonic development duration was not related to treatment for the subsample of individuals for which we assessed oxidative status ($F_{2,20} = 0.439$, $p = 0.649$). Development duration was negatively linked to SOD ($F_{2,20} = 6.84$, $p = 0.015$) and thiols ($F_{2,20} = 4.62$, $p = 0.048$). We did not find any interaction between markers and treatment (all $p > 0.363$) except for a marginal interaction with SOD/(catalase+GPx) ($F_{1,20} = 6.88$, $p = 0.056$).

Hatching success was influenced by treatment in the individuals for

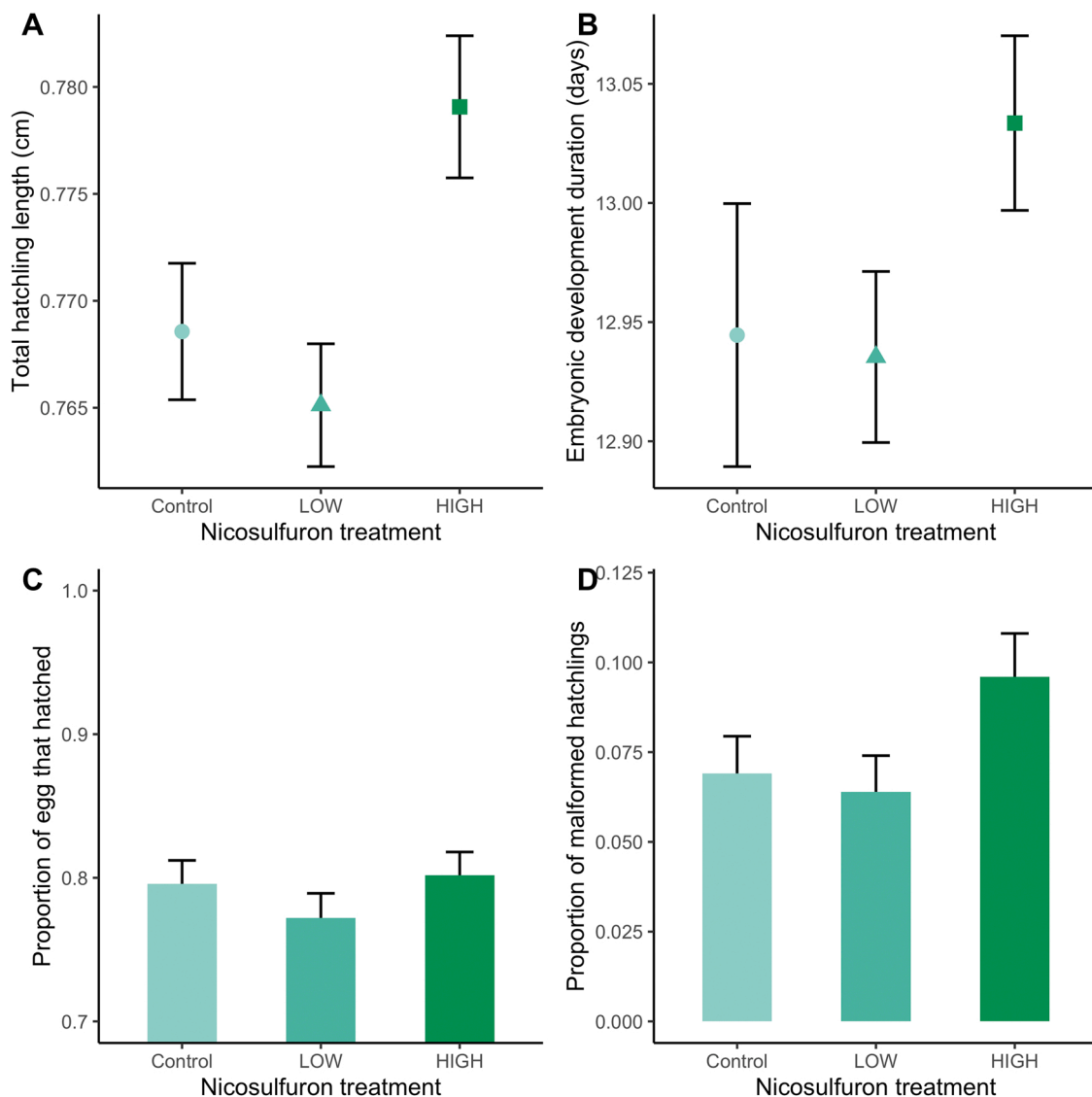


Fig. 1. A) Hatchlings total length at Gosner 22 (cm, upper left), B) Embryonic development duration (days, upper right) C) Proportion of eggs that hatched (lower left) D) Proportion of malformed hatchlings (lower right) relative to nicosulfuron treatments in *Bufo spinosus*. Mean \pm SE are represented. Colour and shape represent nicosulfuron treatment (Control: light green-circle; LOW: green-triangle; HIGH: dark green-square). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

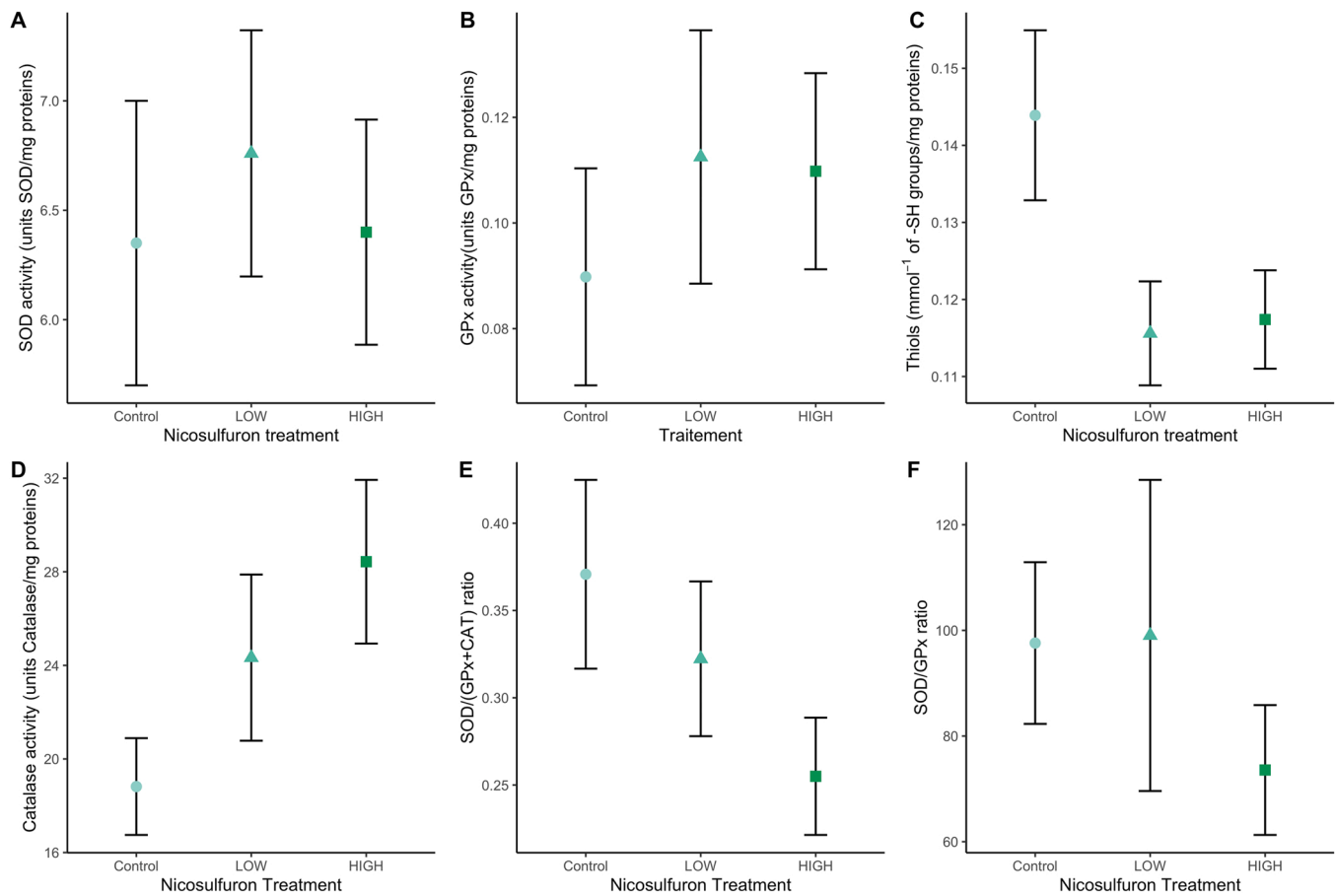


Fig. 2. A) Superoxide dismutase activity (units SOD/mg proteins, upper left), B) Glutathione peroxidase activity (units GPx/mg proteins, upper right) C) Thiols (mmol⁻¹ of -SH groups/mg proteins, middle left) D) Catalase activity (units Catalase/mg proteins, middle right) E/ SOD/(GPx+CAT) ratio (lower left) F) SOD/GPx ratio (lower right) relative to nicosulfuron treatments in *Bufo spinosus* hatchlings. Mean±SE are represented. Colour and shape represent nicosulfuron treatment (Control: light green-circle; LOW: green-triangle; HIGH: dark green-square). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

Table 3

Comparisons of outputs of linear mixed models (LMER, "lmerTest" package) of markers of oxidative status amongst treatment of nicosulfuron. Clutch identity was used as random factor in all our models.

Variable	Effect	df	F-value	p-value	Clutch identity	
					Variance	Residuals variance
<i>SOD</i> ^a	Intercept	1,9	120.50	< 0.001	0.433	2.913
	Treatment	3,38	0.17	0.843		
<i>Thiols</i>	Intercept	1,9	298.73	< 0.001	< 0.001	< 0.001
	Treatment	3,38	5.14	0.017		
<i>Catalase</i> ^a	Intercept	1,9	36.48	< 0.001	28.92	68.17
	Treatment	3,38	3.41	0.055		
<i>GPx</i> ^a	Intercept	1,9	18.03	< 0.001	< 0.001	< 0.001
	Treatment	3,38	0.422	0.661		
<i>SOD/(GPx+CAT)</i>	Intercept	1,9	68.50	< 0.001	< 0.001	0.020
	Treatment	3,38	1.68	0.214		
<i>SOD/GPx</i>	Intercept	1,9	47.88	< 0.001	< 0.001	1989.76
	Treatment	3,37	0.970	0.399		

SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: Catalase

Values in bold are considered significant P < 0.05

^a (units/mg protein)

which we assessed oxidative status ($X^2 = 21.09$, $p = 0.001$). The interaction with treatment was significant for hatching success and SOD ($X^2 = 10.86$, $p = 0.004$). Control individuals displayed a positive relationship between hatching success and SOD, which was marginally different from the relationships found in the High group ($p = 0.054$) and significantly different from Low group ($p = 0.03$) with both groups of treated

individuals displaying negative relationships between hatching success and SOD. Catalase influenced hatching success between treatments ($X^2 = 25.41$, $p < 0.01$). Control individuals displayed a positive relationship between hatching success and Catalase which was different from High group hatchlings which displayed negative relationship ($p < 0.01$) and Low group individuals which displayed positive relationship ($p < 0.01$).

Hatching success was related to thiols ($X^2 = 5.442$, $p = 0.019$) and the interaction with treatment was also significant ($X^2 = 7.815$, $p = 0.020$). Control individuals displayed a positive relationship between hatching success and thiols that was significantly different from Low individuals that displayed a negative relationship ($p = 0.016$).

Finally, the number of malformed tadpoles did not differ among treatments ($X^2 = 1.836$, $p = 0.340$). We found a negative relation between the occurrence of malformed hatchlings and thiols ($X^2 = 12.012$, $p < 0.005$, Fig. 3) and a positive relation between the occurrence of malformed hatchlings and catalase levels ($X^2 = 20.313$, $p < 0.001$, Fig. 3) with no interactions with the treatment (all $p > 0.07$).

4. Discussion

Overall, we found that environmentally relevant concentrations of nicosulfuron altered embryonic development of *Bufo spinosus*, a species that occurs in agricultural areas. Most notably, exposure to nicosulfuron increased embryonic development duration and influenced hatchling morphology. In addition, exposure to nicosulfuron caused lower levels

of thiols and marginally higher levels of catalase activity. Interestingly, lower levels of thiols were linked to higher embryonic mortality, higher proportion of malformed individuals and longer embryonic development duration; and higher catalase activity were also linked to higher proportion of malformed individuals, suggesting that the effects of exposure to nicosulfuron induced teratogenic effects.

The highest concentration of nicosulfuron we tested ($0.83 \mu\text{g}\cdot\text{l}^{-1}$) is very similar to concentrations found in surface waters (Battaglin et al., 2009). Although sublethal, this concentration influenced embryonic development of *Bufo spinosus*, which is exemplified by increased development durations and larger body size. Interestingly, the pattern of covariation between these two traits was expected given the widespread relationship between embryonic development time and body size (Gillooly and Dodson, 2000). Whether these responses can have consequences for tadpoles developing under natural conditions is complicated to assess. Delayed hatching may potentially increase the vulnerability of immobile embryos to predators (Zamudio et al., 2016) or increase the vulnerability of tadpoles to desiccation if spawning occurs in temporary water bodies (Lindgren et al., 2018; Székely et al., 2017). Conversely,

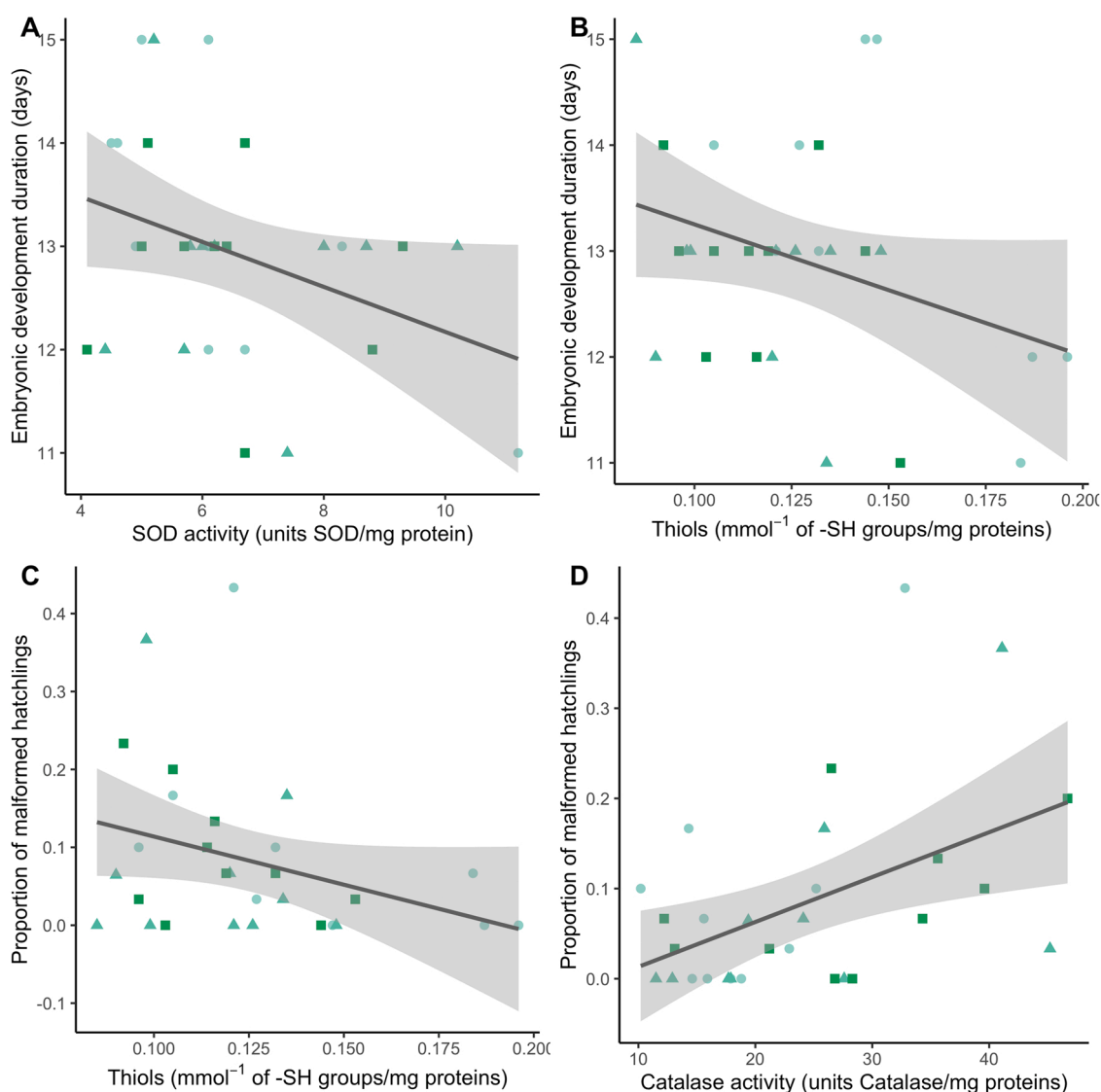


Fig. 3. A) Linear regression between embryonic development duration (days) and Superoxide dismutase activity (units SOD/mg proteins) regardless of nicosulfuron treatment, B) Linear regression between embryonic development duration (days) and thiols (mmol^{-1} of -SH groups/mg proteins). C) Linear regression between proportion of malformed hatchlings and thiols (mmol^{-1} of -SH groups/mg proteins) regardless of nicosulfuron treatment, D) Linear regression between proportion of malformed hatchlings and catalase activity (units catalase/mg proteins). Colour and shape represent nicosulfuron treatment (Control: light green-circle; LOW: green-triangle; HIGH: dark green-square). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

increased body size may improve mobility and thus foraging ability while decreasing susceptibility to predation (Broomhall and Shine, 2003; Cheron et al., 2021b; Hoff and Wassersug, 2000). Such hypotheses will require thorough testing, especially as post-embryonic survival might be more critical to population persistence than egg survival (Touchon et al., 2013; Vonesh and De la Cruz, 2002). Surprisingly, these effects were detectable for our “high” treatment solely suggesting that the lower concentration we tested did not trigger visible effects on embryonic development, as expected from classical concentration-response relationships (Vandenberg et al., 2012). Indeed, a previous study on Goldfish showed an alteration of swimming behaviour due to exposure to nicosulfuron and further highlighted an increase in swimming alterations along with exposure to increasing nicosulfuron concentrations (Saglio et al., 2001). Because our “high” treatment is situated at the lower range of maximum concentrations assessed in surface water (see “Materials and methods section”), it is plausible that free ranging *Bufo spinosus* embryos are exposed to higher concentrations which could induce strong consequences on hatching success. Future studies including a larger range of tested concentrations are required to describe reaction norms of embryos exposed to nicosulfuron.

Regardless of the concentration tested, nicosulfuron influenced thiol levels with lower levels - hence higher oxidation of thiols - in hatchlings exposed during their embryonic development. Thiols are relevant indicators of the antioxidant capacity of an organism (Lushchak, 2011), so that their concentration decreases when there are strong perturbations to the oxidative status (Baba and Bhatnagar, 2018; Ulrich and Jakob, 2019). As a consequence, our results suggest that exposure to nicosulfuron altered oxidative status. We also found a trend for catalase to increase across treatments. The enzymatic antioxidant protection occurs through two major steps: first superoxide dismutase (SOD) breaks down O_2^- into H_2O_2 , then H_2O_2 is converted into H_2O by catalase and/or glutathione peroxidase (GPx). However, when H_2O_2 is produced at high concentrations, catalase is more efficient in the conversion of H_2O_2 in H_2O than GPx which can explain the trend we detected (Halliwell and Gutteridge, 2015). In keeping with the results on development duration (see above), it is noteworthy that a rapid development may increase oxidative damage, with possible long-lasting effects (Janssens and Stoks, 2018). In this context, slowing down embryonic development, as observed in individuals exposed to the highest nicosulfuron treatment might constitute a strategy to avoid a disproportional increase of oxidative damages linked to both exposure to nicosulfuron and rapid development.

Finally, we found that development-related traits (i.e., embryonic development duration, number of malformed hatchlings, hatching success) were correlated to some markers of oxidative status (SOD, thiols and catalase). The negative relationship between embryonic development duration and SOD or thiols is consistent with other studies that suggested that enhanced metabolism linked to accelerated development can increase cellular oxidative stress (Burraco et al., 2020; Smith et al., 2016). Thiol levels were negatively correlated with proportion of malformed hatchlings, and catalase activity was positively correlated to higher proportion of malformed individuals. Such results strongly suggest that disruption in oxidative status might be related to teratogenic effects (Wu et al., 2017; Xie et al., 2016). Indeed, occurrence of malformations is known to be related to pesticide-induced oxidative stress (Liendro et al., 2015; Rutkoski et al., 2020; Wells et al., 2005), suggesting that, in our study, these teratogenic effects may be linked to nicosulfuron-induced oxidative imbalance. Interestingly, occurrence of physical malformations in anuran tadpoles is known to be frequent in agricultural habitats (Lajmanovich et al., 2003; Mann et al., 2009; Taylor et al., 2005), and it has also been shown that such malformations are detrimental for mobility and performance (Sotomayor et al., 2012), growth and survival (Herek et al., 2020; Ma et al., 2019; Ruiz et al., 2010; Schuytema and Nebeker, 1998).

5. Conclusions

Our results reinforce the fact that pesticides constitute a major threat to amphibians and point out to the importance of testing the effects of novel pesticides on wildlife. Studies on the impact of nicosulfuron on wildlife are lacking in the literature, although the substance is known to alter behaviour and have noticeable effect on AchE activity in fish at sublethal concentrations (Bretaud et al., 2000; Saglio et al., 2001). To our knowledge, this is the first study to show the effects of nicosulfuron on biochemical markers and development-related traits in a non-target aquatic species. Given the fact that post metamorphic fitness is mostly explained by quality of larval development in amphibians (Bekhet et al., 2014; Boonekamp et al., 2018; Bredeweg et al., 2019), future studies should usefully investigate potential effects of nicosulfuron on larval development and metamorphosis. Finally, we emphasize that the sublethal effects of nicosulfuron have been overlooked to date (but see Hackenberger et al., 2018) and require further investigations on other non-target taxa occurring in agricultural landscapes.

Ethics approval

All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities (COMETHEA ethic committee and Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation) under permit #APAFIS#13477-2018032614077834 v7.

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CRedit authorship contribution statement

F.B. & M.C. conceived and designed the study, M.C. performed the experiment and collected the data, D.C performed oxidative stress markers assays, M.C, D.C & F.B. interpreted the data, M.C. & F.B. designed the figures and wrote the initial draft, M.C, D.C & F.B. revised the paper. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data are available upon request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2022.113277](https://doi.org/10.1016/j.ecoenv.2022.113277).

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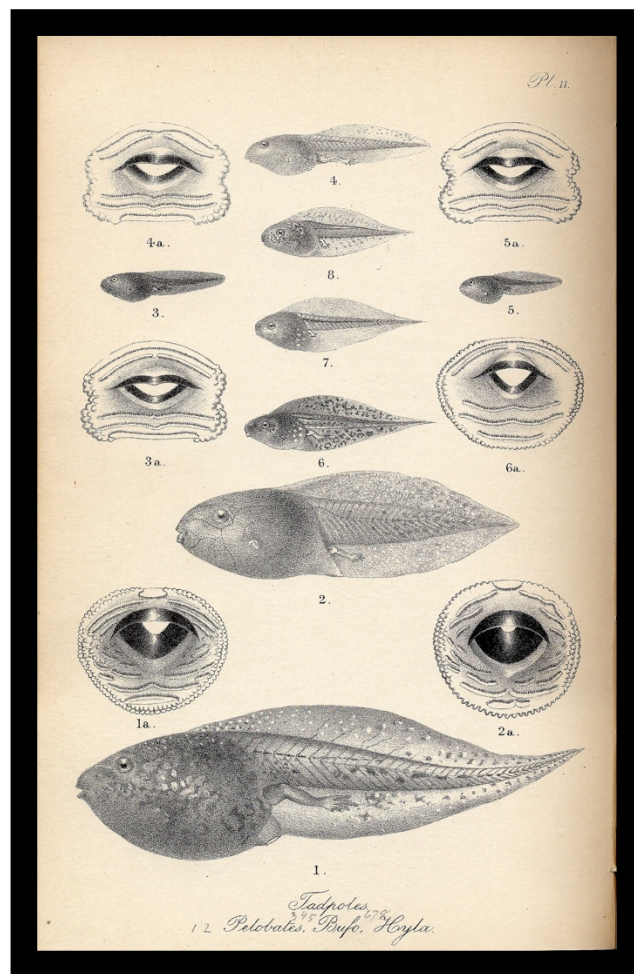
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Article VII

Marion Cheron, Léa Raelison, Akiko Kato, Yan Ropert-Coudert, Xavier Meyer, Andrew J. J. Macintosh & François Brischoux

Ontogenetic changes in activity, locomotion and behavioural complexity in tadpoles

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Ontogenetic changes in activity, locomotion and behavioural complexity in tadpoles

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Metamorphosis is a widespread developmental process that involves considerable changes in morphology, habitat use, ecology and behaviour between early developmental (larval) stages and adult forms. Among amphibians, anuran larvae (tadpoles) undergo massive morphological and ecological changes during their development, with early stages characterized by somatic growth, whereas more conspicuous changes (i.e. metamorphosis) occur later during development. In this study, we examined how locomotor and behavioural traits covary with morphology (body size) and metamorphosis (hindlimb and forelimb development) across developmental stages in spined toad (*Bufo spinosus*) tadpoles. As expected, we found that locomotion and behaviour undergo significant changes during tadpole development. These changes are curvilinear across developmental stages, with a phase of increasing activity and locomotion followed by a phase of stasis and/or reduction in locomotion and behavioural complexity. All the metrics we investigated indicate that the peak of activity and associated behaviour is situated at a pivotal stage when somatic growth decreases and significant morphological changes occur (i.e. hindlimb growth). Future studies that aim to investigate determinants of locomotion should include developmental stages as covariates in order to assess whether the sensitivity of locomotion to environmental variables changes across developmental stages.

ADDITIONAL KEYWORDS: activity – amphibian – behaviour – fractal analysis – metamorphosis – swimming speed.

INTRODUCTION

Metamorphosis is a key developmental process that is widespread across animal phylogeny (insects, fish, amphibians, molluscs, crustaceans, cnidarians, echinoderms and tunicates; Heyland & Moroz, 2006). This biological process involves massive organismal modifications after birth between early developmental (larval) stages and adult forms. Metamorphosis is not limited to changes in the body architecture, but often involves considerable shifts in other life-history traits, such as habitat use, trophic ecology and behaviour (Mueller *et al.*, 2015; Truman & Riddiford, 2019).

Among vertebrates, amphibians are well-known examples of taxa that undergo metamorphosis. Many amphibian species are biphasic: they live on land but

reproduce in freshwater environments, where eggs and larvae develop (Dodd, 2010). In most urodeles (salamanders), larvae broadly resemble adults, and metamorphosis mainly involves a shift from aquatic to terrestrial life and a consequent modification from branchial aquatic respiration to pulmonary aerial respiration (Vitt & Caldwell, 2014). Anuran (frog and toad) larvae undergo comparatively more massive morphological and ecological changes during their development (Vitt & Caldwell, 2014). In these species, eggs hatch as tadpoles, characterized by a globose body and an elongated tail used for locomotion, which are very different from adults both morphologically and ecologically. In tadpoles, early developmental stages are mainly marked by significant somatic growth, whereas more conspicuous changes occur later during development (i.e. hindlimb and forelimb development), ending with the atrophy of the tail and gills and the

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acquisition of aerial respiration and a feeding system that allows foraging on mobile terrestrial prey (Vitt & Caldwell, 2014).

These two different phases of tadpole development are expected to influence several parameters of their day-to-day life, including activity levels, locomotor performance (i.e. speed; Arnold, 1983) and behaviour (Kurali *et al.*, 2018). During early developmental stages, tadpoles are expected to increase activity levels and locomotion with increasing body size, because tadpole locomotion involves the entire anteroposterior axis, from the snout to the tail (Hoff & Wassersug, 2000). Conversely, at later stages of development, hindlimb and forelimb development is expected to influence mobility negatively (increased drag) and thus to decrease locomotor performance (but see Liu *et al.*, 1996). Finally, during the ultimate steps of the metamorphosis, tail atrophy is expected to decrease locomotor performance, because tail undulations are used as the main propulsive force in tadpoles (Liu *et al.*, 1996, 1997; Fabrezi *et al.*, 2014).

An important body of literature has investigated how environmental parameters (i.e. water temperature, predation, contamination or a combination thereof) can influence tadpole activity, performance or behaviour at specific developmental stages (Capellán & Niecieza, 2007; Touchon *et al.*, 2013; Johnson *et al.*, 2015; Kurali *et al.*, 2018; Lee-Jenkins & Robinson, 2018; Moreira *et al.*, 2019; Gallo *et al.*, 2020; Zhang *et al.*, 2020). Yet, to our knowledge, no study has investigated comprehensively the ontogenetic trajectories in activity and behaviour across the entirety of tadpole development and the relationships between body size, morphological changes linked to metamorphosis and locomotor and behavioural traits across the entirety of tadpole development. Thus, in this study, we examined how locomotor and behavioural traits covary with morphology (body size) and metamorphosis (hindlimb and forelimb development) across developmental stages in spined toad (*Bufo spinosus*) tadpoles.

Bufo spinosus tadpoles are benthic tadpoles, usually found foraging on the substrate, but also less frequently crossing the water column for ‘bobbing’ (Hoff *et al.*, 1999). Tadpoles swim continually and have remarkable endurance, even against slow currents (Wassersug & Feder, 1983). Swimming abilities fall into two categories: rapid bursts to evade predators and slow, continuous swimming while foraging or ‘bobbing’ (Hoff *et al.*, 1999). We investigated various metrics of locomotion and behaviour, including activity, distance travelled, swimming speeds and number of bouts, in undisturbed tadpoles across five discrete ontogenetic stages spanning the entire tadpole development, from hatching to forelimb appearance and tail atrophy. We also implemented an analysis of long-range correlations in locomotion sequences as an index of behavioural complexity using detrended fluctuation

analysis (DFA; Peng *et al.*, 1994; MacIntosh *et al.*, 2013). These analyses used the same developmental stages as those listed above to assess whether changes in body size and locomotion were associated with changes in behavioural complexity throughout development.

We predicted that all studied parameters (i.e. swimming speed, distance travelled, activity, number of activity bouts, and behavioural complexity) should increase with body and tail length during somatic growth and decrease in later developmental stages during metamorphosis.

MATERIAL AND METHODS

TADPOLE SAMPLING AND MAINTENANCE






Bufo spinosus egg strings ($N = 10$) were collected immediately after laying from a pond near the study site (Centre d’Etudes Biologiques de Chizé, France) in February 2019. For each string, 30 eggs were randomly selected and kept for the experiment. The remaining eggs (i.e. 3000–5000) were released at the site of capture. For each string, the 30 eggs were placed in 2 L glass tanks that contained dechlorinated tap water until hatching. Upon hatching, two tadpoles from each clutch were selected randomly and kept for the experiment (all other tadpoles were released in the pond of capture). The selected tadpoles were individually placed in individual 2 L tanks ($N = 20$), and their development was monitored until the end of the metamorphosis. Such husbandry methods allow for the development of each tadpole to be monitored individually and for the locomotion and behaviour of each individual to be assessed repeatedly throughout larval development. Importantly, maintaining the tadpoles individually in aquaria should not generate stressful conditions (e.g. Bókony *et al.*, 2021). All the experiments took place in a thermally controlled room with the temperature set at 17 °C (both air and water). The photoperiod was controlled (12 h dark–12 h light) using light-emitting diodes that were placed evenly across the room. Water was changed once a week. Tadpoles were fed *ad libitum* with organic spinach.

DEVELOPMENTAL STAGES AND MORPHOLOGICAL MEASUREMENTS

We used morphological features to classify and select specific stages according to Gosner (1960). We selected Gosner stages 25, 30, 37, 41 and 42 (hereafter, GS 25, GS 30, GS 37, GS 41 and GS 42, respectively) because those stages correspond to specific changes in the morphology of tadpoles (Table 1).

For each of the stages, we measured total length and tail length, following Watters *et al.* (2016), in order to

Table 1. Gosner stages (Gosner, 1960) selected to investigate morphological, locomotor and behavioural changes over time in developing *Bufo spinosus* tadpoles and corresponding pictures

Stages	Features	Pictures
25	Free-swimming tadpoles and feeding behaviour	
30	Hindlimb bud fully shaped	
37	Beginning of toe differentiation and development	
41	Cloacal tail pieces lost; fully grown hindlimbs	
42	Forelimb emergence; tail atrophy	

assess the overall growth of tadpoles. Each tadpole was put into a Petri dish with the water from its own tank, and photographs were taken using a fixed camera (Panasonic Lumix DC-TZ55) placed above the Petri dish. Morphological measurements were performed with the software IMAGEJ (Schneider *et al.*, 2012).

ACTIVITY AND LOCOMOTOR PERFORMANCE

At each developmental stage, tadpoles were removed from their tank and placed in an individual arena (Petri dish, diameter 13.5 cm, water level 1.5 cm) placed above a white background. After 15 min of acclimation (Dayton *et al.*, 2005), tadpoles were filmed for 30 min using a video camera [GoPro HERO (2018); GoPro, San Mateo, CA, USA] placed above the arena. Camera settings were as follows: 1080 pixel resolution, 60 frames s⁻¹ and narrow field of view. Codec for video was H.264 to produce MP4 files, and image stabilization was built into the GoPro (electronic image stabilization). A narrow field of view was used to limit the fish-eye effect, which was corrected further using *ToxTrac* during calibration. Operators left the room during experiments. Videos were analysed with the software *ToxTrac* (Rodriguez *et al.*, 2018). The contrast between the white arena and the dark tadpole allowed *ToxTrac* to detect and follow the tadpoles during the whole video. The successive positions of a tadpole (defined from the *x*- and *y*-calibrated coordinates within the Petri dish) were then extracted per frame, and the distance moved between two consecutive frames was calculated. We defined the tadpole as inactive when the distance moved between two consecutive frames was < 0.25 mm (Touchon & Wojdak, 2014; Hänzi & Straka, 2017). Initially, we calibrated the cameras to correct for lens distortion. We tested different calibration models for lens distortion, resulting in selection of ‘radial 3 + tangential 2’ distortion parameters. The calibration image was recorded in the same conditions

as the experiment and was a black-and-white chessboard pattern made using editing tools (GIMP, 2019). Calibrations were performed before each video. Definition of the arena was performed manually for each video to ensure that the entire arena was captured during the process. The animal detection threshold and minimal and maximal object size were adapted to the size of the tadpole for each video. We used the tutorial explained by Rodriguez *et al.* (2018). Using ‘Tracking RealSpace’ data and RSTUDIO scripts, we determined the following variables: number of activity bouts; total duration of activity; and total distance travelled. We measured total distance travelled as both the absolute distance (in millimetres) and relative to body size (per total length). We also extracted locomotion as the mean and maximal swimming speeds, calculated both as absolute speeds (in millimetres per second) and relative to total length (total lengths per second) (Wassersug, 1989). To minimize observer bias, blinded methods were used when all behavioural data were recorded and analysed.

BEHAVIOURAL COMPLEXITY

Detrended fluctuation analysis was conducted to measure long-range dependence as an index of temporal complexity in tadpole activity sequences (Peng *et al.*, 1994, 1995). By measuring fractal properties, animal time-series data can be defined as ranging from stochastic (uncorrelated) behaviour to deterministic (autocorrelated) behaviour that persists across measurement scales (see MacIntosh, 2014). The degree of complexity depends on both the internal conditions (e.g. stress, disease; Alados *et al.*, 1996; MacIntosh *et al.*, 2011; Cottin *et al.*, 2014) and external conditions (e.g. environment; MacIntosh *et al.*, 2011; Meyer *et al.*, 2017) of an individual.

To prepare the locomotion data for DFA, we followed the methodology as reported by Touchon & Wojdak (2014) and Hänzi & Straka (2017) to extract bouts of activity and inactivity. The process of DFA is described in detail elsewhere (Peng *et al.*, 1994, 1995). Briefly, the derived binary activity sequences were transformed into a time series, in which minus one corresponded to inactivity and one to activity (see previous subsection, ‘Activity and locomotor performance’). Time series were then integrated (through cumulative summation) to create an ‘activity walk’ sequence $[y(k)]$. Next, equally sized windows (n) were aligned along the sequence, and a least squares line was fitted within each to reflect the trend $[y_n(k)]$. Local trends in each box were then subtracted from the sequence, so that the average fluctuation as a function of window size $[F(n)]$ could be calculated. The process was repeated across a range of increasing window sizes (from 2^{10} to $2^{28/2}$), described by the following formula:

$$F(n) = \sqrt{\frac{1}{N} \sum_{k=1}^N [y(k) - y_n(k)]^2}$$

This analysis produces a sequence of values that produce a positive linear relationship on a double logarithmic plot of $F(n)$ against n , the slope of which is used to calculate the scaling exponent of the sequence (α DFA). We calculated α DFA using the package ‘fractal’ (Constantine & Percival, 2014) in R statistical software v.4.0.0 (R Core Team, 2020). Values of α DFA range between {0,1} for fractional Gaussian noise (fGn) and between {1,2} for fractional Brownian motion (fBm) (Eke *et al.*, 2000; Seuront, 2009). Crucial to our study aims, α DFA of 0.5 for fGn and 1.5 for fBm reflect noise in time series data (random uncorrelated sequences), whereas values between {0.5,1} and {1.5,2} reflect persistence and those between {0,0.5} and {1,1.5} reflect antipersistence. Thus, values of α DFA approaching the extremes reflect higher long-range dependence and thus greater determinism in behavioural sequences, whereas values approaching the midpoints (0.5, 1.5) reflect increased stochasticity (MacIntosh, 2014). Thorough details of the analytical approach used, including DFA calculation, validation of scaling and its relationship to other fractal dimension estimates and illustrations, can be found in the studies by MacIntosh *et al.* (2013) and Burgunder *et al.* (2018).

STATISTICAL ANALYSES

We retained in our analyses only the individuals for which the entire duration of the behavioural experiments (30 min) was recorded and successfully extracted using *ToxTrac*. Given that *ToxTrac* did not detect some individuals satisfactorily during the whole duration of the behavioural experiments and because some mortality occurred at later stages of development ($N = 4$), we were able to analyse data on 13 individuals from GS 25, 18 individuals from GS 30, 17 individuals from GS 37, 14 individuals from GS 41 and 12 individuals from GS 42.

All statistical analyses were conducted with R statistical software v.4.0.0 (R Core Team, 2020) and RSTUDIO v.1.2.5042 (RStudio, 2020, Inc.). All residuals were tested for homogeneity of variance and normality with Bartlett’s test and the Shapiro–Wilks test, respectively. We also checked the normality of the residuals using diagnostic plots. Overall, clutch identity always influenced measured parameters significantly (all $P < 0.001$), hence we kept clutch identity as a random factor in all our analyses. Given that we assessed locomotion and behaviour in the same individuals throughout development, we nested the identity of the tadpole in their respective clutch. We tested a random intercept model and a random slope

model and kept the models with the lowest Akaike information criterion corrected for small sample size (AICc; Supporting Information, Table S1).

We fitted linear mixed-effect models (LMERs; package ‘lme4’; Bates *et al.*, 2015) to analyse differences in total activity time, total distance, number of activity bouts, swimming speed (maximum, mean and relative) and α DFA across developmental stages (Supporting Information, Table S2). We fitted LMERs to analyse the relationship between total activity time, total distance, number of activity bouts, swimming speed (maximum and mean) and α DFA with total length and another round of models for tail length (Supporting Information, Tables S3 and S4). In order to extract random effects variances, model estimates and R^2 , we used the ‘parameter’ package with 1000 bootstrap iterations (Lüdecke *et al.*, 2020) and the ‘lme4’ package (Supporting Information, Tables S5–S7). Tukey’s *post hoc* (‘multcomp’ package) tests were carried out to analyse the differences. Finally, to investigate whether behavioural traits were correlated together, we calculated the Pearson correlation coefficient using the `cor.test` function in the `corr.plot` package.

ETHICAL APPROVAL

All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities (COMETHEA ethical committee and Ministère de l’Enseignement Supérieur, de la Recherche et de l’Innovation).

DATA AVAILABILITY

Data will be made available upon reasonable request.

RESULTS

All behavioural traits at each stage are summarized in Table 2.

TADPOLE SIZE

Total length ($F_{4,69} = 160.70$, $P < 0.0001$; Fig. 1) and tail length ($F_{4,69} = 97.62$, $P < 0.0001$; Fig. 1) differed among all stages. Both variables increased across the first three stages before reaching a plateau at GS 41 (Fig. 1). A slight but significant ($P < 0.001$) decrease was visible at GS 42, when tail atrophy occurs (Fig. 1).

ACTIVITY AND DISTANCE

Total activity time varied across developmental stages ($F_{4,51} = 8.69$, $P < 0.0001$; Fig. 2). It increased by 72% from GS 30 [190 s, 95% confidence interval

Table 2. Summary statistics (mean ± SE) of behavioural and morphological traits

Trait	Developmental stage				
	GS 25 (n = 13)	GS 30 (n = 18)	GS 37 (n = 17)	GS 41 (n = 14)	GS 42 (n = 12)
Behavioural trait					
Total activity time (s)	132.15 ± 29.46	319.27 ± 17.62	459.27 ± 38.52	398.24 ± 30.40	341.67 ± 40.52
Number of activity bouts	4899.62 ± 844.55	3126.78 ± 234.71	11 175.47 ± 1244.28	13 715.21 ± 781.76	12 085.00 ± 1213.61
Total distance (mm)	3314.59 ± 976.64	14 510.81 ± 1021.44	14 904.87 ± 1269.47	10 155.46 ± 1170.18	8360.61 ± 1220.93
Absolute mean swimming speed (mm s ⁻¹)	22.14 ± 1.24	44.10 ± 1.37	31.87 ± 1.32	23.77 ± 1.15	22.80 ± 0.80
Absolute maximal swimming speed (mm s ⁻¹)	65.16 ± 4.45	248.78 ± 13.17	254.94 ± 18.99	130.42 ± 12.70	144.20 ± 15.31
Relative mean swimming speed (total lengths s ⁻¹)	-8.96 ± 1.24	13.75 ± 1.36	2.12 ± 1.31	-5.86 ± 1.14	-7.08 ± 0.81
Relative maximal swimming speed (total lengths s ⁻¹)	-62.46 ± 4.41	81.84 ± 13.36	56.30 ± 19.41	-74.70 ± 13.29	-47.70 ± 14.35
α DFA	0.97 ± 0.00	0.91 ± 0.01	0.90 ± 0.01	0.97 ± 0.00	0.98 ± 0.00
Morphology					
Total length (cm)	1.04 ± 0.02	1.90 ± 0.03	2.59 ± 0.04	2.73 ± 0.06	2.45 ± 0.11
Tail length (cm)	0.63 ± 0.01	1.15 ± 0.02	1.56 ± 0.03	1.63 ± 0.04	1.43 ± 0.09

Abbreviation: α DFA, scaling exponent of the sequence.

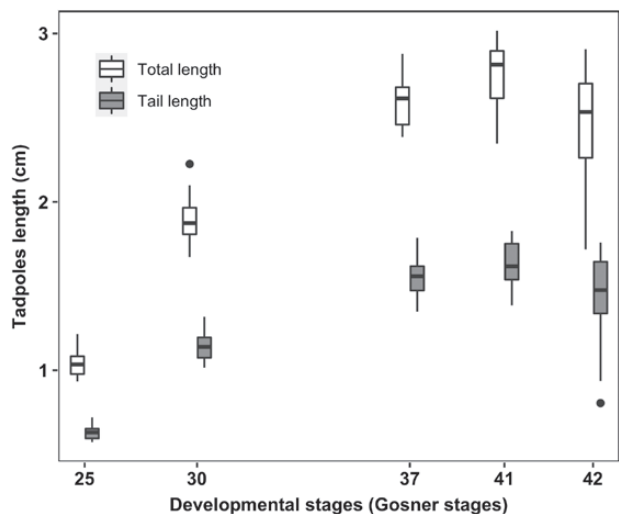


Figure 1. Total length (white boxes) and tail length (grey boxes; both in centimetres) relative to Gosner developmental stages in *Bufo spinosus* tadpoles. The top and bottom of the boxes represent the first and last quartiles, the horizontal line within the box represents the median, the whiskers represent the fifth and 95th percentiles, and the circles represent outliers. Dots represent the distribution of all individuals. Intervals between Gosner developmental stages were calculated using the mean number of days between two Gosner stages for all individuals.

(CI) = 77–310] to reach maximal values at GS 37 (328 s, 95% CI = 213–440) and subsequently decreased by 19% at GS 41 (266 s, 95% CI = 138–400) and again by 19% upon metamorphosis (216 s, 95% CI = 86–345), although all confidence intervals overlapped.

The number of activity bouts differed significantly between developmental stages ($F_{4,51} = 14.35$, $P < 0.0001$), with tadpoles at GS 25 and GS 30 being significantly less active than in all other stages (all $P < 0.001$, Fig S1). The total distance travelled was also significantly different between stages $F_{4,51} = 10.62$, $P < 0.0001$; Fig. 2). Early developmental stages (GS 25) moved more than three times less than GS 30 (11.2 cm, 95% CI = 7.1–15.5) and GS 37 (11.7 cm, 95% CI = 7.2–15.8), when maximal values were obtained; and total distance travelled gradually decreased at GS 41 (6.8 cm, 95% CI = 2.0–11.4) and at GS 42 (5.3 cm, 95% CI = 0.3–9.9), although all confidence intervals overlapped.

MEAN AND MAXIMAL SWIMMING SPEED

Both swimming speed variables differed between developmental stages (all $P < 0.001$, Fig S1). GS 25 displayed lower swimming speeds than GS 30 (all $P < 0.04$). After GS 37, maximal and mean swimming speeds decreased along development stages (all $P < 0.02$). Absolute maximal swimming speed increased

by almost 300% for GS 30 (183 mm s⁻¹, 95% CI = 130–237) to reach maximal values at GS 37 (190 mm s⁻¹, 95% CI = 135–248) and returned to the same value as GS 25 for GS 41 (66 mm s⁻¹, 95% CI = 7–125) and finally increased by 17% upon metamorphosis (216 s, 95% CI = 86–345), although all confidence intervals overlapped.

BEHAVIOURAL COMPLEXITY

Scaling exponent differed significantly between developmental stages ($F_{4,51} = 10.34$, $P < 0.0001$; Fig. 2), with GS 30 and GS 37 displaying higher complexity (lower α DFA, all $P < 0.006$) than other stages.

RELATIONSHIPS BETWEEN LOCOMOTION AND MORPHOLOGY

Total activity time was positively correlated with both total length and tail length across stages (total length, $F_{1,72} = 23.44$, $r^2 = 0.23$, $P < 0.0001$; tail length, $F_{1,72} = 24.85$, $r^2 = 0.24$, $P < 0.0001$), but not within stages (all $P > 0.14$). The number of activity bouts was positively correlated with total length and tail length across stages (total length, $F_{1,72} = 28.01$, $r^2 = 0.27$, $P < 0.0001$; tail length, $F_{1,72} = 26.65$, $r^2 = 0.26$, $P < 0.0001$), but not within stages (all $P > 0.19$), except for a positive correlation with tail length at GS 41 ($F_{1,12} = 6.85$, $r^2 = 0.31$, $P = 0.02$). Total distance travelled was positively correlated with total length and tail length across stages (total length, $F_{1,72} = 7.60$, $r^2 = 0.08$, $P = 0.007$; tail length, $F_{1,72} = 8.73$, $r^2 = 0.09$, $P = 0.004$), but not within stages (all $P > 0.2$).

Absolute mean swimming speed was not correlated with total length or with tail length across (all $P > 0.67$) or within stages (all $P > 0.09$), whereas absolute maximal swimming speed was positively correlated with total length and tail length across stages (total length, $F_{1,72} = 5.59$, $r^2 = 0.06$, $P = 0.02$; tail length, $F_{1,72} = 6.39$, $r^2 = 0.07$, $P = 0.01$), but not within stages (all $P > 0.1$).

Scaling exponent was not correlated with total length either across ($P = 0.07$) or within stages (all $P > 0.2$), except for a positive relationship at GS 37 ($F_{1,15} = 4.59$, $r^2 = 0.18$, $P = 0.04$). However, α DFA was negatively correlated with tail length across stages ($F_{1,72} = 4.29$, $r^2 = 0.04$, $P = 0.04$), but not within stages (all $P > 0.07$).

RELATIONSHIPS BETWEEN BEHAVIOURAL TRAITS

Most behavioural traits were correlated with one another (all $P < 0.001$; Table 3). The α DFA was negatively correlated with all locomotion traits except for the number of activity bouts ($P > 0.05$; Table 3), indicating that as locomotion increased, behavioural sequences exhibited greater complexity.

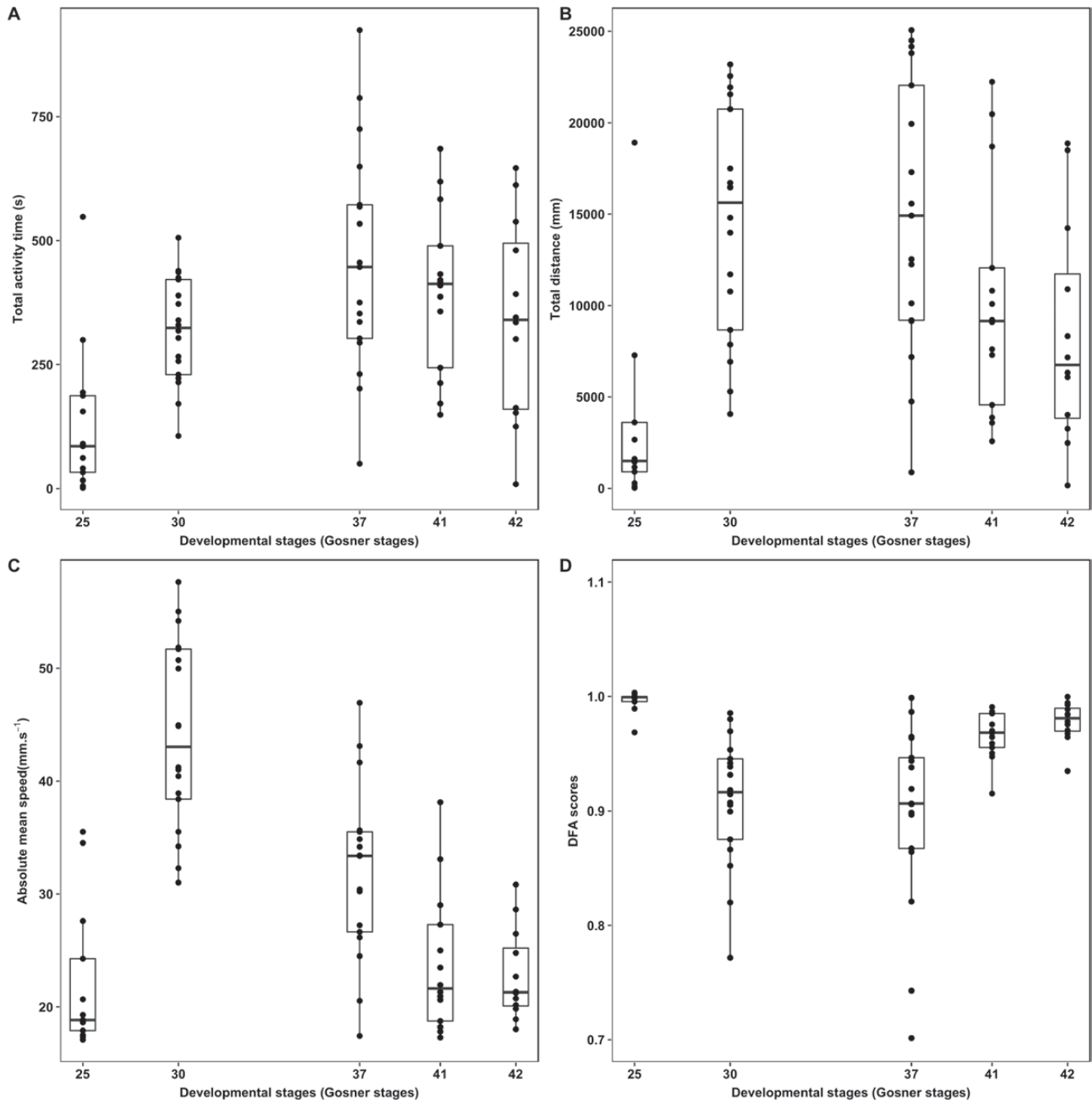


Figure 2. Total activity duration (in seconds; A), total distance travelled (in millimetres; B), absolute maximal swimming speed (in millimetres per second; C) and α DFA (unitless scaling index inversely related to behavioural complexity; D) relative to Gosner developmental stages in *Bufo spinosus* tadpoles. The top and bottom of the boxes represent the first and last quartiles, the horizontal line within the box represents the median, the whiskers represent the fifth and 95th percentiles, and the circles represent outliers. Dots represent the distribution of all individuals. Intervals between Gosner developmental stages were calculated using the mean number of days between two Gosner stages for all individuals.

DISCUSSION

Overall, we showed that activity levels, locomotion and behaviour undergo massive changes during tadpole development. These changes are not linear, but rather curvilinear across developmental stages, with

a phase of increasing activity followed by a plateau and/or reduction in the values of these parameters. These two different phases dovetail relatively well with the morphological changes associated with metamorphosis (i.e. a phase of intense growth followed

Table 3. Correlations (r) among all behavioural traits in *Bufo spinosus* tadpoles

	Total activity duration (s)	Number of activity bouts	Total distance (mm)	Absolute mean swimming speed (mm s ⁻¹)	Absolute maximal swimming speed (mm s ⁻¹)	Relative mean swimming speed (total lengths s ⁻¹)	Relative maximal swimming speed (total lengths s ⁻¹)	α DFA
Total activity duration (s)	/	0.73	0.87	0.3	0.48	0.32	0.36	-0.63
Number of activity bouts		–	0.33	-0.34	0.01	-0.31	–0.03	–0.06
Total distance (mm)			–	-0.69	0.72	0.71	0.66	-0.8
Absolute mean swimming speed (mm s ⁻¹)				–	0.76	1	0.81	-0.61
Absolute maximal swimming speed (mm s ⁻¹)					–	0.78	0.96	-0.58
Relative mean swimming speed (total lengths s ⁻¹)						–	0.81	-0.62
Relative maximal swimming speed (total lengths s ⁻¹)							–	-0.54
α DFA								–

Numbers in bold indicate significant average intra-individual correlations at $P < 0.05$. Abbreviation: α DFA, scaling exponent of the sequence.

by morphological changes linked to metamorphosis; [Brown & Cai, 2007](#)).

As predicted, the two observed phases of tadpole development strongly influenced all the metrics we studied. The highest values of activity levels, distance travelled, swimming speeds and behavioural complexity (i.e. lowest value of α DFA) were attained between GS 30 and GS 37, which represent the later developmental stages at the onset of the most intensive changes linked to metamorphosis. Our results suggest that, in order to sustain the intensive growth occurring between GS 25 and GS 37, tadpoles increased their activity levels and mobility, presumably to optimize foraging while evading predation ([Van Buskirk & Saxer, 2001](#)). In turn, greater body and tail lengths are likely to improve locomotion ([Wilson & Franklin, 2000](#); [Wilson et al., 2005](#); [Johnson et al., 2015](#); [Lindgren et al., 2018](#)).

During later stages of development (GS 41 and GS 42), the total length of tadpoles remained roughly constant, while locomotion-related traits decreased significantly. These stages are characterized by strong morphological changes linked to metamorphosis, with the end of growth of the hindlimbs (GS 41), the emergence of forelimbs from the body cavity and the onset of tail atrophy (GS 42). Interestingly, a previous theoretical study has suggested that the shape of tadpoles, along with large lateral oscillations at the snout, compensates for the reduced hydrodynamism (increased drag) linked to presence of the hindlimbs ([Liu et al., 1996](#)). Accordingly, it has been suggested that hindlimbs remain bound to the side of the body to improve streamlining ([Stehouwer & Farel, 1984](#)).

In contrast, our results showed that locomotion was strongly reduced at GS 41, when fully developed hindlimbs are present but probably not yet useful and/or used for propulsion ([Wilson & Franklin, 2000](#)). These appendages might have a negative influence on hydrodynamism and/or impede efficient undulatory body and tail movements (e.g. presence of hindlimbs on both sides of the base of the tail might impede tail undulations). Accordingly, hindlimb presence has been shown to influence postural and directional changes and predation avoidance ([Stehouwer & Farel, 1984](#); [Brown & Taylor, 1995](#)). GS 41 is also characterized by the growth of forelimbs that occur in the atrial chambers where gills are located ([Wells, 2007](#)). This process might also limit the drag imposed by limb development at this stage. Yet, comparatively lower activity was also observed at GS 42, when both hindlimbs and forelimbs are present and when tail atrophy begins, all of which seem to constrain aquatic locomotion of tadpoles further ([Calsbeek & Kuchta, 2011](#)). Similar changes in the index of behavioural complexity confirm these hypotheses: α DFA was markedly lower at stages GS 30 and GS 37, indicating greater levels of behavioural complexity (stochasticity) at these stages ([MacIntosh, 2014](#)).

Alternatively, but not mutually exclusively, we can hypothesize that after GS 37 activity is no longer dedicated to acute growth (as suggested by the stabilization of body size), but rather towards organismal maintenance and metamorphosis. In this respect, by decreasing the costs of locomotion (lower activity levels and swimming speeds) tadpoles dedicate energy to metamorphosis ([Beck & Congdon, 2003](#);

Wright *et al.*, 2011; Kirschman *et al.*, 2017; Ruthsatz *et al.*, 2020). Additionally, other major changes linked to metamorphosis are also expected to influence locomotion negatively; for instance, the shift from herbivory and detritivory to carnivory, characterized by significant anatomical modifications in the digestive system and mouth (Vitt & Caldwell, 2014). The feeding apparatus and oral disc disappear, and alterations of the intestinal epithelium preclude efficient nutrient absorption (Hourdry *et al.*, 1996). This might prevent tadpoles at later stages of development from foraging efficiently (e.g. inability to acquire and/or digest food), thereby limiting the necessity of displaying well-developed locomotor performance. Accordingly, it has been suggested that during later stages of development, tadpoles become aphagic and rely, in part, on tail resorption to fuel energetic demands (Gonçalves *et al.*, 2015; Bouchard *et al.*, 2016; Ruthsatz *et al.*, 2018).

To conclude, our study shows that tadpole ontogeny is characterized by strong variations in locomotion and behaviour according to two different phases of development (i.e. somatic growth vs. morphological changes linked to metamorphosis). All the metrics we investigated indicate that the peak of activity and associated behaviour is situated at a pivotal stage when somatic growth decreases and significant morphological changes occur (i.e. hindlimb growth). Future studies that aim to investigate determinants of activity should include developmental stages as covariates in order to assess whether the sensitivity of locomotion to environmental variables (e.g. temperature and predation) changes across developmental stages. Additionally, future studies should investigate the influence of tadpole development on their movements throughout the water column.

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during data curation. L.R., X.M. and A.K. arranged the dataset for fractal analysis. A.K., L.R. and M.C. conducted all fractal analyses. F.B., M.C., L.R., A.K. and Y.R.-C. interpreted the data. F.B., M.C. and L.R., designed the figures and wrote the initial draft. All authors contributed to manuscript revision. All authors read and approved the final manuscript. The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Table S1. Model comparison between random intercept model and random slope (individual identification) model using the Akaike information criterion corrected for small sample size (AICc). We retained the model with the smallest AICc value.

Table S2. Linear mixed model of the difference in several behavioural traits of developmental stages (Gosner stages). We kept individual identification nested in clutches in all our models. σ represents variance of random effect. When possible, marginal and conditional R^2 were used.

Table S3. Linear mixed model of the relationship between several behavioural traits and total length of the tadpoles. We kept individual identification nested in clutches in all our models. σ represents variance of random effect. When possible, marginal and conditional R^2 were used.

Table S4. Linear mixed model of the relationship between several behavioural traits and tail length of the tadpoles. We kept individual identification nested in clutches in all our models. σ represents variance of random effect. When possible, marginal and conditional R^2 were used.

Table S5. Linear mixed model of several behavioural traits and developmental stages. We kept individual identification nested in clutches in all our models. The confidence interval (CI) and P -value were estimated from 1000 bootstrap iterations (‘parameter’ package; Lüdecke *et al.*, 2020).

Table S6. Linear mixed model of several behavioural traits and total length. We kept individual identification nested in clutches in all our models. The confidence interval (CI) and P -value were estimated from 1000 bootstrap iterations (‘parameter’ package; Lüdecke *et al.*, 2020).

Table S7. Linear mixed model of several behavioural traits and tail length. We kept individual identification nested in clutches in all our models. The confidence interval (CI) and *P*-value were estimated from 1000 bootstrap iterations ('parameter' package; Lüdecke *et al.*, 2020).

Figure S1. A, number of activity bouts. B, absolute mean swimming speed (in millimetres per second). C, D, relative mean swimming speed (per total length; C) and relative maximal swimming speed (per total length; D) relative to Gosner developmental stages in *Bufo spinosus* tadpoles. The top and bottom of the boxes represent the first and last quartiles. The line across the box represents the median. The whiskers represent the fifth and 95th percentiles, and the dots represent the distribution of the individuals.

Article VIII

Marion Cheron, François Brischoux

Exposure to low concentrations of AMPA decreases survival during larval development in a widespread amphibian.

In preparation for Aquatic Toxicology



1 **Exposure to low concentrations of AMPA influences morphology and**
2 **decreases survival during larval development in a widespread**
3 **amphibian species.**

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11 Manuscript in consideration for Archives of Environmental Contamination and
12 Toxicology

13

14 **Abstract**

15

16 Glyphosate's primary metabolite, AMPA (aminomethylphosphonic acid), is the main
17 chemical substance detected in surface waters worldwide. However, ecotoxicological
18 studies on the potential effects of this metabolite at environmental concentrations on
19 wildlife are scarce. Yet, due to its chemical properties, AMPA is likely to affect non-
20 target species. In this study we investigated sublethal effects of ecologically relevant
21 concentrations of AMPA on the larval development of a widespread amphibian
22 species, the spined toad *Bufo spinosus*. We performed a factorial experimental design
23 to study the effect of concentration and the timing of exposure (during embryonic
24 development, larval development or both) to AMPA on the morphology, duration of
25 development and survival of tadpoles. AMPA and timing of exposure interactively
26 affected tadpole morphology, but not duration of development. Most of these effects
27 were linked to exposure during embryonic development. Such effects in individuals
28 exposed during embryonic development solely were long-lasting and occurred until
29 the latest larval stages. Finally, we found that exposure to AMPA after hatching
30 (during the larval stage) increased mortality. Exposure to minute AMPA
31 concentrations could have long lasting consequences on fitness and population
32 persistence as it affects survival. These effects are especially important to take into
33 account at a time when multiple compounds can interact to affect wildlife.

34

35 **Key-words** : Aminomethylphosphonic acid – Tadpoles - Development - Sublethal
36 effects – Survival

37

38 **Introduction**

39

40 Modern agricultural practices have intensified in recent years despite international
41 concerns on potential consequences to biodiversity (Brooks et al., 2002; Stoate et al.,
42 2009). For instance, intensive agriculture can affect biodiversity through the
43 widespread use of agrochemicals can also negatively influences wildlife, notably
44 through subtle sublethal effects (Agostini et al., 2020; Garcês et al., 2020). Indeed,
45 although designed to be toxic for specific taxa, phytosanitary compounds can also
46 affect non-target species (de Brito Rodrigues et al., 2019; Hasenbein et al., 2017;
47 Isidori et al., 2005; Relyea, 2009).

48

49 The effects of agrochemicals on non-target species are being increasingly investigated
50 (Baier et al., 2016a; Hasenbein et al., 2017; Thiour-Mauprivez et al., 2019), and can
51 influence wildlife through both acute and chronic exposures (Bernabò et al., 2008;
52 Bókony et al., 2017; Hackenberger et al., 2018). Environmental concentrations are
53 often very low as compared to the values tested under laboratory conditions on
54 model species which define standards of use (e.g., based on LD50, Beiras, 2018;
55 Kramer et al., 2009). As a consequence, although these low, environmentally-
56 relevant concentrations are usually disregarded by regulatory toxicity studies, an
57 increasing body of literature has highlighted their negative effects through several
58 mechanisms affecting DNA structure (Guilherme et al., 2010; Matozzo et al., 2019),
59 oxidative status (Isaksson, 2010; Lushchak, 2016; Pašková et al., 2011), physiology
60 (Hayes et al., 2006; Leemans et al., 2019; Muñoz et al., 2021), development
61 (Lenkowski et al., 2010; Relyea, 2009; Wang et al., 2019), morphology (Baier et al.,

62 2016b; Cheron and Brischoux, 2020) or behaviour (Browne and Moore, 2014; Brunelli
63 et al., 2009; Hellou, 2011), all of which can ultimately influence individual survival
64 (Baker et al., 2013; Herek et al., 2020) and/or reproduction (Adams et al., 2021;
65 Costantini et al., 2014; Hackenberger et al., 2018) and thus population persistence
66 (Blaustein et al., 2011; Hamilton et al., 2016).

67

68 Despite the growing interest of the scientific community to assess the effects of
69 agrochemicals on wildlife, most studies have focused on the active ingredients
70 (Mesnage and Antoniou, 2018). Yet, in natural conditions, most pesticides degrade
71 due to contact with air or water (Kamrin, 1997), exposure to sunlight (Fenoll et al.,
72 2012) and/or because of the activity of microbiota (Sviridov et al., 2015). As a
73 consequence, our knowledge is often limited to the effects parent compounds (i.e.,
74 the listed active substances), and the potential effects of their metabolites has been
75 overlooked to date (de Brito Rodrigues et al., 2019; EFSA, 2012; Lee et al., 2017).

76

77 Such bias is exemplified by one of the most (in)famous herbicide, glyphosate, and its
78 primary metabolite, aminomethylphosphonic acid (AMPA). Glyphosate remains one
79 of the most widely used non-selective herbicide despite the intense scientific and
80 societal debates regarding its toxicity (Clapp, 2021; Helander et al., 2012).

81 Accordingly, the overall effects of glyphosate on wildlife, including investigations of
82 commercial formulations and/or of different surfactants have been relatively
83 thoroughly studied (Berger et al., 2018; Bókony et al., 2017; Cauble and Wagner, 2005;
84 de Brito Rodrigues et al., 2019; Giaquinto et al., 2017; Gill et al., 2018; Guilherme et
85 al., 2010; Herek et al., 2020; Lenkowski et al., 2010; Matozzo et al., 2019; Muñoz et al.,

86 2021; Solomon and Thompson, 2003). Yet, as mentioned above, its primary
87 metabolite, AMPA is arguably the most common compound found in surface and
88 groundwater worldwide (Bonansea et al., 2017; Grandcoin et al., 2017; Medalie et al.,
89 2020; Okada et al., 2020). The greater occurrence and abundance of AMPA in natural
90 environments is likely linked to the extensive use of glyphosate and also because
91 AMPA is an organic phosphonate derived from water treatment facilities, textile
92 industries, and industrial or household detergents (Grandcoin et al., 2017). Because
93 AMPA has a longer half-life than glyphosate in water (Battaglin et al., 2014;
94 Bonansea et al., 2017; Silva et al., 2018), it tends to accumulate in aquatic habitats
95 from contaminated environments via runoff or soil erosion (Solomon and Thompson,
96 2003). Studies on the effects of AMPA at environmental concentrations on wildlife
97 are comparatively fewer (Cheron and Brischoux, 2020; de Brito Rodrigues et al., 2019;
98 Domínguez et al., 2016; Guilherme et al., 2014; Martinez and Al-Ahmad, 2019;
99 Matozzo et al., 2019). Because of such discrepancy, it is crucial to collect data on the
100 effects of AMPA on wildlife if we are to provide a thorough assessment of the
101 potential impacts of glyphosate on biodiversity (Hahn and Sadler, 2020).

102

103 In this study, we assessed the effects of exposure to environmentally-relevant
104 concentrations of AMPA on the development of a widespread amphibian species
105 (spined toad, *Bufo spinosus*). Amphibians are particularly susceptible to the presence
106 of pesticides in surface waters (Hayes et al., 2010; Mann et al., 2009; Quaranta et al.,
107 2009). First, in temperate areas, breeding period often coincide with the use of
108 agrochemicals (Berger et al., 2013; Lenhardt et al., 2015). Second, many amphibians
109 have biphasic life cycle, with terrestrial adults breeding in aquatic sites where eggs

110 and larvae develop (Reading et al., 1991). As a consequence, breeding occurs in
111 lentic systems where concentrations of pesticides can be relatively elevated due to
112 stagnation of water (Battaglin et al., 2009). Third, the skin of aquatic or semi-aquatic
113 amphibian are characterized by high permeability because it is actively involved in
114 gas exchanges and regulation of internal concentration of water and ions (Brischoux
115 et al., 2021; Uchiyama and Konno, 2006). Moreover, surface area to volume ratio is
116 elevated in amphibians compared to other species which influence the
117 transcutaneous transfer of xenobiotics (Quaranta et al., 2009). Fourth, tadpoles often
118 forage on sediment and associated biofilm where pesticides are known to accumulate
119 (Degenhardt et al., 2009). Alteration of sediment load can result in reduced larval
120 abundances or growth and development rates (Gillespie, 2002; Snodgrass et al., 2008;
121 Wood and Richardson, 2009). Early developmental (embryonic and larval) phases
122 bear strong and long-lasting influences on life-history traits such as body size,
123 growth, survival, and reproduction (Kashiwagi et al., 2009; Arrighi et al., 2013). As a
124 consequence, early development alteration through aquatic habitats disturbances
125 might influence post-metamorphic development to adulthood.

126

127 In this study, we investigated the effects of the timing of exposure to AMPA on the
128 larval development until metamorphosis. Using a factorial design in a common
129 garden experiment, we exposed eggs and tadpoles to environmentally relevant
130 concentrations of AMPA at different phases of their development (embryonic
131 development only, larval development only or throughout the whole development).
132 Such approach allowed to test not only for the effects of exposure to AMPA but also
133 to assess the relative susceptibility of different developmental phases (embryonic

134 *versus* larval). Previous studies showed that embryonic development of *Bufo spinosus*
135 is negatively affected by environmentally-relevant concentrations of AMPA which
136 decrease embryonic survival, increase development duration and influence hatchling
137 morphology (Cheron and Brischoux, 2020) through mechanisms affecting oxidative
138 status (Cheron et al., 2022). Herein, we investigated larval developmental alterations
139 using relevant morphological (body size and shape) and developmental (duration of
140 developmental stages) characteristics and survival.

141

142

143 **Materials and Methods**

144

145 *Eggs collection*

146 Spined toad (*Bufo spinosus*) eggs strings (number of egg string=10) were collected
147 from 29/01/2018 to 28/02/2018 in ponds near the Centre d'Etudes Biologiques de
148 Chizé (46°09'N, 0°24'W) and returned to the laboratory. We monitored the ponds
149 twice a day to ensure that the eggs were collected immediately after oviposition to
150 minimize embryonic development in the natural environment. A subset of 120 eggs
151 on each clutch was collected for our experiment, while the remaining eggs (i.e., 3000-
152 5000) were released at their oviposition site. We changed water once a week
153 according to the half-life of AMPA ranging from 7 to 14 days in water (Battaglin et
154 al., 2014). Egg jelly was maintained throughout the experiment.

155

156 *Treatment concentrations and chemical solutions*

157 In this study, we aimed at reproducing the environmental concentrations found in
158 aquatic environments in France. AMPA concentrations range from 0.1 µg.l⁻¹ to 6.6

159 $\mu\text{g.l}^{-1}$ (data from Water Agencies, “Agence de l'eau Loire Bretagne” and “Agence de
160 l’eau Adour-Garonne”) which are similar to concentrations found in aquatic
161 environments in Northern America and Europe (Grandcoin et al, 2017; Coupe et al,
162 2012; Bonansea et al, 2017). We produced three treatments spanning from low to
163 relatively high concentrations (Table 1). We dissolved commercial crystalline
164 powder (Aminomethylphosphonic acid, 99% purity, ACROS ORGANICS™) in
165 dechlorinated aged-tap water in order to obtain stock solutions (0.1g/L) of AMPA
166 which were further diluted. Analytical verifications of the actual concentrations in
167 tanks were performed by an independent accredited analytical laboratory
168 (QUALYSE, Champdeniers-Saint-Denis, France) in triplicate. Determination of
169 AMPA in water samples was assessed using liquid chromatography-mass
170 spectrometry (LC/MS/MS) with 9-fluorenylmethyl chloroformate (FMOC-Cl) used
171 as derivatization agent. The limit of quantification was $0.1 \mu\text{g l}^{-1}$ for the
172 determination of AMPA in water samples. Overall, these verifications showed that
173 actual concentrations were similar to concentrations found in aquatic environments
174 (see above). For clarity, we will refer to the treatments as Low, Medium and High
175 hereafter.

176

177 *Experimental design*

178 Experimental design can be found in Figure 1. We subjected developing *Bufo spinosus*
179 to AMPA concentrations or control conditions (no AMPA) during embryonic
180 development only, larval development only or the entire development (3
181 concentrations X 3 exposures periods X 15 clutches + 1 Control X 15 clutches = 150
182 experimental units)” in 2L tanks filled with dechlorinated aged-tap water. First,

183 segment of 30 eggs were subjected to either one of the AMPA concentrations or a
184 control during the whole embryonic development until hatching (see Cheron et al.,
185 2021). Upon hatching (GS22), we randomly chose 1 hatchling per tank per clutch (4
186 hatchlings for control treatment) and disposed in individual tanks for the larval
187 development phase (Figure 1). Maintaining the tadpoles individually in aquaria
188 should not generate stressful conditions and mimic natural conditions (Bókonyi et al.,
189 2021; Melvin and Houlihan, 2014). We exposed larvae from GS 22 until GS 42. At GS
190 42, at the onset of metamorphosis, tadpoles were transferred to boxes with a small
191 amount of water from their original tank and a ramp which allowed them to climb
192 out of water. When individuals were observed perched on the ramp (GS 46), we
193 removed the water, and added a piece of damp paper to avoid desiccation. We fed
194 the tadpoles with boiled organic spinach and the metamorphs with *Acyrtosiphon*
195 *pisum* and *collembola sp.* ad libitum and kept metamorphs 130 days after
196 metamorphosis in order to test for long term survival as fitness proxy. All
197 experiments took place in a thermally controlled room with the temperature set at
198 17°C (both air and water) and under natural 12:12 hr day/night photoperiod.

199

200 *Measurements*

201 Hatching began at GS 22 after 16.10 ± 0.02 days (Gosner, 1960). We assessed the time
202 between each stages (GS22, GS 25, GS 30, GS 41, GS 42) as well as the morphology
203 using with the software ImageJ (Schneider et al, 2012). We selected these Gosner
204 stages as they represent key developmental stages in our study species (Cheron et al.
205 2021). Each tadpole was placed in a petri dish and photographed from above and
206 side-view using a camera (Panasonic Lumix DC-TZ55). We measured morphological

207 traits that are known to exhibit plasticity in anurans species (Watters et al., 2016):
208 total length (body + tail), body length, tail length, body width, tail height and body
209 height. All measurements were performed by the same person throughout the
210 experiment. Upon metamorphosis and five days after, toadlets were weighted every
211 day and were photographed to measure their morphology (snout-vent length: SVL).
212 Residuals of linear regression between SVL and weight of toadlets were used to
213 calculate body-condition index (BCI). Finally, we checked for survival every day.

214

215 *Statistical Analyses*

216 All data were tested for homogeneity of variance and residuals normality with
217 Barlett's and Shapiro-Wilks tests, respectively. We also checked normality of the
218 residuals using diagnostics plots. All statistical analyses were conducted with R
219 statistical software v.4.0.0 (R Core Team, 2020) and RStudio v 1.2.5042 (RStudio, Inc.).

220

221 First, Principal component analysis (PCA) was used to graphically assess whether
222 the AMPA Concentration and Exposure influenced the morphology of tadpoles since
223 all morphological variables were strongly correlated. PC1 explained 96.54% of the
224 total data variability. The contributions for each variable were: 0.98 for total length,
225 0.978 for body length, 0.99 for tail length, 0.97 for body width, 0.98 for tail height and
226 0.98 for body height. We performed LMER (Linear mixed-effects models, "lme4"
227 packages) on PCA scores of the first axis with the interaction between
228 "Concentration", "Exposure" and "Stage". "Concentration" represented which
229 concentration tadpoles were exposed to, either Control, Low or High. "Exposure"
230 represented timing of exposure either embryonic, larval or both. "Stage" represented

231 each studied stage, GS25, GS30, GS37, GS41 or GS42. In each case, the model was the
232 following " Variable~ Concentration*Exposure*Stage+ (1 | Clutch/id)".

233

234 First for each morphological variable, we did a full model (Linear mixed-effects
235 models-LMER for gaussian data, Generalized linear mixed-effects models-GLMER
236 for non-gaussian data) with the interaction between "Concentration", "Exposure" and
237 "Stage". "Concentration" represented which concentration tadpoles were exposed to,
238 either Control, Low or High. "Exposure" represented timing of exposure either
239 embryonic, larval or both. "Stage" represented each studied stage, GS25, GS30, GS37,
240 GS41 or GS42. In each case, the model was the following " Variable~
241 Concentration*Exposure*Stage+ (1 | Clutch/id)". We chose the model using AIC
242 (Akaike Information Criterion) though stepwise regression (package "stats"). When
243 all predictors were selected, we fitted either linear mixed model or generalized
244 mixed model.

245

246 Second, we fitted linear mixed-effect models to analyse differences in development
247 duration between stage and throughout ontogeny using structure provided by
248 stepwise regression (LMERs, package "lme4" (Bates et al., 2015).

249

250 Third, we performed GLMER (Generalized linear mixed-effects models, binomial
251 family) to study whether larval mortality was influenced by AMPA treatment. The
252 model was the following " Mortality~ Concentration*Exposure*Stage+
253 (1 | Clutch/id)".

254

255 Finally, we performed survival analysis using log-rank method ("survival" packages)
256 to assess tadpoles mortality rate until J+130. This method allowed us to estimate the
257 rate at which death occurs over time and whether death is more susceptible to occur
258 at a specific stage. We examined differences across treatment (Concentration or
259 Exposure) with a Cox model. The test for assumption of proportional hazards for
260 Cox regression which show that our model was appropriate.

261

262

263 **Results**

264

265 *Tadpoles*

266 First, neither total development duration nor durations of each developmental stages
267 were affected by our experimental treatment (concentration or timing of exposure,
268 Table 2, Figure 2).

269

270 However, morphological score (PCA first axis) was affected by AMPA Exposure
271 throughout ontogeny ($F_{12,400}=1.830$, $p=0.042$, $\eta^2=0.06$, Figure 2).

272

273 By focusing our analyses on morphological variables only at each stage. We found an
274 effect of timing of exposure on total length at GS25 ($F_{2,132}=3.6218$, $p=0.029$, $\eta^2=0.06$).

275 Tadpoles treated during larval development only were smaller than tadpoles

276 exposed during the embryonic development only (-0.034 ± 0.014 , $p=0.019$) and

277 tadpoles exposed throughout development (-0.033 ± 0.014 , $p=0.095$). We found an

278 effect of timing of exposure solely on GS25 on tail length ($F_{3,132}=4.484$, $p=0.005$).

279 Tadpoles exposed during larval phase were smaller than tadpoles exposed during
280 embryonic phase (-0.033 ± 0.02 , $p=0.003$) or those exposed throughout development ($-$
281 0.026 ± 0.003 , $p=0.003$).

282

283 Moreover, while we did not detect an effect of AMPA during the somatic growth
284 phase (GS30 to GS41). At GS 42, for tadpoles exposed during embryonic
285 development, we found that individuals exposed to our High treatment were longer
286 than those from the Medium treatment (0.283 ± 0.089 , $p=0.01$).

287 We found a significant Concentration X Timing of exposure interaction ($F_{3,111}=2.616$,
288 $p=0.039$, Figure 2) showing a long-lasting effect of AMPA concentration in tadpoles
289 exposed during embryonic development solely. Tadpoles exposed as embryos to the
290 high concentration had longer tails than those exposed as embryos to the medium
291 concentration (0.201 ± 0.07 , $p=0.015$).

292 Finally, we found an effect of AMPA on tail height at GS42 ($F_{3,111}=2.813$, $p=0.029$,
293 Table 2) showing an effect of concentration in tadpoles exposed during embryonic
294 development. Tadpoles exposed as embryos to the high concentration had higher
295 tails than those exposed as embryos to the medium concentration (0.07 ± 0.002 ,
296 $p=0.013$).

297

298

299 *Post metamorphic toads*

300 We did not find any influence of AMPA concentration or Timing of exposure on the
301 body mass, SVL or BCI of metamorphs either upon metamorphosis or 5 days after
302 metamorphosis (Table 2, Figure 4).

303

304 *Survival*

305 We found an effect of the timing of exposure on larval mortality but no effect of the
306 concentration (Concentration $\chi^2=1.57$, $p=0.67$; Exposure: $\chi^2=6.48$, $p=0.039$, Figure 5
307 A). Mortality was higher in individuals exposed to AMPA during larval
308 development solely.

309 There were no differences in survival between groups after metamorphosis (log-rank
310 test, $\chi^2=10.9$, $p=0.3$, Figure 5B)

311

312

313 **Discussion**

314

315 Overall, our results suggest that low, environmentally-relevant concentrations of
316 AMPA influence embryonic and larval development in *Bufo spinosus* tadpoles.
317 AMPA and timing of exposure interactively affected tadpole morphology, but not
318 duration of development. Most of these effects were linked to exposure during
319 embryonic development, in accordance with results from previous studies (Cheron
320 and Brischoux 2020, Cheron et al. 2022). Yet, in the current study, we show that these
321 effects in individuals exposed during embryonic development solely were long-
322 lasting and occurred until the latest larval stages at the onset of metamorphosis.
323 Finally, we found that exposure to AMPA after hatching during the larval stage
324 increased mortality.

325

326 We found that exposure to AMPA during embryonic and larval development had
327 small, but significant effects on tadpole morphology. Specifically, we found that
328 individuals treated during a very short period of time during their larval
329 development (i.e., between GS22 and GS 25 which corresponds to ~5 days) displayed
330 smaller tail length than their counterparts from all other timing of exposure. Such
331 result indicates that exposure to AMPA just after hatching can rapidly affect growth
332 rates and relative energetic investments in different body parts. Importantly, we
333 found that these effects were transitory and were not detectable at later
334 developmental stages, suggesting that tadpoles may have compensated for these
335 altered early growth rates and/or have acclimated to exposure to AMPA. Indeed,
336 mechanisms of compensatory growth has been shown in amphibians in response to
337 different environmental stressors such as desiccation (Altwegg & Reyer, 2003),
338 density (Jasienski, 2008) and predation (Vonesh & De la Cruz, 2002), presumably
339 through variations in feeding behaviour (Broomhall and Shine, 2003). We can
340 hypothesize that similarly to these other environmental stressors, environmental
341 contamination may potentially affect several traits linked to growth in tadpoles,
342 allowing them to achieve similar body size than control individuals. Although we
343 did not quantify the amount of the food ingested during our experiment, it is
344 possible that feeding rate is one of the variables that would allow for compensation
345 in tadpoles exposed during larval stages. Future studies should investigate the
346 influence of AMPA on feeding rates, metabolism, and/or food-to-tissue conversion
347 rates in order to test for this hypothesis. Alternatively, but not exclusively, it is
348 noteworthy that amphibians can display detoxification mechanisms which allows
349 them to cope with exposure to contamination (Pašková et al., 2011). In our context, it

350 is possible that exposure to AMPA during larval development induced such
351 mechanisms that allowed these individuals to cope with water contamination at later
352 developmental stages. Whatever the mechanisms involved in these compensatory
353 responses (i.e., increased feeding behaviour, detoxification mechanisms or a
354 combination thereof), they seem to occur at a cost for developing tadpoles, as
355 survival appeared jeopardized in this group of individuals. Indeed, we found that
356 survival was lower in individuals exposed to AMPA after hatching, while no such
357 effect was detectable for those individuals exposed to AMPA during embryonic
358 development. This result dovetails relatively well with those from our previous
359 studies (Cheron et al., 2021; Cheron and Brischoux, 2020). In these studies, the
360 embryos that hatched were those that displayed elevated antioxidant defences,
361 suggesting a selective mortality of individuals less able to cope with AMPA
362 exposure. Our current results further support these findings and show that mortality
363 was higher in individuals exposed to AMPA after hatching presumably because
364 selective mortality processes linked to AMPA exposure during embryonic
365 development. Future studies are required to assess the survival costs of exposure to
366 environmental contaminants according to developmental stages in amphibians.

367

368 Interestingly, we found that individuals exposed to the highest concentration of
369 AMPA during embryonic development solely displayed altered morphologies at the
370 latest larval stages (e.g., GS 42 corresponding to the onset of actual metamorphosis).
371 Such results raise important questions regarding the possible long-lasting effects of
372 an early and temporary exposure to contamination in developing vertebrates.
373 Indeed, although these individuals have been exposed to AMPA during embryonic

374 development (i.e., ~16 days), they were raised in control conditions during the
375 whole larval development (i.e., ~74 days). The latest larval stages at which the
376 effects of earlier exposure to AMPA were detectable correspond to strong
377 morphological and physiological changes associated to metamorphosis (Beck and
378 Congdon, 2003; Brown and Cai, 2007; Wright et al., 2011). Specifically, at this stage,
379 tadpoles cease feeding as their feeding and digestive apparatus changes to shift from
380 grazing and detritivory underwater to feeding on mobile animal prey on land
381 (Hourdry et al., 1996; Vitt and Caldwell, 2014). The respiratory system also shifts
382 from branchial respiration underwater to pulmonary respiration on land (Vitt and
383 Caldwell, 2014). Finally, pattern of energy use include tail resorption that is used to
384 fuel, at least in part, morphological changes associated to metamorphosis and
385 organismal maintenance (Bouchard et al., 2016; Gonçalves et al., 2015). All of these
386 morphological changes could be affected by an early exposure to contamination, and
387 presumably associated mechanisms that induce lower survival in a contaminated
388 environment (e.g., detoxification mechanisms, altered oxidative status, selective
389 mortality, Cheron et al., 2021). Clearly, future studies are required to decipher the
390 mechanisms that underlie such long-lasting effects of early contamination in
391 developing amphibians.

392

393 Despite these different effects of exposure to AMPA according to different timing of
394 exposure and developmental stages, we did not find any significant effects of
395 exposure to AMPA on toadlets after metamorphosis. Such lack of effect may support
396 the hypothesis that compensatory mechanisms may be involved and continue to
397 occur throughout metamorphosis (see above) as well as selective mortality which

398 may have favoured individuals less susceptible to AMPA exposure. Despite this lack
399 of direct influence of our treatment on young toads, and given the long-lasting effects
400 we found for individuals exposed during embryonic development solely (see above),
401 it is plausible that long-term effects could be detectable later in life (Awkerman and
402 Raimondo, 2018). Such long-term effects should usefully be investigated by raising
403 experimental toadlets under similar conditions (i.e., common garden) until
404 adulthood.

405

406 It is also noteworthy that experimental tadpoles were raised under optimal
407 conditions with food available *ad libitum*, constant thermal conditions and absence of
408 predators and competitors. Such optimal conditions are clearly different to what is
409 observed *in natura*, where competition, predation, and/or food shortage can occur
410 (Broomhall and Shine, 2003; Goater, 1994; Jones et al., 2011). It is possible that the
411 conditions we offered to tadpoles during their growth were optimal enough for them
412 to compensate for putative effects of AMPA (Capellán and Nicieza, 2007). In keeping
413 with this idea, it is important to highlight that the toadlets from our experiment were
414 ~1.5 times larger and ~4 times heavier than wild toadlets captured at the same stage
415 (SVL=1.15±0.08 cm; Mass=0.19±0.03 g for experimental metamorphs *versus*
416 SVL=0.78±0.07 cm; Mass=0.05±0.01 g for n=16 toadlets captured in the field). These
417 strong differences suggest that tadpoles raised under natural conditions produce
418 smaller and skinnier toadlets. We can further hypothesize that under relatively
419 harsher natural conditions, the influence of anthropogenic contaminants such as
420 AMPA may be much more pronounced than under optimal experimental conditions.
421 Future studies should aim at assessing the influence of environmental contamination

422 on developing tadpoles under constraining environmental conditions by
423 manipulating food availability, competition, predation and a combination thereof
424 (Hua et al., 2017; Relyea, 2001).

425

426 **Conclusion**

427 Overall, our results show that minute concentrations of AMPA (100 to 6000 times
428 lower than the official “Predicted No Effect Concentration”, Ineris, 2013) can affect
429 development and survival of spined toads. Even though AMPA is the main
430 substance found in aquatic habitats, other xenobiotics are likely to affect the
431 development of amphibians. It seems particularly urgent to investigate such effects
432 in the context of the multiple threats that are known to affect amphibian populations
433 worldwide (IUCN, 2020).

434

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440

441

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771

772 **Captions to figures**

773

774 **Figure 1:** Experimental design used to study the effects of both AMPA concentrations
775 and timing of exposure on *Bufo spinosus* tadpoles. Colour represents concentration
776 (Blue = Control, light orange = Low, range = Medium, dark orange =High). Line
777 shape represent Exposure phase (Solid : Entire development, long dashed : Larval
778 exposure only, dashed : Embryonic exposure only)

779

780 **Figure 2:** Mean Δ development duration days between treatment and control
781 throughout ontogeny (GS25, GS30, GS41, GS42, GS46,(Cheron et al., 2021) according
782 to the timing of exposure to AMPA (control, embryonic development solely, larval
783 development solely or total exposure) and AMPA concentrations (Blue = Control,
784 light orange = Low, range = Medium, dark orange =High) in *Bufo spinosus* tadpoles.
785 Data represent mean \pm SE.).

786

787 **Figure 3:** PCA scores (first axis : 96.61 % variability) of the morphological variables
788 (Total length, tail length, body length, body length, body width, tail height, body
789 height) throughout ontogeny (GS25, GS30, GS41, GS42) according to the timing of
790 exposure to AMPA (control, embryonic development solely, larval development
791 solely or total exposure) and AMPA concentrations (Blue = Control, light orange =
792 Low, range = Medium, dark orange =High) in *Bufo spinosus* tadpoles. The top and
793 bottom of the boxes represent the first and last quartiles, the horizontal line within
794 the box represents the median, the whiskers represent the fifth and 95th percentiles,
795 and the circles represent outliers. Dots represent the distribution of all individuals.

796 **Figure 4:** Mass at metamorphosis (A, g) and snout-vent length at metamorphosis (B,
797 cm) according to the timing of exposure to AMPA (control, embryonic development
798 solely, larval development solely or total exposure) and AMPA concentrations (Blue
799 = Control, light orange = Low, range = Medium, dark orange =High) in *Bufo spinosus*
800 tadpoles. Data represent mean \pm SE.

801

802 **Figure 5.** Larval mortality (A) relative to the timing of exposure to AMPA. Larval
803 exposure yielded significantly higher mortality. Colors represent different
804 concentrations (i.e Blue = Control, light orange = Low, range = Medium, dark orange
805 =High). Survival during 130 days post metamorphosis according to the timing of
806 exposure to AMPA (control, embryonic development solely, larval development
807 solely or total exposure) and AMPA concentrations (Blue = Control, light orange =
808 Low, range = Medium, dark orange =High) in *Bufo spinosus* tadpoles.

809

810

811 **Table 1** Concentrations ($\mu\text{g.l}^{-1}$) measured in the experimental tanks (Mean \pm SD)

Treatment	AMPA concentrations
Control	0
Low	0.07 \pm 0.01
Medium	0.32 \pm 0.052
High	3.57 \pm 0.153

812

813

814

815 **Table 2:** Anova output of linear mixed models and generalized mixed models.
816 "Concentration" represents AMPA concentration with 4 levels (Control, Low, Medium,
817 High). "Exposure" represents timing of exposure with 4 levels (Control, Embryonic exposure,
818 Larval exposure, total exposure). "Stage" represents all studied Gosner stages with 4 levels
819 for Morphology (GS25, GS30, GS41, GS42).

Variable	Model						
	Main effect			Interaction			
	Concentration	Exposure	Stage	Concentration X Stage	Exposure X Stage	Concentration X Exposure	Concentration X Exposure X Stage
Developmental related traits							
<i>Time from hatching to metamorphosis</i>	F _(3, 114) =0.707, p=0.550	F _(2, 114) =0.295, p=0.767				F _(4, 114) =0.407, p=0.803	
<i>Time between stages</i>	F _(2, 653) =0.00, p=1.00	F _(2, 653) =0.00, p=1.00	F_(4, 536)=522.78, p<0.001	F _(12, 534) =0.576, p=0.862	F _(8, 537) =0.621, p=0.760	F _(4, 653) =0.00, p=1.00	F _(16, 538) =0.859, p=0.618
Morphology							
<i>PCA scores</i>	F _(3, 500) =0.12, p=0.945	F _(2, 500) =0.048, p=0.953	F_(3, 396)=581.74, p<0.001	F _(9, 394) =1.601, p=0.111	F_(4, 397)=2.33, p=0.032	F _(4, 500) =0.187, p=0.935	F _(12, 401) =1.694, p=0.066
<i>Total length</i>	F _(3, 513) =0.03, p=0.992	F _(2, 513) =0.272, p=0.762	F_(3, 396)=647.96, p<0.001	F_(9, 394)=2.069, p=0.032	F_(6, 399)=2.276, p=0.036	F _(4, 513) =0.08, p=0.988	F _(12, 403) =1.677, p=0.069
<i>Body length</i>	F _(3, 513) =0.424, p=0.734	F _(2, 513) =0.163, p=0.850	F_(3, 396)=533.07, p<0.001	F _(9, 394) =1.636, p=0.078	F _(6, 399) =0.648, p=0.737	F _(4, 513) =0.259, p=0.904	F _(12, 403) =1.122, p=0.330
<i>Body height</i>	F _(3, 513) =0.172, p=0.916	F _(2, 513) =0.47, p=0.954	F_(3, 396)=239.22, p<0.001	F _(9, 394) =0.962, p=0.472	F_(6, 399)=2.123, p=0.050	F _(4, 513) =0.078, p=0.989	F _(12, 403) =0.801, p=0.649
<i>Body width</i>	F _(3, 513) =0.018, p=0.997	F _(2, 513) =0.059, p=0.946	F_(3, 397)=255.16, p<0.001	F _(9, 395) =0.289, p=0.978	F _(6, 399) =1.731, p=0.112	F _(4, 513) =0.477, p=0.753	F _(12, 403) =1.091, p=0.366
<i>Tail length</i>	F _(3, 513) =0.002, p=1.00	F _(2, 513) =0.047, p=0.954	F_(3, 396)=32.85, p<0.001	F_(9, 394)=2.700, p=0.005	F_(6, 399)=2.762, p=0.012	F _(4, 513) =0.004, p=0.999	F _(12, 403) =1.796, p=0.068
<i>Tail height</i>	F _(3, 513) =0.328, p=0.805	F _(2, 513) =0.038, p=0.963	F_(3, 396)=244.72, p<0.001	F_(9, 394)=2.81, p=0.003	F _(6, 399) =1.562, p=0.157	F _(4, 513) =0.131, p=0.971	F_(12, 403)=2.24, p=0.009
<i>Toadlets mass J0</i>	F _(2, 114) =0.130, p=0.942	F _(2, 114) =1.162, p=0.317				F _(4, 114) =0.582, p=0.677	
<i>Toadlets SVL J0</i>	F _(3, 114) =1.635, p=0.185	F _(2, 114) =0.121, p=0.886				F _(4, 114) =0.339, p=0.851	
<i>Toadlets BCI J0</i>	F _(3, 104) =0.127, p=0.944	F _(2, 114) =1.165, p=0.316				F _(4, 114) =0.407, p=0.804	

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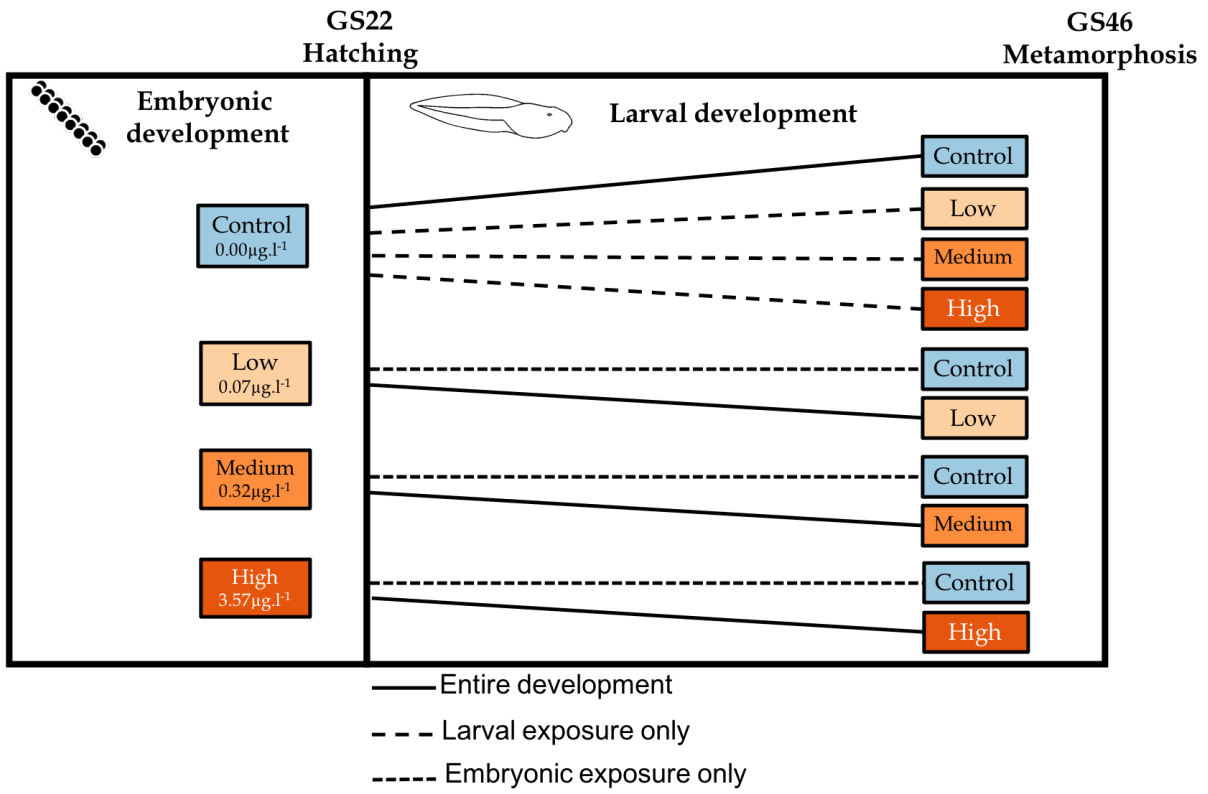
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823 Figures

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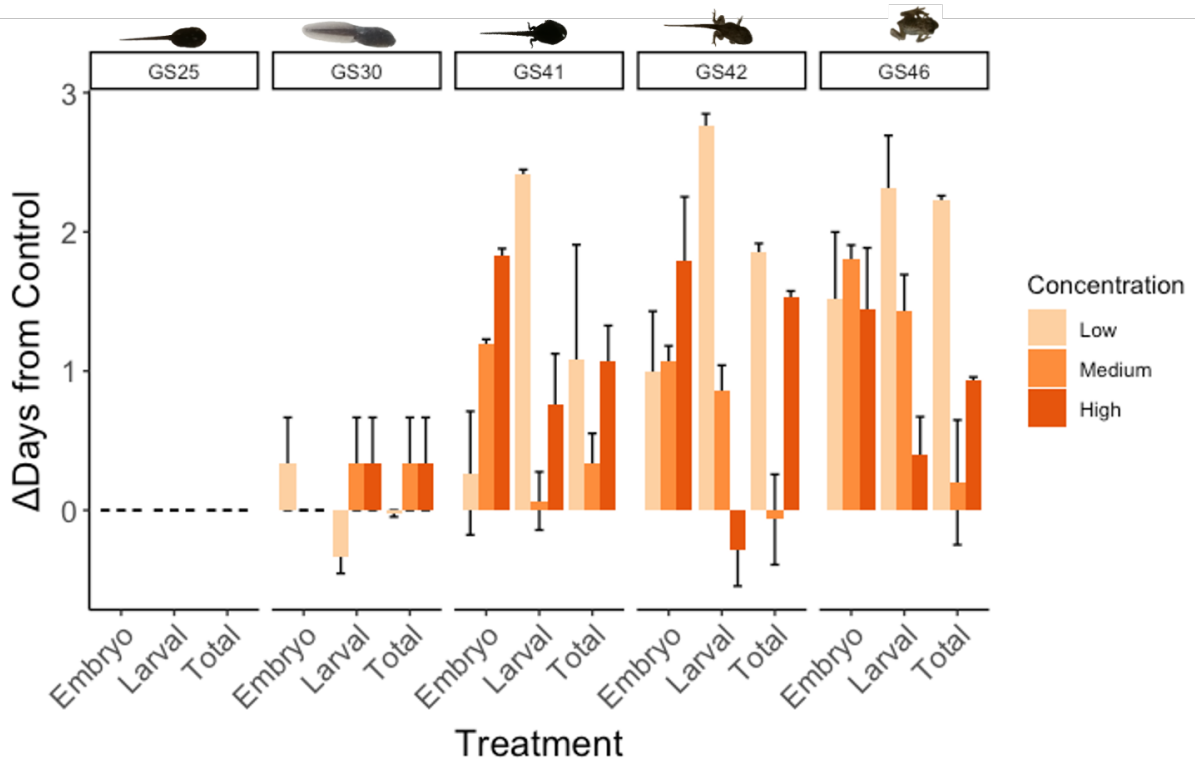
825 Figure 1

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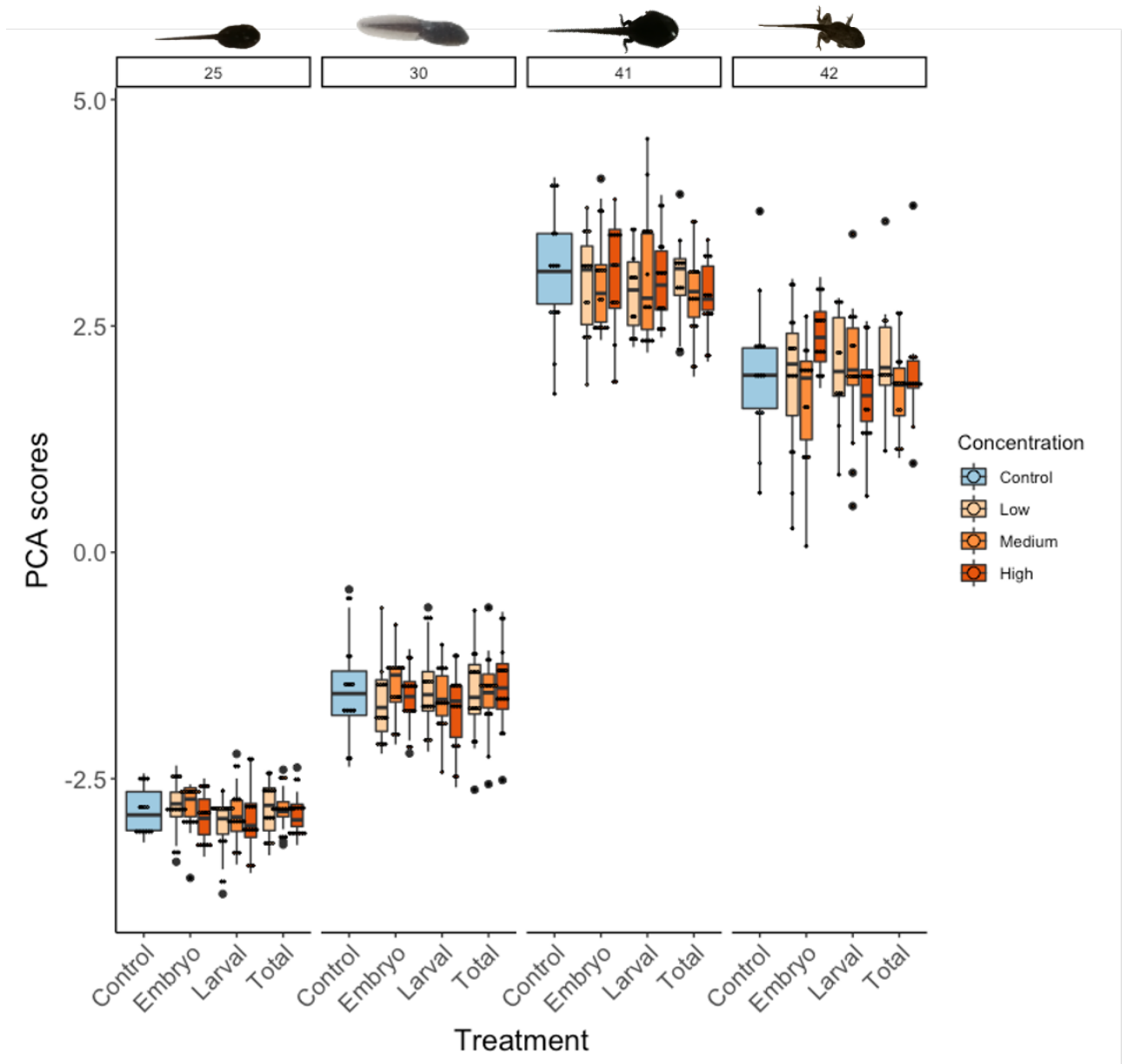
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828 **Figure 2**



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831 **Figure 3**



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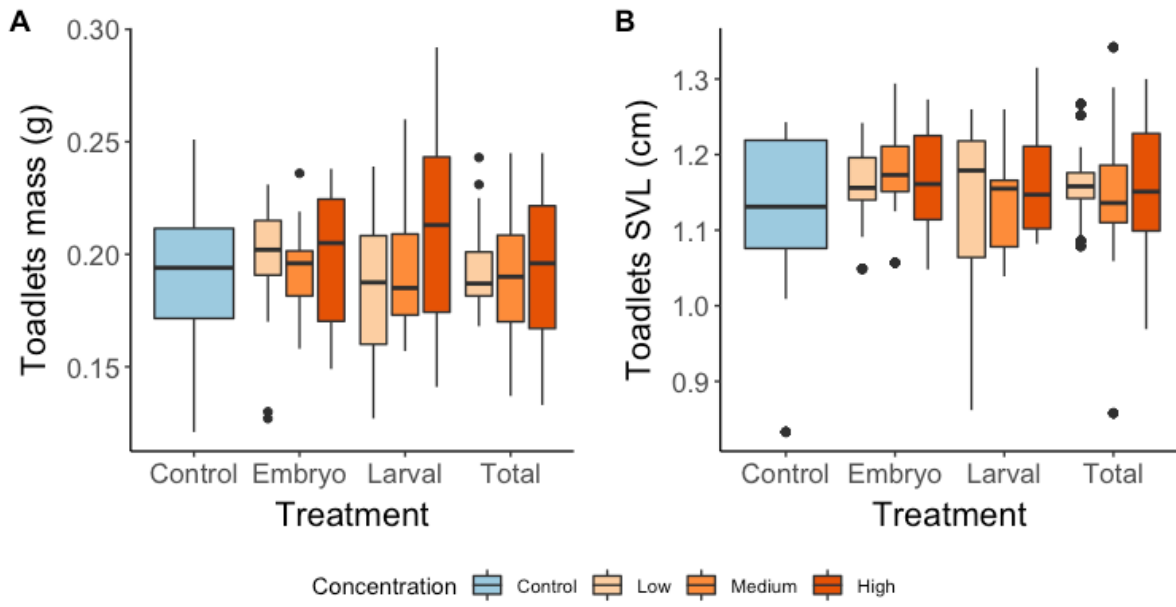
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839 **Figure 4**



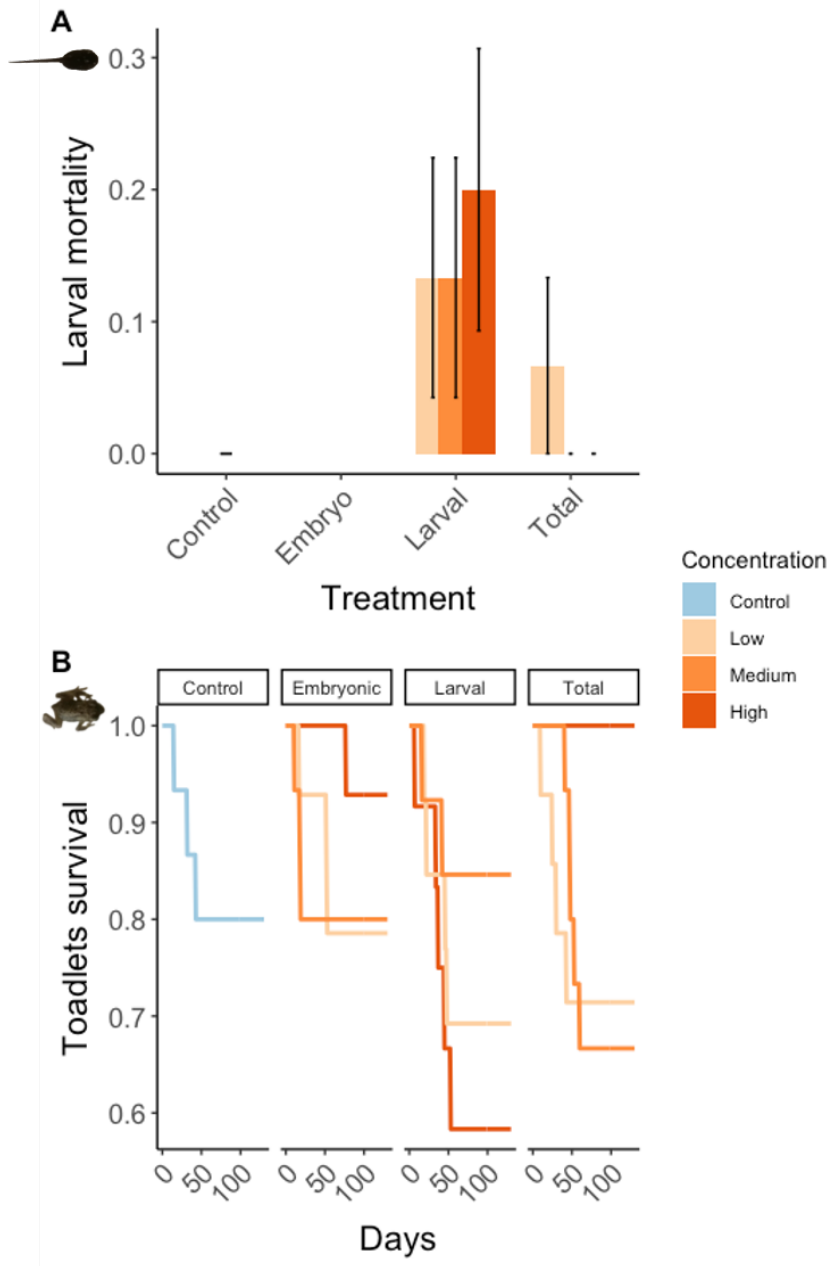
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844 **Figure 5**
845



846

Article IX

Marion Cheron, Léa Raelison, Akiko Kato, Yan Ropert-Coudert, Xavier Meyer, Andrew J. J. Macintosh & François Brischoux

Exposure, but not timing of exposure, to a sulfonyleurea herbicide alters larval development and behaviour in an amphibian species

In preparation for **Environmental Pollution**



1 **Exposure, but not timing of exposure, to a sulfonylurea herbicide**
2 **alters larval development and behaviour in an amphibian species**

3

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17

18 Manuscript in consideration for *Environmental Pollution*

19

20 **Abstract**

21

22 Environmental contamination is one of the major causes of biodiversity loss. Wetlands are
23 particularly susceptible to contamination and species inhabiting these habitats are subjected
24 to pollutants during sensitive phases of development. In this study, tadpoles of a widespread
25 amphibian, the spined toad (*Bufo spinosus*), were exposed to environmentally-relevant
26 concentrations of nicosulfuron, a sulfonylurea herbicide, during different phases of
27 development. Tadpoles were exposed during embryonic or larval development, or
28 throughout both phases, and we quantified development duration, morphological traits and
29 behavioural features as responses to exposure. Developing tadpoles exposed to nicosulfuron
30 were larger, but with smaller body, and had shorter but wider tail muscles. They were also
31 more active and swam faster than control tadpoles and showed diverging patterns of
32 behavioural complexity. We showed that higher concentrations had greater effects on
33 individuals than lower concentrations, but the timing of nicosulfuron exposure did not
34 influence the metrics studied; i.e., exposure to nicosulfuron triggered similar effects
35 irrespective of the developmental stages at which exposure occurred. These results further
36 indicate that transient exposure (e.g., during embryonic development) can induce long-
37 lasting effects throughout larval development to metamorphosis. Our study confirms that
38 contaminants at environmental concentrations can have strong consequences on non-target
39 organisms. Our results emphasize the need for regulation agencies and policy makers to
40 consider sublethal concentrations of sulfonylurea herbicides, such as nicosulfuron, as a
41 minimum threshold in their recommendations.

42

43 Key-words: herbicide, amphibian, behaviour, DFA, morphology

44

45 **Introduction**

46

47 Environmental contamination is now recognized as a major threat to biodiversity (Bernhardt
48 et al., 2017; Kendall et al., 2016). Although the sources and compounds involved in
49 environmental contamination are diverse (Prakash et al., 2018), modern agricultural
50 activities induce the use of various agrochemicals (Lushchak et al., 2018) and are thus
51 responsible for the spread of these compounds in the environment (Vitousek et al., 1997). For
52 instance, compounds that aim to protect crops from insect pests, fungi or weeds (i.e.,
53 pesticides) are widely used (Prakash et al., 2018), but can enter non-target environments
54 (Backhaus et al., 2012; Battaglin et al., 2014) where they can negatively affect non-target
55 species (Hasenbein et al., 2017; Thiour-Mauprivez et al., 2019; Ware, 1980).

56

57 The effects of pesticides on wildlife are being increasingly documented but most studies
58 focus on legacy pesticides that have been used for decades (e.g., glyphosate, Annett et al.,
59 2014; Gill et al., 2018), some of which are now banned because of their infamous effects on
60 wildlife and human health (e.g., DDT, neonicotinoids, atrazine, Lee-Jenkins & Robinson,
61 2018; Lenkowski et al., 2010). Novel replacement compounds are regularly being developed
62 and used, both to counteract adaptive resistance of pests to existing compounds and in
63 response to growing societal concerns (Bell, 2018; Deblonde et al., 2011; Kendall et al., 2016).
64 Yet, in most cases, these “emerging” pesticides (*sensu* Deblonde et al., 2011) are lacking
65 regulatory status and/or detailed assessments of their effects on wildlife and human health
66 (Bunke et al., 2019; Geissen et al., 2015).

67

68 Among the various new pesticides that are regularly employed, the use of sulfonylurea
69 herbicides has increased greatly (de Lafontaine et al., 2014; Food and Agriculture
70 Organization, 2018). This group of herbicides is used to control weeds in a large variety of

71 crops, including wheat, oat, soybean and maize. Despite their increasing importance in
72 agriculture (de Lafontaine et al., 2014), detailed studies on their potential effects on non-
73 target species at environmentally-relevant concentrations are comparatively scarce.
74 Although sulfonylurea herbicides are usually considered to display low toxicity to non-
75 target species (Centers for Disease Control and Prevention, 2017; Kamrin, 1997), there is
76 growing evidence for their potential role in inducing abnormal development, teratogenic
77 effects, biochemical disruptions and behavioural alterations in various organisms (Park et al.,
78 2020; Pretto et al., 2011; Saglio et al., 2001).

79

80 Toxicology studies employ metrics, such as median lethal doses (LD50), to assess the short-
81 term toxicity of agrochemicals and are traditionally used by regulation agencies and policy
82 makers to determine standards of use (i.e., Predicted no-effect concentration, (EFSA, 2007)
83 and/or tolerances for residues in food (Hotchkiss, 1992)). Yet, deleterious effects of
84 pesticides on wildlife and human health occur at low concentrations through subtle
85 dysregulations of developmental and physiological mechanisms (i.e., sublethal effects,
86 Beiras, 2018). Such sublethal effects often occur at environmentally-relevant concentrations
87 that are usually much lower than the concentrations tested in acute lethality tests (Hasenbein
88 et al., 2017). These sublethal effects can arise via a wide range of mechanisms, such as
89 genotoxicity (Guilherme et al., 2014, 2010), endocrine disruption (Vandenberg et al., 2012),
90 cellular damage (Lushchak, 2016; Pašková et al., 2011), altered development (Baker et al.,
91 2013; Burraco and Gomez-Mestre, 2016), morphological change (Baier et al., 2016; Cheron
92 and Brischoux, 2020; Da Silva et al., 2020; Rutkoski et al., 2020) or behaviour modification
93 (Browne and Moore, 2014; Brunelli et al., 2009; Zhang et al., 2020); all of which can ultimately
94 influence population viability (Hamilton et al., 2016).

95

96 Among the various sublethal effects, alterations of behaviour are ecologically relevant
97 indicators of physiological stress (Ford et al., 2021; Scott and Sloman, 2004). Importantly,
98 behavioural changes linked to agrochemical exposure can be useful indicators insofar as they
99 provide earlier warnings than standard test methods (Scott and Sloman, 2004). In this
100 context, sulfonylurea herbicides have been shown to disrupt acetylcholinesterase (AChE)
101 activity and oxidative status markers in fish, which in turn affected their behavioural
102 responses (Bretaud et al., 2000; Saglio, Olsén & Bre, 2001).

103

104 Amphibians are well suited to assess sublethal effects of environmental contamination
105 (DeGarady and Halbrook, 2006; Sansiñena et al., 2018), as they are particularly sensitive to
106 environmental contamination given their complex biphasic life cycle. In most species, adults
107 are relatively terrestrial but reproduction occurs in aquatic environments where sensitive
108 early-life stages (embryos and larvae) develop. In temperate areas, the timing of
109 reproduction in aquatic environments coincides with agrochemical application (Berger et al.,
110 2013). Taking into account the effects of environmental contamination on aquatic stages is
111 especially relevant as environmental conditions (including contamination) experienced
112 during early-life stages can influence fitness later in life (Greulich and Pflugmacher, 2003;
113 Knutie et al., 2017). Finally, sulfonylurea herbicides can diffuse through the jelly matrix
114 protecting the eggs, which leads to direct exposure during embryonic development
115 (Greulich et al., 2003).

116

117 Here, we aimed to assess sublethal effects of a scarcely studied sulfonylurea herbicide,
118 nicosulfuron, on the development of a widespread anuran known to inhabit agricultural
119 landscapes (*Bufo spinosus*). Nicosulfuron is detected in surface waters in North America and
120 Europe (Battaglin et al., 2009, de Lafontaine et al., 2014) in low concentrations yet only few
121 studies determined the potential effects on wildlife. Hence, we exposed eggs and tadpoles to

122 environmentally relevant concentrations of nicosulfuron at different phases of their
123 development (embryonic development only, larval development only or throughout the
124 whole developmental period). This approach allowed us to test not only for the effects of
125 exposure to nicosulfuron but also the relative susceptibility of different developmental
126 phases (embryonic *versus* larval). We assessed developmental alterations using relevant
127 morphological (body size and shape), developmental (duration of developmental stages) and
128 behavioural traits (Cheron et al., 2021). We predicted that (i) tadpoles exposed at the
129 embryonic stage would show altered development throughout ontogeny that would last
130 until metamorphosis. Indeed, nicosulfuron is known to alter embryonic development
131 through perturbation of oxidative status and teratogenic effects (Cheron et al., 2022).
132 Moreover, we predicted that (ii) exposure would lead to behavioural alterations such as
133 increased activity due to erratic swimming, as has been shown previously in species exposed
134 to this herbicide (Saglio, Olsén, Bre, 2001).
135

136 **Materials and methods**

137

138 *Tadpole sampling and maintenance*

139 Egg spawn of *Bufo spinosus* (N=10) were collected upon laying from a pond near the study
140 site (Centre d'Etudes Biologiques de Chizé, France) in February 2019. We randomly selected
141 3 segments of 30 eggs (i.e. 90/3 treatments, see below) from each clutch and kept them for
142 the experiment. The remaining eggs (i.e., 3000-5000) were released at the site of capture. The
143 segments of eggs in each spawn were placed in 2L glass tanks filled with dechlorinated tap
144 water.

145

146 Each segment of eggs was assigned an experimental condition (Fig. 1). One segment was
147 clear from contamination (Control), one segment was exposed to low concentrations of
148 nicosulfuron (see below) and the remaining segment was exposed to high concentrations of
149 nicosulfuron (see below). This step is referred to as "Embryonic exposure" hereafter (Fig. 1).
150 We determined the stage of development following Gosner (1960). Hatching occurred at
151 Gosner stage 22 (hereafter GS 22) after 12.98 ± 0.90 days.

152

153 Upon hatching, individuals of each segment were re-allocated to new experimental groups
154 (Fig. 1). For each clutch, six individuals from the initial control groups were randomly
155 selected and individually placed in a 2L glass tank. Two remained in control conditions (no
156 contaminant), two were assigned to low contamination, and two were allocated to high
157 contamination (Fig. 1) until the end of the development (metamorphosis).

158 We followed the same procedures for the individuals that were initially under low and high
159 concentration treatments, but we randomly selected four tadpoles from each clutch and
160 reallocated them as follow: two tadpoles were assigned to control conditions and the

161 remaining two individuals were maintained in their initial conditions (either low or high
162 concentrations, Fig. 1). The remaining tadpoles were released in the pond of capture.

163

164 This design allowed us to monitor the development of each tadpole individually and to
165 repeatedly assess morphology and behaviour for each individual during the whole period of
166 larval development. Importantly, maintaining the tadpoles individually in aquaria does not
167 impose undue stress (Bókonyi et al., 2021) and represents the best method available for
168 individual monitoring while avoiding density effects on development (Melvin and
169 Houlahan, 2014). All the experiments took place in a thermally controlled room with the
170 temperature set at 17°C (both air and water). Photoperiod was controlled in a 12:12h cycle
171 using LEDs placed evenly across the room. Water was changed once a week. Tadpoles were
172 fed *ad libitum* with organic spinach.

173

174 *Treatment concentrations and chemical solutions*

175 Near our study site, mean nicosulfuron concentrations range from 0.005 µg l⁻¹ to
176 0.920 µg l⁻¹ (mean: 0.043 ± 0.076 µg l⁻¹) and maximum values range from 0.005 µg l⁻¹ to
177 10.72 µg l⁻¹ (mean of maximum concentrations: 0.142 ± 0.076 µg l⁻¹, data from Agence de
178 l'Eau Loire-Bretagne). Hence, to mimic the range of concentrations found in the wild, we
179 produced two treatments, low and high concentration (Table 1). Stock solutions (0.1 g.l⁻¹) of
180 nicosulfuron were obtained by dissolving commercial crystalline powder in dechlorinated
181 tap water. These stock solutions were further diluted with dechlorinated tap water to reach
182 each of the targeted concentrations (Table 1). We produced three additional water samples
183 for each treatment to perform analytical verifications of each concentration. Analytical
184 verifications of the concentration were performed in triplicate by an independent accredited
185 analytical laboratory (QUALYSE, Champdeniers-Saint-Denis, France). Determination of
186 nicosulfuron in water samples was assessed using liquid chromatography-mass

187 spectrometry (LC/MS/MS) by direct injection with a limit of quantification of 0.1 µg.l⁻¹.

188 Overall, these verifications showed that actual concentrations were similar to concentrations
189 found in aquatic environments and that the differences between low and high concentration
190 treatments were significant (Table 1).

191

192 For clarity, we refer to the treatments as “Control”, “Low” and “High” hereafter. *A posteriori*
193 investigation confirmed that these concentrations were actually sublethal, as we did not
194 observe differences in survival rates between treatments ($\chi^2=1.7$, $df=6$, $p=0.9$).

195

196 *Developmental stages and morphological measurements*

197 To classify morphological and behavioural features, we selected Gosner stages 25, 30, 37, 41
198 and 42 (hereafter GS 25, GS 30, GS 37, GS 41 and GS 42, respectively) as those stages
199 correspond to specific changes in the morphology of tadpoles, and coincide with significant
200 changes in activity, locomotion and behaviour (Cheron et al., 2021). For each stage, we
201 measured morphological features following Watters et al. (2016) : total length (from the tip of
202 the snout to the tip of the tail), body length, width and height, tail length and height, tail
203 muscle length and height (at the base of the tail)(Figure S1).

204

205 Each tadpole was put in a petri dish with the water from its own tank and pictures were
206 taken using a fixed camera placed above the petri dish. Morphological measurements were
207 performed with the software ImageJ (Schneider et al., 2012). Upon metamorphosis,
208 individuals were weighed every day and were photographed every 5 days to measure their
209 snout-vent length (SVL). Residuals of linear regression between SVL and weight of toadlets
210 were used to calculate body-condition index (BCI).

211

212 *Activity and locomotion*

213 Behavioural trials were measured following Cheron et al. (2021). Briefly, tadpoles were
214 placed in a Petri dish (diameter 13.5cm, water level 1.5cm) at each Gosner stages of interest.
215 Following 15 minutes of acclimation (Dayton et al., 2005), we recorded their behaviour
216 during 30 minutes using a camera (GoPro HERO (2018), GoPro, Inc., San Mateo, CA, USA)
217 placed above the arena. Videos were analysed with the software ToxTrac (Rodriguez et al.,
218 2018), which allows us to extract the frame-to-frame successive positions of a tadpole
219 (defined from the X and Y calibrated coordinates within the petri dish). Using these
220 successive coordinates and R scripts, we determined the following variables: number of
221 activity bouts, total duration of activity, total distance travelled, mean and maximum
222 swimming speeds ($\text{mm}\cdot\text{s}^{-1}$). To minimize observer bias, blinded methods were used when all
223 behavioural data were recorded and analysed. Further details can be found in Cheron et al.
224 (2021).

225

226 *Behavioural complexity*

227 Temporal complexity of tadpole activity sequences were assessed using Detrended
228 Fluctuation Analysis (DFA, Peng et al., 1995; Peng et al., 1994), adapted to amphibian larvae
229 following Cheron et al. (2021). This approach defines animal time-series as ranging from
230 stochastic (uncorrelated) behaviour to deterministic (long-range autocorrelated) behaviour,
231 across measurement scales (see MacIntosh, 2014). The degree of complexity depends on
232 internal (e.g., stress, disease: Alados et al., 1996; Cottin et al., 2014; MacIntosh et al., 2011) and
233 external conditions (e.g., environment : MacIntosh et al., 2011; Meyer et al., 2017) faced by an
234 individual. Briefly, we extracted bouts of activity and inactivity that were transformed into a
235 time series in which -1 corresponded to inactivity and 1 corresponded to activity. Time series
236 were then integrated (through cumulative summation) to calculate average fluctuation
237 across a range of increasing time-window sizes, allowing calculation of the scaling exponent
238 for each sequence (α DFA). We calculated α DFA using the package 'fractal' (Constantine &

239 Percival, 2014) in R statistical software v.4.0.0 (R Core Team, 2020). Thorough details of this
240 analytical approach applied to animal behavior, including DFA calculation, validation of
241 scaling and its relationship to other fractal dimension estimates and illustrations can be
242 found in MacIntosh et al. (2013) and Burgunder et al. (2018). Further details on the
243 calculation procedures and validation of this approach in tadpoles can be found in Cheron et
244 al. (2021).

245

246 *Statistical Analyses*

247

248 All statistical analyses were conducted with R statistical software v.4.0.0 (R Core Team, 2020)
249 and RStudio v 1.2.5042 (RStudio, Inc.). All residuals were tested for homogeneity of variance
250 and normality with the Barlett's test and the Shapiro-Wilks test, respectively. We also
251 checked the normality of the residuals using diagnostic plots. Overall, clutch identity always
252 significantly influenced measured parameters (all $p < 0.001$), and thus we kept clutch identity
253 as a random factor in all analyses. Because we assessed locomotion and behaviour in the
254 same individuals throughout development, we nested tadpole identity in their respective
255 clutch.

256

257 For each feature, we built a full model (LMER for gaussian data, GLMER for non-gaussian
258 data) with the interaction between "Concentration", "Exposure" and "Stage". "Concentration"
259 represented the concentration to which tadpoles were exposed, either Control, Low or High
260 (Fig. 1). "Exposure" represented the timing of exposure, either embryonic, larval or
261 throughout development (Fig. 1). "Stage" represented each studied stage, GS25, GS30, GS37,
262 GS41 or GS42 (Cheron et al., 2021). In all cases, the model was "Variable~
263 Concentration*Exposure*Stage+ (1 | Clutch/id)". We performed stepwise regression using
264 the "stats" packages to choose the best model by AIC for each feature. When all predictors

265 were selected, we fitted either a linear mixed model or a generalized mixed model. We
266 performed effect size tests with statistical power analysis to assess the magnitude of the
267 difference between treatments (Cohen, 2013). The magnitude of the main effect was
268 measured using partial η^2 . Effect sizes were considered negligible when $\eta^2 < 0.01$, small
269 when η^2 between 0.01 and 0.06, medium when η^2 between 0.06 and 0.14 and large when
270 $\eta^2 > 0.14$ as suggested by Cohen (2013).

271
272 First, we fitted LMER models to analyze differences in morphological traits using stepwise
273 regression (package "lme4", Bates et al., 2015). We included morphological traits as
274 dependent variables and predictors variables same as above. Tukey's post hoc ("multcomp"
275 package) tests were carried out to analyze the differences. Finally, to investigate whether
276 behavioural traits were correlated, we calculated Pearson correlation coefficients using the
277 cor.test function in the corr.plot package (Wei and Simko, 2017) (Table S1).

278
279 Second, we fitted LMER to analyze differences in behavioural traits (i.e., total activity time,
280 total distance, swimming speed (maximum, mean, relative) and scaling component (α DFA)
281 across development stages. We fitted GLMER using the package "lme4" (Bates et al., 2015) to
282 analyze the number of activity bouts, which followed a Poisson distribution. Because
283 ToxTrac did not successfully detect some individuals, we retained in our analyses only the
284 individuals for which we recorded 30 consecutive minutes of behavioural sequences, i.e. on
285 N=132 tadpoles at GS30, N=122 tadpoles at GS37, N=112 tadpoles at GS41, N=103 tadpoles
286 at GS42.

287

288 **Results**

289

290 *Effect of Nicosulfuron on developmental traits*

291 Developmental stage was always a significant determinant of the variables we analyzed (all
292 $p < 0.001$, Table 2). We did not find any effect of nicosulfuron on development duration,
293 neither across the entire development period ($F_{6,72} = 1.12$, $p = 0.358$) nor during each stage
294 ($F_{6,72} = 0.568$, $p = 0.841$).

295

296 However, concentrations of nicosulfuron did influence the total length of tadpoles, both
297 directly (Table 2, $\eta_p^2 = 0.03$) and through an interaction with stage (Table 2, $\eta_p^2 = 0.05$, Fig 2).
298 Significant differences were found only at stage GS42, with individuals exposed to high
299 concentrations being longer than those exposed to low concentrations (0.140 ± 0.036 ,
300 $p < 0.001$).

301

302 Concentration of nicosulfuron significantly influenced body length of tadpoles, both directly
303 (Table 2, $\eta_p^2 = 0.04$) and through an interaction with stage (Table 2, $\eta_p^2 = 0.04$, Fig 2).

304 Differences were found only at stage GS42, with Control individuals having marginally
305 longer bodies than those exposed to Low concentrations (0.051 ± 0.022 , $p = 0.058$) and
306 individuals exposed to High concentrations having significantly longer bodies than those
307 exposed to Low concentrations (0.045 ± 0.014 , $p = 0.005$).

308

309 Nicosulfuron concentration significantly influenced tail muscle length, again both directly
310 (Table 2, $\eta_p^2 = 0.03$) and through an interaction with stage (Table 2, $\eta_p^2 = 0.06$, Fig 2).

311 Differences were found only at stage GS42, where Control tadpoles had marginally longer
312 tail muscles than those exposed to Low concentrations (0.173 ± 0.051 , $p = 0.002$) and tadpoles
313 exposed to High concentrations had significantly longer tail muscles than those exposed to
314 Low concentrations (0.133 ± 0.032 , $p < 0.001$).

315

316 Nicosulfuron concentration influenced tail muscle height. However, in this case there was no
317 significant main effect of Concentration (Table 2, $\eta_p^2=0.02$), only a significant interaction
318 between Concentration and stage (Table 2, $\eta_p^2=0.04$, Fig 2). We found differences at GS30,
319 where Low tadpoles had marginally shorter tail muscle than those of the Control ($-0.082 \pm$
320 0.013 , $p=0.071$) and the High tadpoles (-0.019 ± 0.001 , $p=0.080$). At GS37, Control tadpoles
321 had wider tail muscle than Low tadpoles (-0.037 ± 0.013 , $p=0.014$). At GS42, Control tadpoles
322 had marginally longer tail muscle than those exposed to Low ($p=0.002$) and High ($p<0.001$)
323 concentrations.

324

325 All other morphological traits of tadpoles (tail length and height, body width and height)
326 were not influenced by treatment or exposure, but by developmental stages only (Table 2).

327

328 Finally, we found a marginal effect of nicosulfuron concentrations on body mass ($\eta^2=0.08$,
329 Table 2) and body condition of metamorphic individuals ($\eta^2=0.06$, Table 2, Fig.3), but not on
330 body size (SVL, Table 2). For body mass, metamorphic individuals from the High group
331 were heavier than those exposed to Low concentrations (0.015 ± 0.01 , $p=0.042$). The
332 interaction between Exposure and Concentration was not significant for body mass, SVL or
333 body condition (Table 2).

334

335 *Effect of Nicosulfuron on behavioural traits*

336 We did not find any relationship between total activity time and exposure. However,
337 concentrations of nicosulfuron did significantly influence total activity time of tadpoles
338 (Table 2, $\eta_p^2=0.08$). The interaction between concentration and stage was only marginally
339 significant (Table 2, $\eta_p^2=0.03$, Fig 4). At GS37, we found that High tadpoles had higher
340 activity time than individuals exposed to Low concentrations (107.1 ± 37.6 , $p=0.013$). At GS41,

341 Control tadpoles had lower activity time than tadpoles exposed to High concentration (-
342 156.8 ± 59.3 , $p=0.023$).

343

344 Mean swimming speed was influenced by nicosulfuron treatment, with significant main
345 effects of both Concentration (Table 2, $\eta_p^2=0.05$) and Exposure (Table 2, $\eta_p^2=0.05$).

346 Furthermore, the interaction between exposure and stage was also significant (Table 2,
347 $\eta_p^2=0.04$, Fig 4). Conversely, neither Concentration nor Exposure influenced max speed,
348 which instead was solely affected by developmental stage ($p<0.001$, Table 2).

349

350 Nicosulfuron concentration influenced the total distance moved (Table 2, $\eta_p^2=0.06$).

351 However, the interaction between concentration and stage was not significant (Table 2, Fig
352 4). Nicosulfuron concentration influenced the total distance moved, with tadpoles exposed to
353 high concentrations moving greater distances than those exposed to low concentrations and
354 those in the control group.

355

356 Scaling exponents were influenced by Nicosulfuron treatment through an interaction with
357 stage (Table 2, $\eta_p^2=0.11$, Fig 4D). There was no significant main effect of Concentration itself
358 (Table 2, $\eta_p^2=0.01$). At GS37, control tadpoles showed greater behavioural complexity (lower
359 α DFA values) than tadpoles exposed to low concentration (-0.052 ± 0.015 , $p=0.002$) or high
360 concentration (-0.070 ± 0.015 , $p<0.001$). At GS41, on the contrary, control tadpoles showed
361 lower behavioural complexity (higher α DFA values) than tadpoles exposed to low
362 concentration (0.065 ± 0.016 , $p<0.001$) or high concentration (0.063 ± 0.016 , $p<0.001$).

363

364 Finally, the number of activity bouts performed by tadpoles was influenced by nicosulfuron
365 treatment with a main effect of Exposure ($X^2=11.93$, $p=0.003$) as well as an interaction
366 between Concentration, Exposure and stage ($X^2=3959.81$, $p<0.001$).

367

368 **Discussion**

369

370 Overall, we found that exposure to environmentally relevant concentrations of nicosulfuron
371 altered several morphological and behavioural traits of developing tadpoles. The most
372 salient result of our study is the lack of effect of the timing of exposure as compared to the
373 effects of the concentrations tested. Such results suggest that exposure to nicosulfuron can
374 trigger similar effects irrespective of the developmental stage at which exposure occurs (e.g.,
375 embryonic development solely, larval development solely, or both). This indicates that the
376 mechanisms through which nicosulfuron alters development are similar between embryos
377 and larvae. More importantly, temporally restricted exposure (e.g., embryonic development
378 solely) can induce long-lasting effects throughout larval development up to metamorphosis.

379

380 The environmentally relevant concentrations that we tested were sublethal, as they did not
381 induce elevated mortality and did not alter developmental durations. Yet, these
382 concentrations influenced tadpole morphology at pivotal developmental stages between the
383 end of somatic growth and the onset of actual metamorphosis (Cheron et al., 2021). At these
384 stages, individuals from the control group had larger bodies and had longer and wider tail
385 muscles. Similarly, these morphological effects were dependent on the concentration tested,
386 with individuals from the lower concentration showing altered morphology compared to
387 individuals from the higher concentration. This suggests that exposure to nicosulfuron
388 influenced the patterns of growth of – and thus energetic investments in – different body
389 parts (body *versus* tail), and notably influenced tail musculature. The latter is critical for
390 locomotion, as tadpoles with smaller and/or thinner tail muscles need greater swimming
391 effort (Touchon and Wojdak, 2014).

392

393 Accordingly, exposure to nicosulfuron strongly influenced all the behavioural metrics we
394 investigated. This result was expected given the well-known influences of sulfonylurea
395 herbicides on behaviour in other species (Saglio, Olsén, Bre, 2001); effects that are probably
396 mediated by disruption of acetylcholinesterase (AChE, Bretaud et al., 2000; Pretto et al.,
397 2011). Indeed, links between locomotor behaviour and cholinesterase have been shown in
398 rainbow trout (*Oncorhynchus mykiss*), with decreased brain AChE activity being related to
399 decreased swimming behaviour (Beauvais et al., 2001). In our species, study showed that
400 nicosulfuron at sublethal concentrations lead to increase of antioxydant defenses and
401 teratogenic effect (Cheron et al., 2022). Hence, we suspect a nicosulfuron-mediated
402 disruption of AChE activity in the brain of tadpoles, which is known to increase throughout
403 metamorphosis (Franceschini et al., 1987; Sasaki et al., 1985).

404

405 Tadpoles exposed to nicosulfuron were overall more active, swam faster and traveled longer
406 distances. Moreover, behavioural complexity (as assessed through DFA, Cheron et al., 2021)
407 was also affected by exposure to nicosulfuron with a peak of behavioural complexity
408 occurring earlier in the development of Control individuals (e.g., GS 37) compared with
409 exposed individuals (e.g., GS 41). Such behavioural effects are expected to bear consequences
410 on traits related to locomotion, such as foraging efficiency (Browne and Moore, 2014),
411 predator avoidance and escape ability (Moreira et al., 2019). Whether these behavioural
412 alterations are consequences of diverging morphologies (see above), disruption of
413 behaviour-related physiological mechanisms (i.e., AChE) or a combination thereof, remains
414 to be tested.

415

416 Finally, exposure effects tended to persist through metamorphosis, with post-metamorphic
417 individuals from the higher concentration group displaying larger body mass than their
418 counterparts from the low concentration or control groups. Again, such results may be

419 linked to different investments in body parts (body *versus* tail), behavioural alterations
420 (which in turn can affect foraging efficiency and thus energy acquisition), or a combination
421 thereof. Another, non-mutually exclusive hypothesis would be linked to the pattern of
422 energetic mobilization occurring at the end of metamorphosis. At this stage, tadpoles
423 become aphagic and rely, at least in part, on tail resorption to fuel energetic requirements of
424 ultimate stages of metamorphosis (Bouchard et al., 2016; Gonçalves et al., 2015; Ruthsatz et
425 al., 2018). In our study, at the latest stages of development corresponding to tail atrophy and
426 resorption, tadpoles from the control group display both longer and higher tail muscles,
427 suggesting that the dynamics of tail resorption and associated energetic recovery may
428 strongly diverge between individuals exposed to nicosulfuron or not. Whatever the
429 underlying mechanism, this result reinforces the notion that a transient and/or early
430 exposure to nicosulfuron can bear long-lasting influences on developing amphibians.

431

432 **Conclusion**

433 Exposure to sublethal concentrations of an increasingly used herbicide altered both the
434 morphological and behavioural development of a widespread amphibian species that
435 inhabits agricultural areas. Most notably, the lack of effect of the timing of exposure of
436 different development stages (embryonic versus larval) on the traits we investigated
437 suggests that similar mechanisms are at play during embryonic and larval development.
438 Future studies are required to identify such mechanisms. Sulfonylurea herbicides affect
439 activity and behaviour through disruption of normal AChE activity (Bretaud et al., 2000;
440 Saglio, Olsén, Bre, 2001), and whether the same mechanisms occur in the developing
441 embryos of amphibians remains to be tested (de Llamas et al., 1985). Given the temporal
442 variation in agrochemical fate due to local variation in climatic conditions (Cabrerizo et al.,
443 2018), and given that the timing of application coincides with the reproduction of
444 amphibians and other non-target species in temperate areas (Berger et al., 2013), our results

445 reinforce the need for regulatory toxicology to take into account the effects of low,
446 environmentally relevant concentrations of agrochemicals on non-target wildlife occurring in
447 agricultural areas.

448

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450

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455

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462

463 **Ethics approval**

464 All applicable institutional and/or national guidelines for the care and use of animals were
465 followed. This work was approved by the French authorities (COMETHEA ethic committee
466 and Ministère de L'Enseignement Supérieur, de la Recherche et de L'innovation) under
467 permit #APAFIS#17821-2018112614459058 v5.

468

469 **Competing interests**

470 We declare we have no competing interests.

471

472 **Authors' contributions**

473 F.B, M.C, L.R, A.K, Y.R-C conceived and designed the study. M.C, L.R. performed the
474 experiment and collected the data. F.B, M.C, L.R, A.K, Y.R-C, X.M helped during data

475 curation. L.R, X.M, A.K arranged the dataset for fractal analysis. F.B, M.C, L.R, A.K, Y.R-C,
476 interpreted the data, F.B, M.C, designed the figures and wrote the initial draft. A.K, L.R, M.C
477 conducted all fractal analyses. All authors contributed to manuscript revision. All authors
478 read and approved the final manuscript.

479

480

481

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744 [02953-3](https://doi.org/10.1007/s00128-020-02953-3)
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746

747 **Table 1:** Concentrations ($\mu\text{g l}^{-1}$) measured in the experimental tanks (Mean \pm SD)

Treatment	Nicosulfuron concentrations
Control	0.00 \pm 0.00
Low	0.15 \pm 0.05
High	0.83 \pm 0.04

748

Table 2: Anova output of linear mixed models or generalized mixed models (for number of activity bouts only). "Concentration" represents nicosulfuron concentration with 3 levels (Control, Low, High). "Exposure" represents phase of exposure with 4 levels (Control, Embryonic exposure, Larval exposure, Embryonic+Larval exposure). "Stage" represents all studied Gosner stages with 5 levels for Morphology (GS25, GS30, GS37, GS41, GS42) and 4 levels for behaviour (GS30, GS37, GS41, GS42).

Variable	Model						
	Main effect			Interaction			
	Concentration	Exposure	Stage	Concentration X Stage	Exposure X Stage	Concentration X Exposure	Concentration X Exposure X Stage
Morphology							
<i>Total length</i>	F_(2, 420)=7.661, p<0.001	F _(2, 406) =0.03, p=0.968	F_(4, 356)=277.51, p<0.001	F_(6, 353)=2.951, p=0.008	F _(6, 341) =0.549, p=0.771	F _(2, 396) =0.493, p=0.611	F _(6, 341) =0.856, p=0.528
<i>Body length</i>	F_(2, 431)=5.715, p=0.004	F _(2, 431) =1.183, p=307	F_(4, 349)=270.56, p<0.001	F_(6, 343)=2.470, p=0.024	F _(6, 345) =1.332, p=0.242	F _(2, 426) =1.458, p=0.234	F _(6, 344) =0.853, p=0.530
<i>Body height</i>	F _(2, 409) =0.947, p=0.389	F _(2, 406) =0.107, p=0.899	F_(4, 337)=89.50, p<0.001	F _(6, 332) =1.168, p=0.323	F _(6, 334) =1.064, p=0.384	F _(2, 399) =0.396, p=0.673	F _(6, 332) =0.195, p=0.978
<i>Body width</i>	F_(2, 438)=4.957, p=0.007	F _(2, 438) =0.954, p=0.386	F_(4, 352)=138.92, p<0.001	F _(6, 346) =1.206, p=0.302	F _(6, 348) =1.285, p=0.264	F _(2, 436) =0.979, p=0.377	F _(6, 346) =0.824, p=0.552
<i>Tail length</i>	F _(2, 421) =1.032, p=0.357	F _(2, 421) =0.171, p=0.843	F_(4, 346)=139.51, p<0.001	F _(6, 341) =0.471, p=0.828	F _(6, 343) =0.340, p=0.932	F _(2, 414) =0.607, p=0.545	F _(6, 341) =0.332, p=0.920
<i>Tail height</i>	F _(2, 431) =1.074, p=0.343	F _(2, 432) =0.166, p=0.847	F_(4, 347)=60.61, p<0.001	F _(6, 340) =0.837, p=0.542	F _(6, 343) =0.355, p=0.907	F _(2, 430) =0.519, p=0.595	F _(6, 340) =0.763, p=0.600
<i>Tail muscle height</i>	F _(2, 435) =1.577, p=0.208	F _(2, 435) =0.056, p=0.946	F_(4, 347)=22.00, p<0.001	F_(6, 341)=2.437, p=0.025	F _(6, 343) =1.429, p=0.203	F _(2, 432) =0.356, p=0.701	F _(6, 340) =0.777, p=0.588
<i>Tail muscle length</i>	F_(2, 424)=3.407, p=0.034	F _(2, 422) =1.668, p=0.190	F_(4, 341)=145.07, p<0.001	F_(6, 335)=2.217, p=0.041	F _(6, 337) =1.772, p=0.104	F _(2, 417) =0.108, p=0.898	F _(6, 334) =1.167, p=0.323
<i>Metamorphic mass</i>	F_(2, 74)=3.07, p=0.052	F _(2, 74) =0.082, p=0.922				F _(1, 70) =0.309, p=0.735	
<i>Metamorphic SVL</i>	F_(2, 74)=0.645, p=0.424	F _(2, 74) =2.056, p=0.118				F _(1, 70) =0.661, p=0.517	
<i>Metamorphic BCI</i>	F_(2, 74)=2.545, p=0.082	F _(2, 74) =0.252, p=0.778				F _(1, 70) =0.163, p=0.850	
Behaviour							
<i>Total activity time</i>	F_(2, 133)=5.28, p=0.006	F _(2, 131) =1.605, p=0.205	F_(3, 359)=45.35, p<0.001	F_(6, 354)=2.121, p=0.050	F _(2, 128) =1.48, p=0.183	F _(2, 128) =1.48, p=0.231	F _(6, 341) =0.856, p=0.528
<i>Total distance</i>	F_(2, 133)=4.600, p=0.011	F _(2, 131) =2.469, p=0.086	F_(3, 359)=7.469, p<0.001	F _(6, 354) =0.774, p=0.509	F _(2, 128) =1.705, p=0.086	F _(2, 128) =0.766, p=0.465	F _(6, 341) =0.353, p=0.908
<i>Absolute mean swimming speed</i>	F _(2, 421) =2.056, p=0.129	F_(2, 424)=11.72, p<0.001	F_(3, 359)=7.469, p<0.002	F _(6, 334) =0.339, p=0.916	F_(6, 339)=2.81, p=0.011	F _(2, 422) =2.876, p=0.057	F _(6, 345) =0.858, p=0.526
<i>Absolute maximum swimming speed</i>	F _(2, 421) =0.815, p=0.367	F _(2, 424) =1.084, p=0.356	F_(3, 359)=27.833, p<0.001	F _(6, 334) =0.985, p=0.399	F _(6, 339) =1.328, p=0.220	F _(2, 422) =1.246, p=0.289	F _(6, 345) =0.710, p=0.642
<i>Number of activity bouts</i>	X ₂ =4.881, p=0.087	X₂=11.93, p=0.003	X₃=97219.69, p<0.001	X₆=6563.49, p<0.001	X₆=9271.14, p<0.001	X ₂ =2.65, p=0.266	X₆=3959.81, p<0.001
<i>αDFA</i>	F _(2, 421) =0.251, p=0.778	F _(2, 424) =833, p=0.435	F_(3, 340)=8.548, p<0.001	F_(6, 340)=5.283, p<0.001	F _(6, 339) =0.110, p=0.995	F _(2, 422) =0.426, p=0.653	F _(6, 345) =1.084, p=0.372

Figure Captions

Figure 1: Experimental design to study the effects of timing of exposure and concentration of nicosulfuron on *Bufo spinosus* tadpoles.

Figure 2: Total length (in cm; A), Body length (in cm; B), Tail muscle length (in cm; C) and Tail muscle height (in cm; D) relative to Gosner developmental stages in *Bufo spinosus* tadpoles. Data represent mean \pm SE. Intervals between Gosner developmental stages were calculated using the mean number of days between two Gosner stages for all individuals. Colors represent different concentrations (i.e., Control = light blue, Low= blue, High= dark blue).

Figure 3: Body mass (g) of metamorphic individuals of *Bufo spinosus* between Nicosulfuron treatment (Timing of exposure or concentration). Data represent mean \pm SE. Colors represent different concentrations (i.e., Control = light blue, Low= blue, High= dark blue).

Figure 4: Total activity time (in s; A), Total distance travelled (in mm; B), Mean swimming speed (in mm s⁻¹; C) and α DFA (unitless scaling index inversely related to behavioural complexity; D) relative to Gosner developmental stages in *Bufo spinosus* tadpoles. Data represent mean \pm SE. Intervals between Gosner developmental stages were calculated using the mean number of days between two Gosner stages for all individuals. Colors represent different concentrations (i.e., Control = light blue, Low= blue, High= dark blue).

Figures

Figure 1

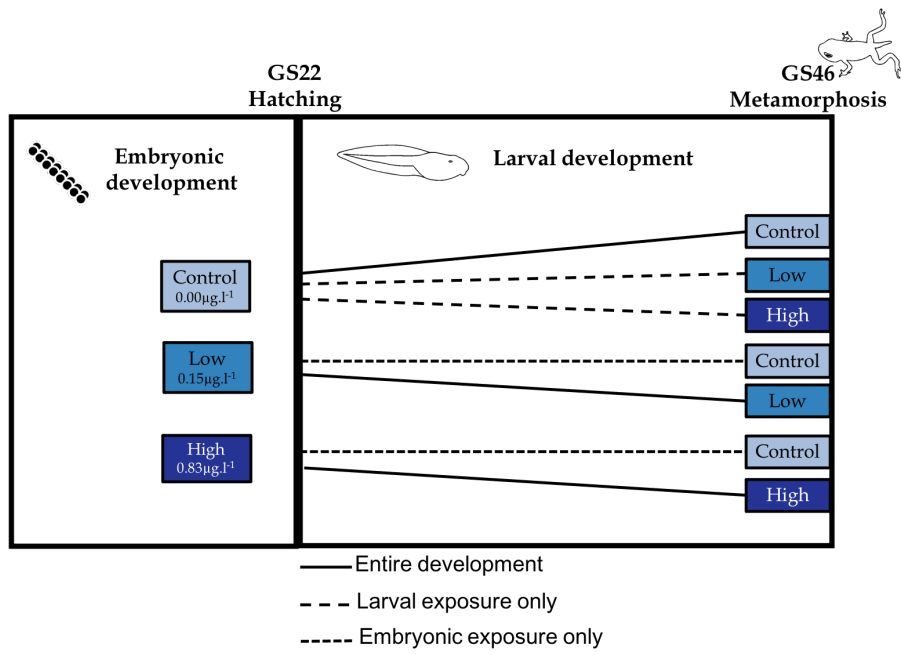


Figure 2

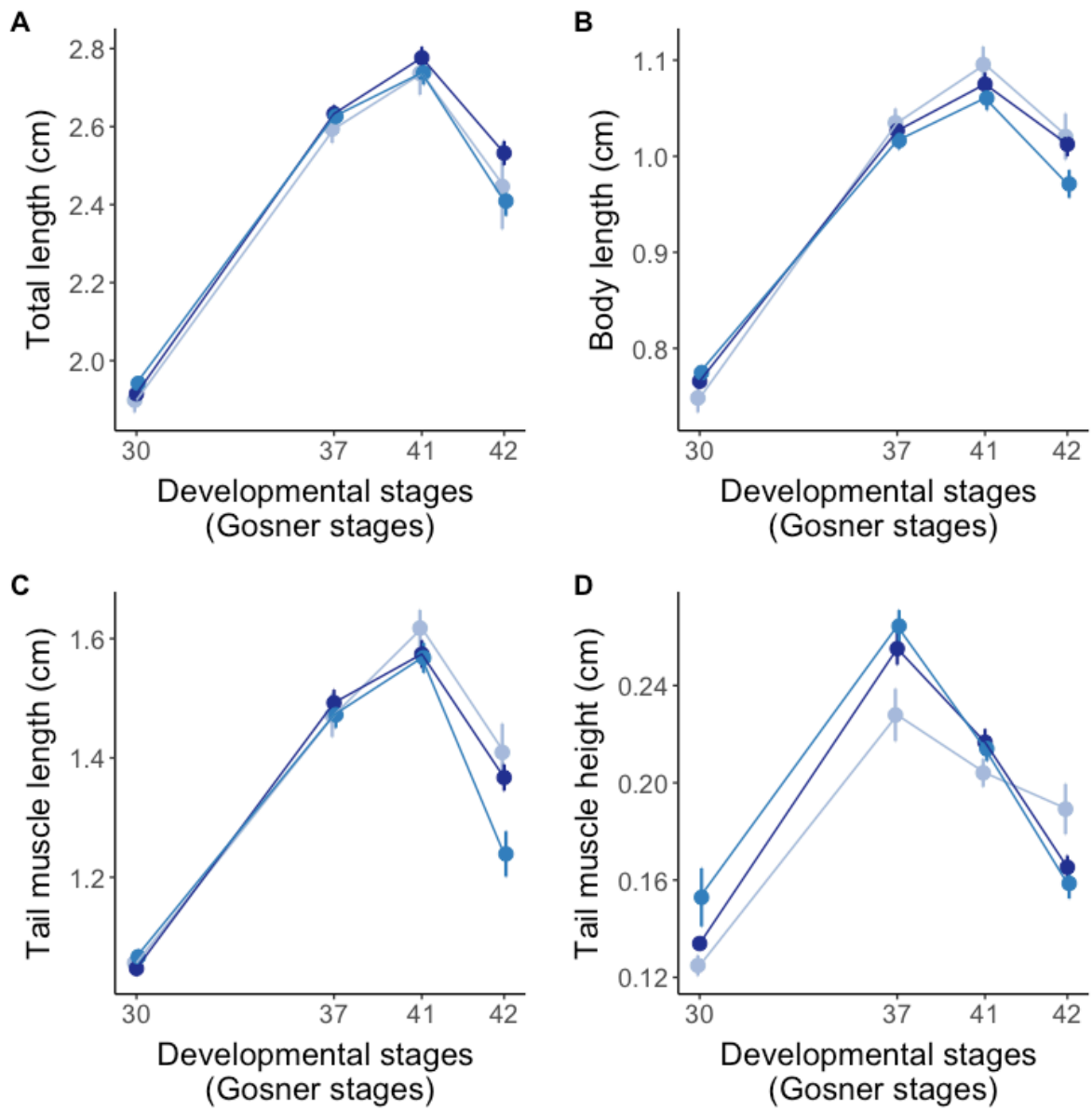


Figure 3

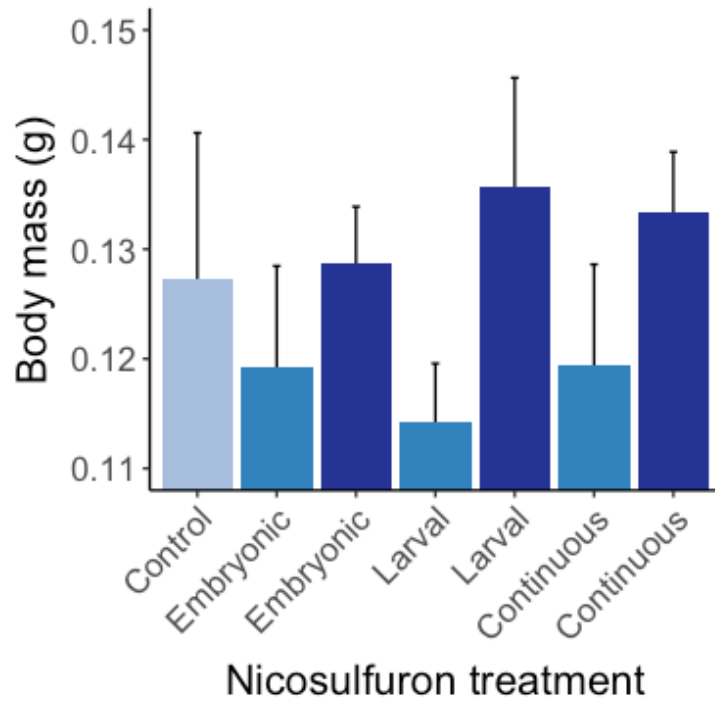
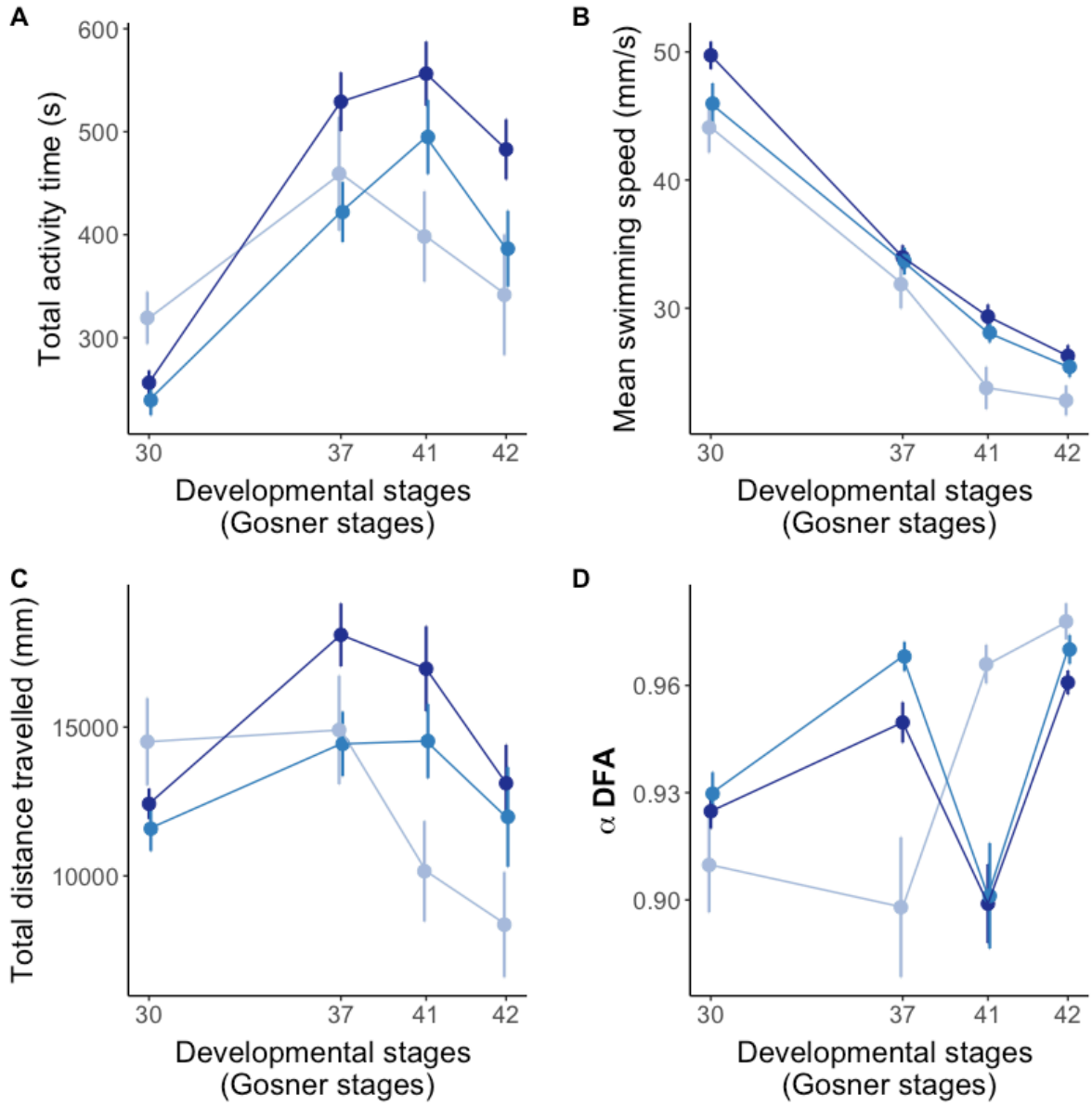


Figure 4



Annexe I

Brischoux, F., & Cheron, M

Osmotic ‘cost’ of reproduction in breeding male toads.

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Research



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Physiology

Osmotic 'cost' of reproduction in breeding male toads

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Shifts between habitats during reproduction can induce costs that are independent of the reproductive effort and that often apply to both sexes. Such shifts can also illustrate physiological costs complementary to those involving energetic currencies. In this study, we investigated osmotic consequences of reproduction in a context where reproduction induces a shift from terrestrial habitats to freshwater environments. During reproduction, toads migrate to breeding ponds where males remain for several weeks, while females leave shortly after egg-laying. We assessed plasma osmolality of male spined toads during the whole reproductive period (approx. 30 days) in conjunction with markers of individual condition. We found that osmolality decreases during the protracted period of immersion in freshwater during reproduction, presumably through water influx as indicated by body mass changes. Hormonal markers of metabolism and sexual activity were positively correlated with osmolality. Recent research has highlighted hydric 'costs' of reproduction when access to water is limited. Our study adds to this growing field of investigation, yet with an opposite perspective, where water availability linked to reproduction provokes hyperhydration rather than dehydration.

1. Introduction

Trade-offs among components of reproductive effort involve both ecological and physiological costs [1]. Among physiological costs, direct costs (i.e. energy and nutrient demands) have attracted considerable attention, probably because they involve the currencies that satisfy demands of the reproductive event itself [2–4]. Yet, physiological costs of reproduction can also be indirect [2–4]. These indirect physiological costs can be further divided into optional compensatory or consequential costs. Compensatory costs involve reduced investment into components of physiology (e.g. immune system or oxidative shielding) to increase resource allocation to reproduction, while consequential costs are inevitable consequences (e.g. hyperthermia or bone loss) of the reproductive event [4].

This latter category is particularly interesting because these consequential costs are largely independent of the reproductive effort (i.e. the number of offspring produced [5]). In this respect, the consequences of shifts between habitats during reproduction are remarkable because they often apply to both sexes, contrarily to other costs that tend to affect mainly the maternal organism [4]. For instance, moving to reproductive grounds can require an energetically demanding migration for both males and females [6–8]. In addition, large-scale movements to reproductive grounds may also increase susceptibility to predation in both males and females [9,10]. Importantly, shifts between habitats during reproduction also illustrate other physiological processes linked to consequential costs of reproduction. This is typically the case when the reproductive habitat differs strongly from the habitat used in the non-reproductive life of organisms. For instance, euryhaline European eels shift from freshwater to marine environments to reproduce [11]. In addition to the other migratory costs mentioned above, such

a shift involves remodelling of the osmoregulatory apparatus that aim to maintain the osmotic balance in the hyperosmotic marine environment [12–14].

Yet, such osmotic consequences of reproduction are likely not restricted to shifts to hyperosmotic habitats. Indeed, reproductive habitat changes can also involve migration to hyposmotic environments such as freshwater habitats (e.g. anadromous fish [15]). In this study, we investigated osmotic consequences of reproduction in a context where reproduction induces a shift from terrestrial habitats to freshwater environments. Many amphibian species are biphasic and live most of their life on land but reproduce in freshwater environments where eggs and larvae develop [16]. On land, many amphibians dehydrate rapidly and display specific adaptations to evade dehydration which are exemplified by their ability to absorb water cutaneously and using their urinary bladder as a water reserve [17,18]. Accordingly, skins of aquatic species have lower permeability to water than those of terrestrial species [17]. Yet, this remarkable ability for water absorption through the skin may become a constraint when terrestrial amphibians are shifting to aquatic environments during reproduction. Spined toads (*Bufo spinosus*) are particularly well suited to investigate potential osmotic consequences of reproduction. On land, this species can be found in a wide variety of habitats including relatively xeric environments [19]. Their ability to absorb water cutaneously is well developed and has been previously coined as ‘cutaneous drinking’ [20]. During reproduction, toads migrate to breeding ponds where males remain for several weeks, while females leave shortly after egg-laying [21], suggesting that possible osmotic consequences of reproduction may apply more strongly to males than to females. In this study, we assessed plasma osmolality of male spined toads during the whole reproductive period to test whether a protracted immersion in freshwater dilutes body fluids. We conjointly monitored physiological markers, such as body condition and hormonal levels (corticosterone (CORT) and testosterone (T) which are pertinent markers of metabolism and sexual activity during reproduction in this species [22]), in order to test whether a change in plasma osmolality influences markers of individual condition.

2. Methods

(a) Study species and sampling

The spined toad, *B. spinosus*, is one of the most common anuran species in Western Europe. Reproduction occurs in late winter (February) and toads massively migrate towards aquatic breeding sites [21], usually within a few days [22]. Males remain at the breeding site for several weeks, while females leave shortly after mating and egg-laying [21]. As a consequence, we focused our investigations on male toads specifically.

Sampling took place between early February and mid-March 2019 in a pond situated nearby the Centre d’Etudes Biologiques de Chizé (46°09’ N, 0°24’ W) in France, during five sessions covering a period of 32 days. Importantly, this sampling period spanned the whole breeding period, from the arrival of the first breeding males (first session) to departure of the last breeding males (last session). Sampling was conducted at night using headlights to locate male toads at the breeding site. Upon sighting, each toad (total $N = 100$, 19–21 different individuals per sampling sessions) was captured with a net, and a blood sample was collected (approx. 100 μ l) via cardiocentesis using a 1 ml syringe and a 30-G heparinized needle [22]. Blood samples were collected within 5 min of capture ($2.73 \pm$

1.07 min, range: 1.13–5.00) in order to quantify baseline hormonal levels [22]. All individuals were weighed (± 0.1 g), and their snout–vent length was measured with electronic calipers (± 0.01 mm). We quantified a body condition index using residual scores from the linear regression between body size and body mass [22]. All individuals were marked with a small puncture on the foot web with a medical trocar (a different web per sampling session) in order to avoid multiple sampling of the same individuals across sessions (for ethical reasons). This marking procedure is temporary (lasting approx. two months, FB and MC 2018, personnel observation) and less invasive than toe-clipping. Recaptures of marked individuals showed that toads captured during the first session were still present at the reproductive pond during the last sampling session. All individuals were released at their location of capture after measurements and blood collection. A final session performed 39 days after the arrival of the first individuals verified that all toads had left the breeding pond.

(b) Osmolality and hormonal assays

Blood was centrifuged, and the plasma was separated and stored at -20°C for one month until assays were conducted. Plasma osmolality (mOsmol kg^{-1}) was measured from 10 μ l aliquots on a Vapro2 osmometer (Elitech group). Plasma concentrations of T and CORT were determined by radioimmunoassay at the CEBC, as described previously [23]. The minimum detectable levels were 0.30 and 0.28 ng ml^{-1} , respectively, for T and CORT, and the intra- and inter-assay coefficients of variation were 13.16% and 17.56% and 10.66% and 11.77%, respectively, for T and CORT (that were run twice both within and between assays).

3. Results

Osmolality was significantly different between sessions ($F_{4,95} = 3.95$, $p = 0.005$; figure 1) and decreased with the time elapsed since the arrival of the first individuals at the breeding site ($F_{1,98} = 12.35$, $p = 0.0006$, $r^2 = 0.10$).

Body condition was also significantly different between sessions ($F_{4,94} = 11.19$, $p < 0.0001$; figure 1) but was only marginally negatively related to the time elapsed since the first session ($F_{1,98} = 3.31$, $p = 0.07$, $r^2 = 0.02$). A closer examination of the data showed that the body condition of toads sampled during the second and third sessions was higher than that of toads sampled during all the other sessions (Fisher’s LSD, all $p < 0.009$; figure 1). Body condition was not correlated to osmolality across field sessions ($F_{1,98} = 0.006$, $p = 0.94$) but was positively correlated to osmolality during the first ($F_{1,19} = 7.38$, $p = 0.01$, $r^2 = 0.24$) and the last session ($F_{1,18} = 4.76$, $p = 0.04$, $r^2 = 0.16$).

CORT levels were different between sessions ($F_{4,95} = 8.60$, $p < 0.0001$; figure 1) and decreased with the time elapsed since the first session ($F_{1,98} = 32.31$, $p < 0.0001$, $r^2 = 0.24$). CORT levels were positively related to osmolality across sampling sessions ($F_{1,98} = 6.51$, $p = 0.01$, $r^2 = 0.05$; figure 2) but not within sessions (all $p > 0.19$). Similarly, T levels were different between sessions ($F_{4,95} = 26.61$, $p < 0.0001$; figure 1) and decreased with the time elapsed since the first session ($F_{1,98} = 93.32$, $p < 0.0001$, $r^2 = 0.48$). T levels were positively related to osmolality across sampling sessions ($F_{1,98} = 13.62$, $p = 0.0003$, $r^2 = 0.11$, figure 2) but not within sessions (all $p > 0.11$).

4. Discussion

The most salient result of our study shows that male toads cannot remain normosmotic during the protracted period of

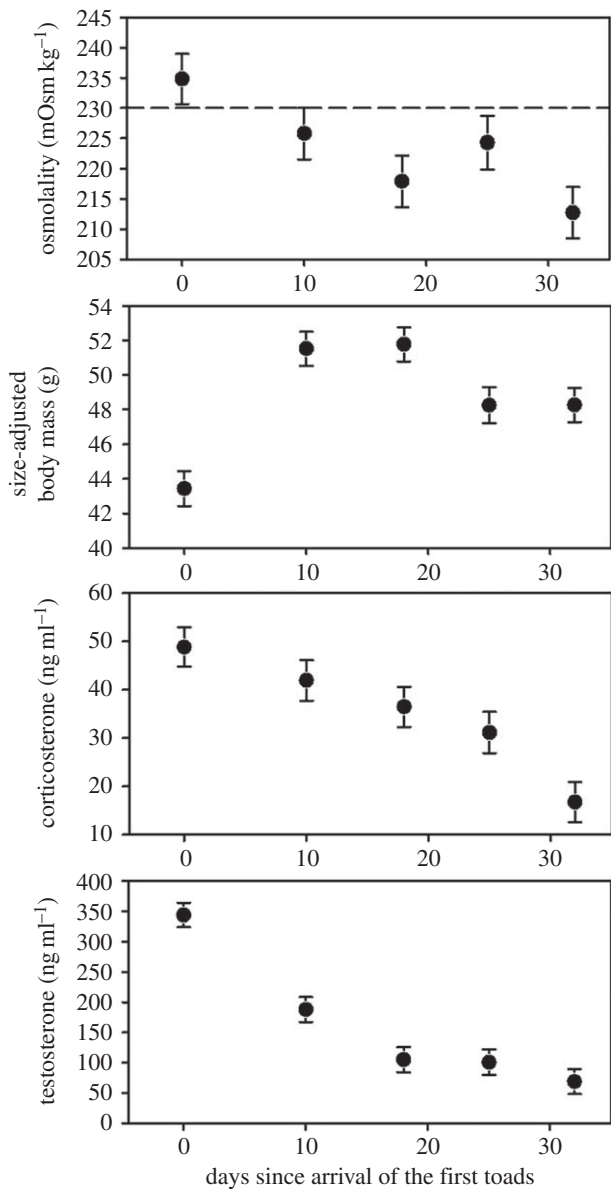


Figure 1. Plasma osmolality, size-adjusted body mass, corticosterone levels and testosterone levels in relation to the time since arrival of the first toads at the breeding site. Dashed line in the upper panel represents normosmolality in toads (approx. 230 mOsm kg^{-1} [17]). Each sampling session represents 19–21 individuals, and data are presented as mean \pm s.e.

immersion in freshwater during reproduction. Indeed, upon arrival at their reproductive pond, male toads displayed plasma osmolality similar to normosmolality in terrestrial amphibians (e.g. approx. $230 \text{ mOsmol kg}^{-1}$ [17]). During the following weeks of immersion in water (up to 32 days for our study site), plasma osmolality decreased and attained the smallest values during our last sampling session (mean approx. $210 \text{ mOsmol kg}^{-1}$, minimum value $188 \text{ mOsmol kg}^{-1}$). Despite the overall decrease in osmolality we detected, male breeding toads were clearly not osmoconformers as they maintained elevated plasma osmolality (greater than $200 \text{ mOsmol kg}^{-1}$) even after prolonged periods of immersion in freshwater [24]. Future studies are required to elucidate the mechanisms underlying the slow decrease of osmolality we detected [25].

Our results on body condition further suggest that the decrease of osmolality may have occurred through water influx rather than osmolyte efflux [24]. Male toads significantly gained mass between the first and the second session (approx.

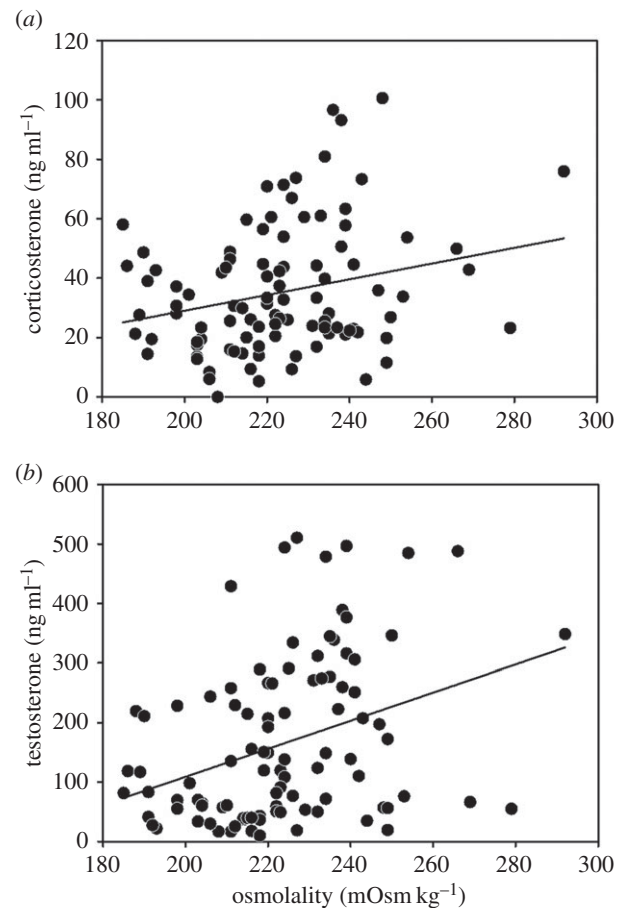


Figure 2. Corticosterone (a) and testosterone (b) in relation to plasma osmolality across sampling sessions. Each dot represents an individual ($N = 100$).

8 g representing 20% of the initial body mass). The reasons why body mass plateaued during the following days or even decreased during the last sampling sessions may be linked to the reproductive anorexia occurring in breeding male toads. During breeding, male toads forgo feeding in order to maximize time spent in their aquatic breeding habitat and thus mating opportunities [26]. In this case, any further mass gain linked to water influx (as suggested by the overall decrease in osmolality we found) would be obscured by the mass loss linked to the metabolic demands of organismal maintenance, rivalry and mating in the breeding pond. Alternatively, but not exclusively, reproductive anorexia may induce protein catabolism resulting in elevated levels of urea [27]. In turn, excreting this metabolic waste could allow toads to remove excess water [28], thereby resulting in decreased body mass during the last sampling sessions. This hypothesis dovetails relatively well with the slight increase of osmolality visible during the fourth sampling session which also corresponds to body mass loss in sampled toads. Clearly, future studies should investigate the temporal dynamics of urea, as well as other organic osmolites and ions, during the breeding season.

The temporal dynamics of the two hormonal markers we monitored seem to be linked to osmolality (see also [24]). CORT, a marker of metabolism and activity during reproduction in this species [22], continuously decreased during the reproductive period in male toads and was correlated with osmolality. This trend was even stronger for T (a marker of sexual activity [22]) for which levels were almost halved between the first and the second sampling sessions. Similarly to CORT, T was positively correlated with osmolality. Yet, we cannot entirely rule-out that these temporal dynamics of

hormones are independent of osmolality (i.e. naturally decreasing across the reproductive period, but see [29,30]). Future studies are required to assess if osmolality and hormonal levels are functionally linked [24], or if both variables simply depend on time within the breeding season. Importantly, these decreasing hormonal levels could bear consequences for the ability of the earliest toads to compete with latecomers for access to mating opportunities. Future studies should investigate whether the timing of arrival on a breeding site, and thus plasma osmolality and hormonal levels, influences male–male competition for access to females.

Whether such decreased osmolality bears consequences for male toads remains complicated to assess. At the cellular level, hyposmolality is well known for provoking alterations of cell volume (swelling), but this is transitory and counter-balanced by intracellular osmolytes extrusion [31], a likely process in hyposmotic toads [24]. Additionally, short-term hyposmolality has been shown to increase brain volume [32], affect neuronal functioning and increase seizure susceptibility [33–35], none of which seems plausible in our system where male toads can spend several weeks in a chronic, hyposmotic state. Alternatively, hyposmolality may even be beneficial during the protracted reproductive period. In humans, hyposmolality has been shown to affect energetic metabolism and results in protein sparing and increased lipolysis [36]. If a similar process holds true in breeding toads (but see above), chronic hyposmolality may allow toads to preserve their protein structure. This would allow for sustained muscular abilities needed to repel rivals and during mating (amplexus) when males clasp females with

their arms for prolonged periods prior to gamete release [26]. Future studies should investigate whether hyposmolality influences the energetic sources (proteins versus lipids) in breeding toads in order to test this hypothesis.

More generally, recent research has highlighted the importance of water balance during reproduction in tetrapods [37–43]. Yet, all of these studies used systems that focussed on limited access to water to explore the hydric ‘costs’ of reproduction. Our study adds to this growing field of investigation, yet with an opposite perspective, where water availability linked to reproduction provokes hyperhydration rather than dehydration. Clearly, our results militate for such field of research to encompass a larger framework.

Ethics. This work was approved by the French authorities (R-45GRETA-F1-10).

Data accessibility. Data are available as electronic supplementary materials.

Authors' contributions. F.B. conceived and designed the study, M.C. and F.B. collected the data, F.B. and M.C. conducted the analyses, interpreted the data, designed the figures and wrote the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Competing interests. We declare we have no competing interests.

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Annexe II

Brischoux, F., Cheron, M., Renoirt, M., Lourdais, O.

Getting ready for a long bath: skin permeability decreases prior to aquatic breeding in male toads

Under review in The Science of Nature – Naturwissenschaften



“I’m the darkness” Alexandre Roux



Getting ready for a long bath: skin permeability decreases prior to aquatic breeding in male toads

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Abstract

Vertebrate skin regulates exchanges between the organism and its environment and notably plays a fundamental role in regulating water fluxes. Dynamic changes of skin resistance to water fluxes are expected to occur in species that regularly shift between habitat types especially if these habitats differ in their hydric properties (e.g., terrestrial versus aquatic). We investigated changes of skin permeability using a study system (terrestrial toads) where reproduction induces a transition from terrestrial to freshwater habitats and a prolonged immersion that can last several weeks in males. In this system, the simultaneity between skin shedding and the onset of breeding suggests that the production of new integument layers prior to immersion for reproduction may regulate water influxes. We found that the skin permeability of male toads decreases significantly prior to breeding, suggesting that skin shedding at the onset of breeding regulates water fluxes to alleviate osmotic costs of immersion during reproduction. The continued decrease of skin permeability detected during breeding suggests that additional mechanisms interact with skin structure to further decrease permeability to water during a prolonged immersion. Future studies are required to assess whether changes in skin permeability to water tradeoffs with other skin characteristics (gas exchanges) relevant to aquatic breeding amphibians.

Keywords *Bufo spinosus* · Water relations · Cutaneous evaporative water loss · Habitat · Reproduction

Introduction

Species that shift between habitats during their lifetime must face environmental constraints that can be highly divergent between habitat types. Typical examples of the consequences of such habitat shifts have been thoroughly described in anadromous or catadromous fish. In these species, reproductive migrations between fresh- and seawater are characterized by highly different chemical compositions (e.g., high salt concentration in seawater) and involve significant changes to the osmoregulatory apparatus that allow osmotic balance either in hyperosmotic marine or hyposmotic freshwater habitats

(Edeline 2007; Tseng and Hwang 2008; Bowerman et al. 2017). Other examples have been documented in amphibious species that commute between aquatic and terrestrial habitats (Mazin and de Buffrénil 2001) which diverging physico-chemical characteristics have been shown to influence several traits such as locomotor performance (Bonnet et al. 2005) or environmental tolerance (Brischoux et al. 2013).

Vertebrate skin provides physical protection and regulates exchanges between the organism and its environment, including the regulation of water fluxes (Lillywhite 2006). Accordingly, the resistance of the skin to water passage has been shown to be highly variable and range from very low in aquatic vertebrates to high values in species adapted to xeric environments (Lillywhite 2006). Dynamic changes in skin resistance to water fluxes are expected to occur in species that regularly shift between habitat types, especially if these habitats differ in their hydric properties (e.g., terrestrial versus aquatic). For instance, many amphibians shift between habitats during reproduction with a transition from terrestrial to aquatic sites.

The integument of amphibians is characterized by very little keratin and a thin stratum corneum (Lillywhite 2006). As a

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consequence, transcutaneous water loss is high, and amphibians tend to dehydrate rapidly under dry conditions (e.g., on land, Burggren and Vitalis 2005). Terrestrial species display specific adaptations to reduce dehydration such as an elevated ability to absorb water cutaneously (Bentley 1971; Hillyard et al. 1998) or increased skin lipids (Withers et al. 1984). Yet, many terrestrial amphibian species reproduce in aquatic habitats where mating occurs and eggs and larvae develop (Dodd 2010). In this context, the ability of terrestrial amphibians to absorb water through the skin can become a constraint when they shift to an aquatic lifestyle for reproduction.

During aquatic reproduction, male toads typically experience an osmotic challenge during the prolonged period (several weeks) of immersion in freshwater, presumably due to water influx (Brischoux and Cheron 2019). Despite a significant decrease in plasma osmolality following immersion, breeding toads can maintain a relatively elevated plasma osmolality even after prolonged periods of immersion suggesting that water influx remains limited (Brischoux and Cheron 2019). In this respect, the integument may well play a significant role in regulating water influxes (Bentley 1971; Shoemaker et al. 1992, Boutilier et al., 1992). Because male bufonid toads shed their skin (i.e., production of new integument layers) just prior to the shift to aquatic reproduction (Jørgensen & Larsen 1961), it is likely that the new skin has specific properties that allow for reduction in water fluxes (Wu et al. 2017).

In this study, we assessed the skin permeability (cutaneous evaporative water loss, CEWL) of reproductive and non-reproductive male spined toads (*Bufo spinosus*) in order to test whether a shift from terrestrial to aquatic lifestyle influences skin permeability in this terrestrial species. First, we hypothesized that skin-shedding prior to reproduction is associated with lower skin permeability during aquatic breeding in order to limit water influxes and thus osmotic costs of reproduction (Brischoux and Cheron 2019). Second, irrespectively of reproduction, we expected that there would be regional variation in CEWL depending on the body area of the toads. We predicted that the dorsal area — presumably exposed to more desiccant conditions than the ventral area, which remains in close contact with relatively humid substrate — should display lower CEWL values than the ventral area or the pelvic area that is involved in transcutaneous water intake (Jørgensen 1994).

Material and methods

Study species and sampling

The spined toad (*Bufo spinosus*) is one of the most common anuran species in Western France. As with most toad species, it is characterized by a biphasic lifestyle with breeding occurring in aquatic habitats (ponds) where eggs and

tadpoles develop, while the remaining cycle occurs in terrestrial habitats (Reading and Clarke, 1983, Brischoux et al. 2018). In late winter (February–March), male toads migrate to breeding ponds where they wait for females during several weeks (Brischoux et al. 2018). Females remain only transitorily at breeding sites, and typically return to land within a few hours once mating and egg-laying has occurred (Brischoux et al. 2018). Because aquatic life (immersion at breeding sites) is disproportionately longer in males, we focused our investigations on this sex only.

In order to comprehensively assess skin permeability of toads both during terrestrial and aquatic (reproductive) phases, toads were sampled during three distinct time periods. First, we captured individuals during the non-reproductive period in early October when climatic conditions allow toads to resume activity after aestivation during drier months (summer). During this period, we opportunistically captured individuals that were foraging on roads situated nearby the pond monitored during the breeding season (see below). Within a few days (4–18 October 2019), we were able to collect 19 adult male toads. Second, at the onset of the reproductive period, during male migration to breeding sites, we captured individuals upon their arrival at the breeding pond, but before they actually entered water. To do so, sampling was conducted at night using headlamps to detect male toads located on land and moving in the direction of the pond. These individuals were located within 10 m of the pond edges. Importantly, male toads shed their skin just before reaching breeding ponds (Jørgensen and Larsen 1961), and the individuals that we captured during this time period were all showing remnants of loose old skin suggesting that skin shedding had occurred. Due to the massive migration of male toads to aquatic breeding sites, all individuals ($N=19$) were captured during a single night (29 January 2020). Finally, we captured individuals that were immersed at the breeding pond for a significant time period (i.e., ~3 weeks after the arrival of the first individuals). Sampling was conducted at night using headlamps, and toads immersed in water were captured with a net. Similarly to the previous capture session, all individuals ($N=20$) were captured during a single night (21 February 2020). For clarity, we will refer to these three time periods as “non reproductive”, “arrival at breeding site”, and “aquatic breeding” hereafter.

After capture, individuals were brought back to the laboratory and maintained in plastic containers with a shelter and either a damp substrate (paper towel) for “non reproductive” and “arrival at breeding site” individuals, or water allowing full immersion for “aquatic breeding” individuals. The snout–vent length (SVL) of each individual was measured with electronic calipers (± 0.01 mm), and CEWL measurements (see below) were performed the day following capture.

All individuals were released at their location of capture after measurements.

CEWL measurements

Measurements were carried out at room temperature (20 °C), and toads were acclimated to this temperature for at least 2 h before measurement. We used an AquaFlux AF200 (Biox, London) and the Bioxsoftware AquaFlux 6.2 to calibrate and compute CEWL rate ($\text{g m}^{-2} \text{h}^{-1}$). We used an in vivo nail cap with rubber O-ring (diameter 2.6 mm) to insure a complete seal between the device and the toad's skin. Contact was maintained by gently restraining the toad and applying steady but slight pressure to the probe against the skin. Trials continued until the CEWL reading stabilized ($\pm 0.02 \text{ g m}^{-2} \text{ h}^{-1}$) for 180 s. If any movement (by the toad or the operator) caused a leak in the seal between the probe and the toad (detected as a sudden change in water flux), the trial was repeated. The AquaFlux unit was calibrated at the beginning of each trial. Each measurement was performed in triplicates at each body area (see below), and we used mean values for analyses.

Because we expected regional variation of CEWL depending on the body area of toads (see above), measurements were performed at three different body regions, namely the dorsal area, the ventral area, and the pelvic patch dedicated to cutaneous drinking (Jørgensen 1994). For dorsal measurements of CEWL, we targeted an area situated between the parotid glands because this area contained fewer warts than the remaining dorsal area, thereby allowing a correct seal between the Aquaflux's probe and the toad's skin. For ventral measurements of CEWL, we targeted an area situated between the forelegs. Finally, the CEWL measurements of the pelvic patch were straightforward to perform because this specific area is easily recognizable by its location (pelvis) and color (pinkish, due to high vascularization, in contrast to the creamy white belly of the toads).

Statistical analyses

Relationships among CEWL values measured at different body regions (dorsal, ventral, or pelvic patch) were assessed using linear models. Differences in CEWL among body regions and time periods were assessed using general linear models, as were relationships between toad size and CEWL within body regions and time periods. All analyses were performed with Statistica 12.

Results

Overall, the values of CEWL measured at different body regions (dorsal, ventral, or pelvic patch) were strongly correlated (dorsal–ventral: $F_{1,75} = 579.74$, $r^2 = 0.88$, $p < 0.0001$,

dorsal–pelvic patch: $F_{1,75} = 322.94$, $r^2 = 0.81$, $p < 0.0001$, ventral–pelvic patch: $F_{1,75} = 1176.60$, $r^2 = 0.94$, $p < 0.0001$, Fig. 1), and similar results were found when restricting analyses to the different time periods (all $r^2 > 0.30$, all $p < 0.008$).

CEWL differed significantly across body regions ($F_{2,165} = 15.05$, $p < 0.0001$, Fig. 2) and time periods ($F_{2,165} = 119.40$, $p < 0.0001$, Fig. 2), with no significant interactions ($F_{4,165} = 0.32$, $p = 0.86$). Dorsal CEWL was lower than both ventral CEWL and CEWL measured from the pelvic patch ($p < 0.008$), and ventral CEWL was higher than CEWL measured from the pelvic patch ($p = 0.006$). For all body areas, the CEWL was the highest during the non-reproductive period and was the lowest during aquatic breeding (all $p < 0.0001$).

Toad size did not influence CEWL within body regions and periods (all $p > 0.11$), but we found a significant interaction between the time period and SVL for dorsal CEWL

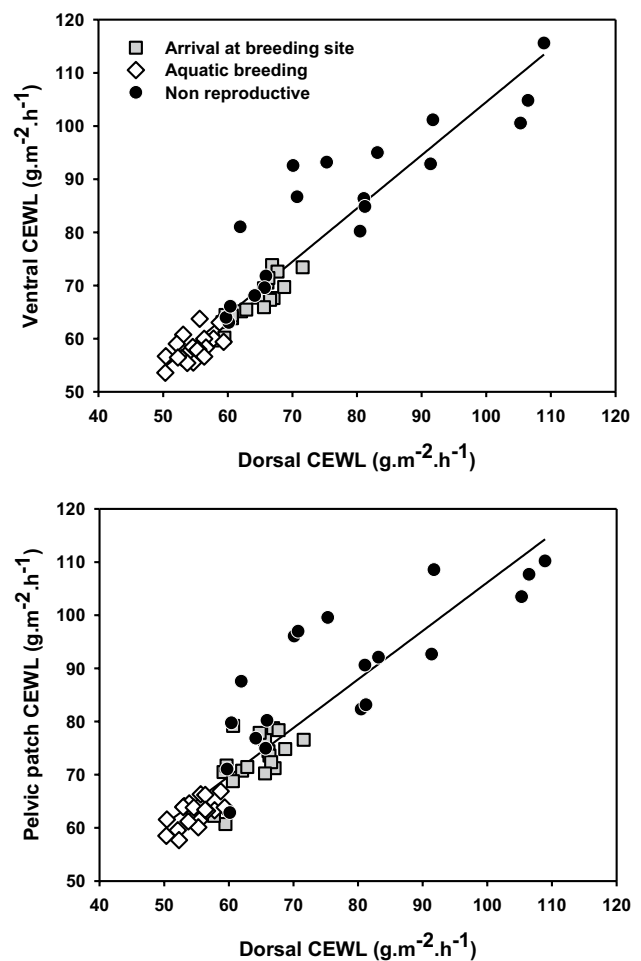


Fig. 1 Relationships between dorsal cutaneous evaporative water loss (CEWL) and ventral CEWL (upper panel) or pelvic patch CEWL (lower panel) during the “non-reproductive” (black circles), “arrival at breeding site” (grey squares) and “aquatic breeding” (white diamonds) sampling periods

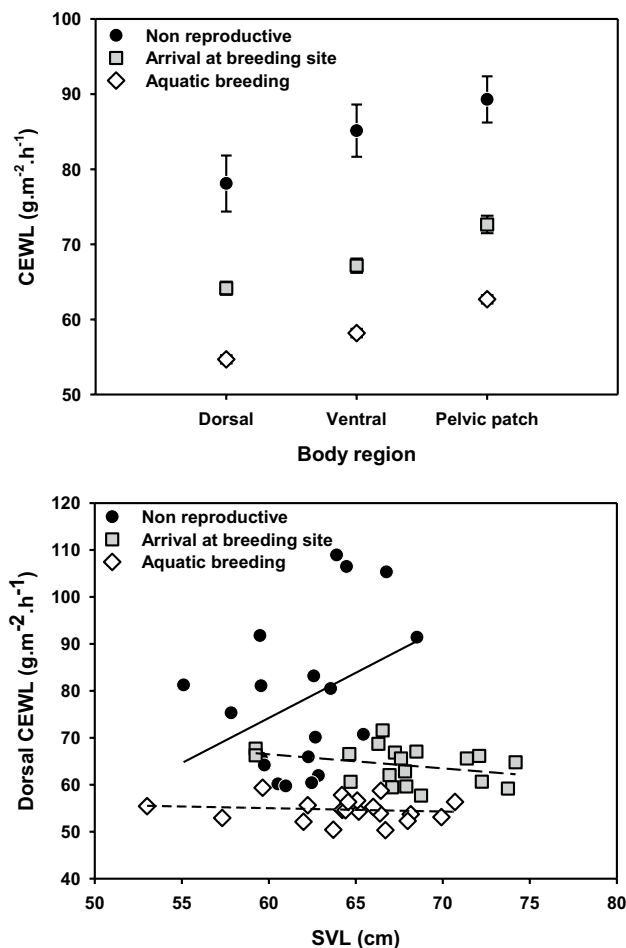


Fig. 2 Cutaneous evaporative water loss (CEWL) measured at three body areas (dorsal, ventral and pelvic patch) during the “non-reproductive” (black circles), “arrival at breeding site” (grey squares) and “aquatic breeding” (white diamonds) sampling periods (upper panel) and relationships between toad size (snout-vent length, SVL) and dorsal cutaneous evaporative water loss (CEWL) during the “non-reproductive” (black circles), “arrival at breeding site” (grey squares) and “aquatic breeding” (white diamonds) sampling periods (lower panel)

($F_{2,52} = 3.71$, $p = 0.03$, Fig. 2) and a similar, but marginal, interaction for ventral CEWL ($F_{2,52} = 2.66$, $p = 0.08$). Interestingly, inter-individual variation of CEWL was almost three times greater during the non-reproductive period (CV ranging from 15.0 to 20.8 depending on body region) than just prior to the arrival at the breeding pond (CV: 5.9–6.9) or during aquatic breeding (CV: 4.0–4.5).

Discussion

We found that the skin permeability of male spined toads decreases significantly prior to breeding. This suggests that the skin shedding that occurs at the onset of breeding (i.e., production of new integument layers with specific properties) may regulate water fluxes in order to alleviate osmotic costs linked to the protracted period of immersion during aquatic breeding (Brischoux and Cheron 2019).

Our results highlighted the regional differences in CEWL, where the dorsal area, which is exposed to more desiccant air, had lower CEWL relative to ventral areas. In addition, the pelvic patch (involved in transcutaneous water intake) had higher CEWL than the two other areas. Yet, the values of CEWL measured at different body areas were highly correlated, suggesting strong regional covariations of this parameter. Interestingly, these regional differences were strongly marked during the non-reproductive period but were also maintained prior to or during aquatic breeding, suggesting that the specific regional properties of skin structure (i.e., keratin, stratum corneum, or lipid layer composition or thickness) were conserved during breeding.

Despite these regional variations, we found that the CEWL of all body areas decreased significantly prior to aquatic breeding and continued to decrease following immersion. Reduced water influxes after shedding may allow individuals to dampen strong deviations in osmotic balance following prolonged immersion in freshwater (Brischoux and Cheron 2019). Importantly, shedding induces a temporary disruption of cutaneous integrity (increased water gain and sodium loss) that lasts a few hours post-sloughing (Jørgensen 1949; Wu et al. 2017). This process lines up particularly well with our field observations, which suggest that skin-sloughing is achieved during terrestrial migration prior to immersion in water. Interestingly, our results highlight that there is a continued decrease in CEWL following immersion in water, suggesting that the affinity for water of the new skin is further reduced during a protracted period of immersion. In addition to possible structural modifications of the skin, a shift to aquatic breeding is likely to involve strong modifications to endocrine regulations (Bentley 1971). In this respect, prolactin is known to influence skin permeability, and notably to induce a reduction in permeability to water (Bentley 1971). Clearly, further studies are required in order to assess how structural skin characteristics (e.g., keratin, stratum corneum, or lipid layer composition or thickness) and endocrine regulation (e.g., prolactin) interact to influence permeability to water during aquatic breeding.

In addition, skin shedding and the subsequent shift to an aquatic lifestyle seem to disrupt the relationship between body size and CEWL. Indeed, during the non-reproductive

period, toad size was positively related to dorsal CEWL and to a lesser extent to ventral CEWL. This result indicates that, during the terrestrial part of their life cycle, smaller individuals — which are presumably more sensitive to dehydration because of a higher surface area to volume ratio — have a lower CEWL which is likely to reduce susceptibility to dehydration. This relationship disappeared during breeding, suggesting that the structural properties of the new skin and/or endocrine regulations interacting to reduce skin permeability are not linked to toad size.

There are some caveats to our investigations notably linked to the fact that we measured CEWL (i.e., water effluxes) rather than water influxes, while aquatic breeding is expected to modify skin permeability to water influxes rather than water effluxes. Yet, both directions of water fluxes are tightly linked and our results are in line with predicted changes. Accordingly, the data on the CEWL of the pelvic patch relative to other body areas suggest that this area, which is dedicated to promote water influxes (cutaneous drinking), also has higher CEWL values and, hence, that the mechanisms responsible for water absorption (e.g., aquaporins, Suzuki et al. 2007) also play a significant role in water loss.

Finally, the breeding strategy of terrestrial toads, and especially the prolonged immersion in water during reproduction, is likely to modify not only water relations but also gas exchanges (Shoemaker et al. 1992, Boutilier et al., 1992, Burggren and Vitalis 2005). For instance, it is likely that submerged breeding toads rely more strongly on cutaneous gas exchange than terrestrial non-reproductive individuals. Future studies should test whether the shift in skin properties that we detected have consequences for the cutaneous gas exchanges of reproductive and non-reproductive toads. In addition, because of the remarkable difference of residence time at breeding ponds between males and females (Brischoux et al. 2018), comparative studies are required to test whether the skin properties of reproductive females undergo similar changes to that of males, in order to assess how divergent reproductive strategies (immersion duration) influence sexual dimorphism in the responses of skin permeability to habitat shifts.

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Author contribution François Brischoux and Olivier Lourdais conceived and designed the study. Marion Cheron and Matthias Renoit performed data collection. François Brischoux performed data analysis and writing of the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Data will be made available upon reasonable request.

Code availability Not applicable.

Declarations

Ethics approval All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities under authorizations number 2015–11-20x-01192, 16–392, and R-45GRETA-F1-10.

Consent to participate Not applicable.

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Competing interests The authors declare no competing interests.

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Annexe III

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Unusual lack of reproduction in toad populations from agricultural habitats

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Unusual lack of reproduction in toad populations from agricultural habitats

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Anthropogenic alterations of habitats can have detrimental consequences for biodiversity. Documenting these effects require monitoring in multiple sites that vary in the degree of alterations over long temporal scales, a task that is challenging. Yet, simple naturalist observations can reveal major ongoing events affecting wild populations, and serve as a basis for further investigations. We quantified breeding parameters of spined toad (*Bufo spinosus*) populations from forested (preserved) and agricultural (altered) habitats. We found that reproduction did not occur at the sites surrounded by agriculture, while it occurred successfully in ponds from forests. Males were present at all sites, but females, amplexus, egg strings and tadpoles remained absent from agricultural sites. Observations made at the same sites indicated that breeding occurred during previous years. Our observations of habitat- and sex-specific lack of reproduction may have critical consequences for the persistence of populations of a widespread amphibian species in agricultural areas.

Keywords: Amphibian, *Bufo spinosus*, breeding, conservation, reproductive success

INTRODUCTION

Biodiversity is dramatically affected by human activities leading to an alteration of ecosystems (Chapin et al., 2000; Myers & Knoll, 2001; Brooks et al., 2002). Human activities, such as intensive farming, generate habitat alteration, fragmentation and simplification (e.g. Maron & Fitzsimons, 2007). In addition, agricultural landscapes often suffer from the massive use of pesticides, which contaminate the environment and the wildlife (Schäfer et al., 2007).

As a consequence, these modern agricultural practices can have detrimental impacts on fauna and flora (Myers & Knoll, 2001; Brooks et al., 2002; Fahrig, 2003; Relyea, 2009). In order to persist in these altered habitats, wildlife must adjust to these ongoing changes. However, the ability of a species to persist in agriculture landscapes can be jeopardised when critical elements necessary to

perform its life-cycle are missing in the environment. For example, the lack of trees or shrubs can impair the ability of some bird species to breed in simplified landscapes (Newton, 1994; Verhulst et al., 2004). Similarly, amphibian populations will disappear if suitable breeding ponds are missing following habitat simplification (Smith & Green, 2005). In addition to habitat alteration, other effects can be linked to the increasing use of chemical inputs that aim to improve crop productivity in agricultural habitats (McLaughlin & Minneau, 1995; Köhler & Triebskorn, 2013). For instance, pesticides are used to control pests (e.g., weeds, insect, fungi) that negatively impact crop productivity. These pesticides can have toxic effects on non-target components. For example, they have been shown to negatively impact reproduction in wildlife species, through various mechanisms that spans from direct toxic or sublethal effects (Mnif et al., 2011; Cheron & Brischoux, 2020) to alterations of ecosystem functioning (e.g., disruption of the food web, Relyea & Hoverman, 2008).

The direct effects of habitat alteration on population persistence are relatively easy to assess (see above). Yet, assessments of indirect effects of agricultural practices on population persistence are more challenging and require population monitoring in multiple sites that vary in their habitat structure (i.e., degree of alteration and fragmentation). To document these effects, simple naturalist observations can be important because they often help to reveal major ongoing and detrimental events that affect wild populations (Sagarin & Pauchard, 2010; Sagarin & Pauchard, 2012; Mauz & Granjou, 2013).

During the course of a study that aimed to compare toad (*Bufo spinosus*) populations between forested (preserved) areas and agricultural (simplified) habitats, we opportunistically quantified breeding parameters (number of males, presence of amplexus, egg strings and tadpoles) in both types of habitat in Western France (Fig. 1). The toad (*Bufo spinosus*) is a widespread species that can live in a variety of habitats and has been previously shown to persist even in highly modified agricultural areas (Arntzen et al., 2014; Guillot et al., 2016). As in most anuran species, *B. spinosus* have a biphasic life-cycle with an extensive use of terrestrial habitats during

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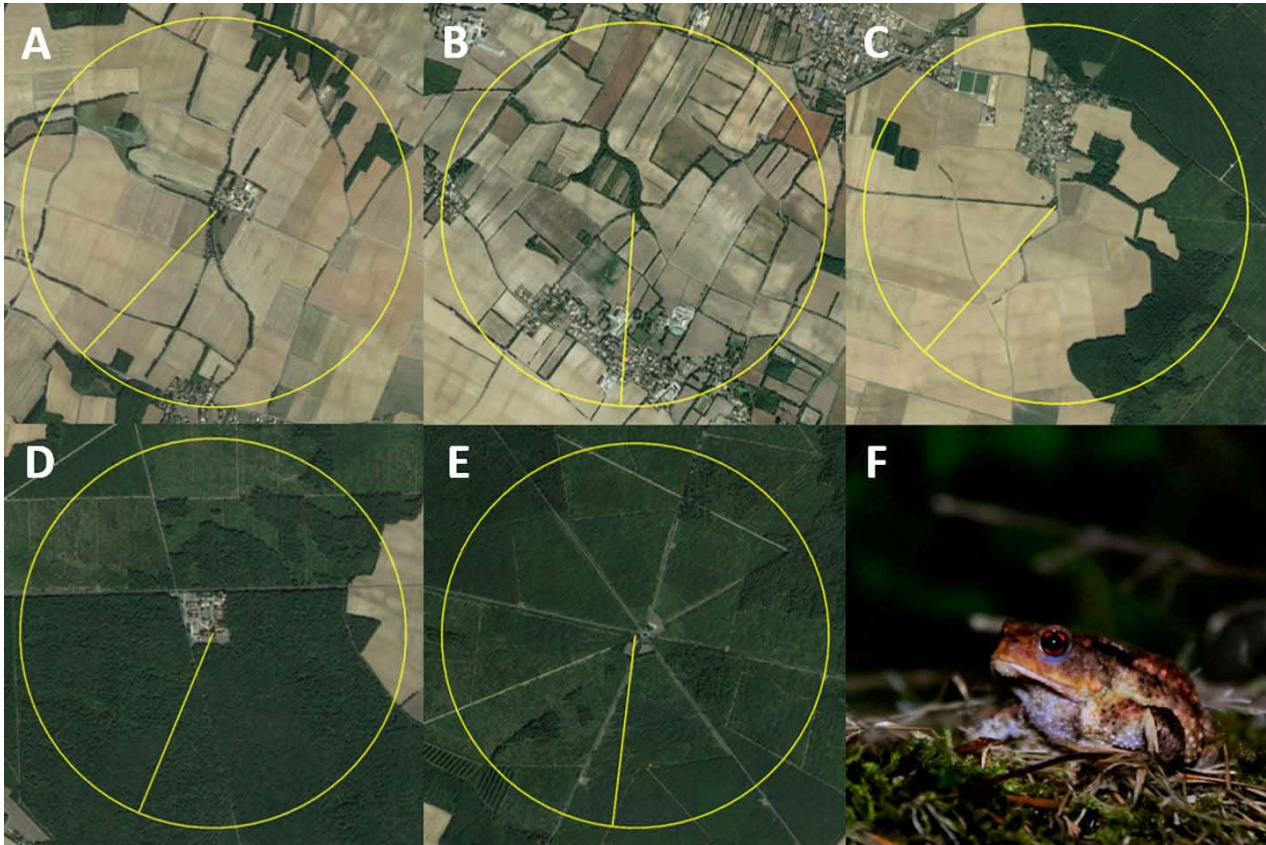


Figure 1. **A-D:** Aerial pictures (Google Earth) of the five study sites with the one km radius surrounding breeding ponds used to illustrate the contrast between three agricultural sites (A, B and C) and two forested sites (D and E). Letters in the pictures relate to site numbers in Table 1. **F:** Picture of an individual *Bufo spinosus* in the field in South Deux-Sèvres, France.

most of the year, and a short breeding season (~1 month) in aquatic sites (ponds) where mating occurs and eggs and tadpoles develop (Reading, 1998; Kelleher et al., 2018; Brischoux & Cheron, 2019). The breeding season occurs at the end of winter (February – March). During this period, male toads migrate towards aquatic breeding sites where they wait for females (Reading, 1998). Males can remain at the breeding site for several weeks, while females leave shortly after mating and egg-laying (Davies & Halliday, 1977). Eggs and tadpoles develop over three to four months before metamorphosis and subsequent dispersal in nearby terrestrial habitats. Reproductive events can be easily assessed later in the season (when breeders have left the breeding site) by monitoring the presence of egg strings and tadpoles.

The terrestrial part of the annual cycle of toads occurs in various environments usually within one km from the breeding pond (Janin et al., 2011; Guillot et al., 2016). Two of our study sites were located in forested areas where forest cover represented > 95 % within a circle of a one km radius centered on the breeding pond; while three sites were located in agricultural areas (composed mainly of large fields) where forest cover was always < 35 % within the same surface area (Fig. 1). Forest and agricultural sites were situated in close proximity (maximum distance 12 km) in order to avoid diverging climatic conditions that may affect timing of reproduction.

Observations were made from early January (week

one) to late June (week 26) 2020. At the onset of the reproductive period (from week one to week 11) all study sites were monitored every night. Observations were stopped from week 12 to week 16 because of the lockdown linked to the COVID-19 pandemic. Observations resumed on week 17 on a monthly basis until late June (week 26) in order to assess the presence of developing tadpoles.

Due to of the diverging reproductive behaviour of males and females (see above), we made the following observations. Males were individually counted when abundances were < 10 individuals and number of individuals was approximated by increment of 10 individuals when abundances were > 10 individuals. Females remain only briefly at the breeding pond, and amplexus occurs in areas where precise quantification is precluded (in highly vegetated areas or deeper water). As a consequence, we assessed female presence through the observation of amplexus and qualified for each site whether amplexus was observed or not (present/absent). When reproduction occurred, large numbers of egg strings and tadpoles precluded direct enumeration and successful reproduction was assessed with the presence/absence of egg strings and tadpoles.

We emphasise that our opportunistic observations are qualitative rather than quantitative for most parameters recorded as they were not directly linked to the primary goal of the surveys we performed (assessment of reproductive success across habitats).

Table 1. Summary of the data collected during our surveys. Male abundances show min-max number of individuals observed for each week. Female presence or absence was assessed through observations of amplexus. The presence of egg strings and developing tadpoles was also documented. “ND” stands for “no data”. “NO” refers to absence of individuals at periods during which presence was expected, while “-” refers to absence of individuals at periods when absence was expected.

Observations	Sites	Habitat	Week number												
			1-4	5	6	7	8	9	10	11	12-16	17	21	26	
Number of males	A	Agriculture	0	1-3	1	0	0	0	0	0	0	ND	-	-	-
	B	Agriculture	0	40	10-40	3-10	1-3	1	1	0	ND	-	-	-	
	C	Agriculture	ND	ND	ND	100	70	50	50	50	ND	-	-	-	
	D	Forest	0	10-20	20	20	10-20	10-20	10	10	ND	-	-	-	
	E	Forest	0	30	30	20	10-20	20	20	10	ND	-	-	-	
Presence of amplexus	A	Agriculture	NO	NO	NO	NO	NO	NO	NO	NO	ND	-	-	-	
	B	Agriculture	NO	NO	YES	YES	NO	NO	NO	NO	ND	-	-	-	
	C	Agriculture	ND	ND	ND	YES	NO	NO	NO	NO	ND	-	-	-	
	D	Forest	NO	YES	YES	YES	YES	YES	YES	YES	ND	-	-	-	
	E	Forest	NO	YES	YES	YES	YES	YES	YES	YES	ND	-	-	-	
Presence of egg strings	A	Agriculture	NO	NO	NO	NO	NO	NO	NO	NO	ND	-	-	-	
	B	Agriculture	NO	NO	NO	NO	NO	NO	NO	NO	ND	-	-	-	
	C	Agriculture	ND	ND	ND	NO	NO	NO	NO	NO	ND	-	-	-	
	D	Forest	NO	NO	YES	YES	YES	YES	YES	YES	ND	-	-	-	
	E	Forest	NO	NO	YES	YES	YES	YES	YES	YES	ND	-	-	-	
Presence of tadpoles	A	Agriculture	-	-	-	-	-	-	-	-	ND	NO	NO	NO	
	B	Agriculture	-	-	-	-	-	-	-	-	ND	NO	NO	NO	
	C	Agriculture	-	-	-	-	-	-	-	-	ND	NO	NO	NO	
	D	Forest	-	-	-	-	-	-	-	-	ND	YES	YES	NO	
	E	Forest	-	-	-	-	-	-	-	-	ND	YES	YES	NO	

Observations are summarised in Table 1. Overall, we found that reproduction did not occur at the three sites from agricultural habitats, while it occurred successfully in breeding ponds from forested areas (presence of egg strings and tadpoles, Table 1).

At all of our study sites, breeding males were present, yet with variable abundances (Table 1). Mean number of adult males was 19.0 ± 28.4 (range 0-100) for agricultural sites and 15.6 ± 8.3 (range 0-30) for forest sites (Table 1). These numbers suggest that abundances of reproductive males did not seem to be related to the surrounding habitat structures. Indeed, some sites from agricultural areas displayed numbers of males that equaled or even exceeded those from forested habitats (Table 1). Importantly, the onset of the reproductive period (first observations of males occurring at the study sites) was similar between habitat types (occurring on week 5, Table 1), suggesting that climatic (micro-) conditions did not significantly influence reproduction between sites. These observations tend to further indicate that the lack of reproduction we recorded (see below) may not be linked to a lack of breeding males (although one agricultural site was characterised by lower abundances, Table 1), but rather to a lack of reproductive females.

Indeed, the most clear-cut difference between our study sites was linked to the presence of females (assessed through the presence of visible amplexus, Table

1) and their reproductive success (assessed through the presence of egg strings and developing tadpoles, Table 1). Amplexus was observed on very few nights (one or two nights) at two of the agricultural sites, and was not observed at the other agricultural site. Conversely, amplectant pairs were observed steadily almost every night over six weeks at the sites surrounded by forest. No egg-strings or developing tadpole were observed at all three sites from agricultural habitats, while egg strings and developing tadpoles were present at the two forest sites. Importantly, these observations suggest that females did not migrate to breed in sites surrounded by agricultural areas and, thus, that habitat-specific and sex-specific responses to habitat perturbations occurred in adult females.

It is important to stress that our observations are unreplicated and preliminary and that we have not observed this phenomenon in previous years. Therefore, these observations do not give any strong clue regarding the mechanisms through which habitat-specific and probably sex-specific lack of reproduction has occurred. Yet, previous observations made at the same study sites (Guillot et al., 2016; MC and FB unpublished data) indicate that breeding successfully occurred at some of these agricultural sites at least in 2015 and in 2019; 2 years during which we monitored reproduction at some of those sites and for which egg strings and developing

tadpoles were observed. Although we acknowledge the limitations of our observational study, we believe it is important to document, at least in a qualitative way, a potential problem for the persistence of the populations of a widespread amphibian species in agricultural areas (Guerry & Hunter, 2002, Boissinot et al., 2019); and we urge other researchers to share similar observations.

Author Contributions

F.B. and F.A. proposed the initial idea and together with M.R. and M.C. contributed to its development. M.R., M.C. and F.B. performed field work. M.R. and M.C. tabulated the resulting data. All authors discussed the results, and substantially contributed to the writing.

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