Perception et apprentissage prérinatal chez la seiche : approche comparative et effet du stress

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THÈSE

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Perception et apprentissage prérinatal chez la seiche : approche comparative et effet du stress

Présentée et soutenue par
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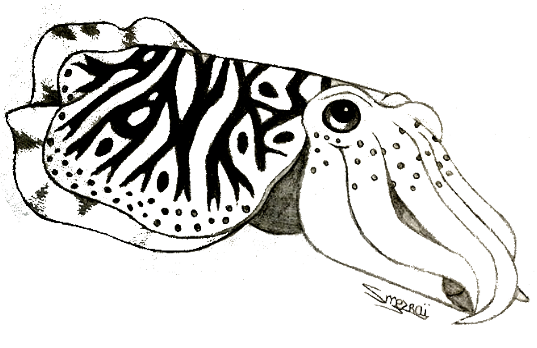

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- Darmaillacq, A.-S., **Mezrai, N.,** O'Brien, C. E., & Dickel, L. (2017). Visual ecology and the development of visually guided behavior in the cuttlefish. *Frontiers in Physiology*, *8* (**Annexe 1**).
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- O'Brien, C. E., Bellanger, C., Jozet-Alves, C., **Mezrai, N**., Darmaillacq, A. S., & Dickel, L. (2018). Stressful conditions affect reproducing cuttlefish (*Sepia officinalis*), reducing egg output and quality. *ICES Journal of Marine Science* (**Annexe 3**).
- O'Brien, C. E., Jozet-alves, C., **Mezrai, N.,** Bellanger, C., Darmaillacq, A.-S., & Dickel, L. (2017). Maternal and Embryonic Stress Influence Offspring Behavior in the Cuttlefish *Sepia officinalis. Frontiers in Physiology,* 8 (**Annexe 4**).
- **Mezrai, N.,** Dickel, L., Chiao, C.-C., & Darmaillacq, A.-S., submitted in *Developmental Psychobiology*. A difference in timing for the onset of visual and chemosensory systems during embryonic development in two closely related cuttlefish species (**Chapitre 3**).
- **Mezrai, N.,** Arduini, L., Dickel, L., Chiao, C.-C., & Darmaillacq, A.-S., submitted in *Learning & Behavior*. Awareness of danger inside the egg: evidence of innate and learned predator recognition in cuttlefish embryo (**Chapitre 4**).
- **Mezrai, N.,** Tchadjiane, C., Dickel L., & Darmaillacq, A.S., in preparation. Prenatal stress effect on simple and associative learning abilities in cuttlefish *Sepia officinalis* (**Chapitre 5**).
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- **SFECA** (Société Française pour l'Etude du Comportement Animal) : *March 22-24, 2016 – Caen, France.* **MEZRAI**, ARDUINI, DICKEL, DARMAILLACQ. Visual and chemosensory perceptual abilities in cuttlefish embryos (*Sepia officinalis*).

- **SFECA** (Société Française pour l'Etude du Comportement Animal) : *May 15-17, 2017 – Gif-sur-Yvette, France.* **MEZRAI**, ARDUINI, CHIAO, DICKEL, DARMAILLACQ. L'embryon de seiche est-il capable d'apprendre dans son œuf ?

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- **SFECA** (Société Française pour l'Etude du Comportement Animal) : *March 22-24, 2016 – Caen, France* **MEZRAI**, HOUDELIER, BERTIN, CALANDREAU, ARNOULD, DARMAILLACQ, DICKEL, LUMINEAU. Le stress prenatal a-t-il toujours des effets délétères sur l'ontogenèse comportementale chez l'oiseau ? **Prix du meilleur poster**.

- **Behaviour 2017** - 35th International Ethological Conference (IEC): *July 30 - August 4, 2017 – Estoril, Portugal.* **MEZRAI**, ARDUINI, CHIAO, DICKEL, DARMAILLACQ. Can cuttlefish embryo recognize predators in egg?

LISTE DES ABREVIATIONS :

RV / VR : rythme ventilatoire / ventilation rate

SA / AS : stress artificiel / artificial stress

SN / NS : stress naturel / natural stress

CRF : corticotropin-releasing factor

ACTH : adrenocorticotropic hormone

GC : glucocorticoïdes

PVF : Perivitelin fluid / liquide périvitellin

Sommaire

Préambule - Contexte scientifique de l'étude

Un peu d'histoire :

L'éthologie est l'étude biologique du comportement animal. Ce terme est issu des deux mots grecs « ethos » qui signifie « mœurs » et « logos », qui signifie « science ». Cette thématique de recherche s'intéresse alors à l'étude des mœurs des animaux. Aristote (-384/-322) est sans doute le premier philosophe à s'intéresser aux animaux et à leurs comportements. C'est le premier à écarter la possibilité que l'âme soit une substance et la définit comme étant une caractéristique fonctionnelle de tous les êtres vivants. Cependant, il sépare tout de même l'humain et l'animal. Selon lui, seul l'humain possède une âme pensante, une raison et un langage. Cette pensée dualiste est partagée par Descartes (1596-1650). Il pense que l'humain est le seul à être doué de raison et que les animaux sont des machines assimilables à des automates. Lloyd Morgan (1852-1936) a quant à lui suivi une approche objective et a décrit le principe de parcimonie (aussi appelé canon de Morgan). Ce principe, décrit en 1894, stipule qu' « en aucun cas nous ne devons interpréter une action comme le produit d'une faculté psychologique supérieure si ces comportements peuvent être expliqués par des mécanismes plus élémentaires ». Puis, Watson (1878-1958), un psychologue américain, fonde le **béhaviorisme** et en établit les bases en 1913 à partir de travaux sur le conditionnement. Pour lui, ainsi que pour tous les behavioristes, les comportements sont prévisibles si l'on connait les stimuli auxquels les animaux sont soumis. Ils utilisent des méthodes et dispositifs très rigoureux et les conditions expérimentales sont strictement contrôlées. En parallèle, les **naturalistes** s'intéressent aux comportements et décrivent les « mœurs » des espèces. C'est en 1854 que Saint Hilaire (1805-1861) utilise le terme « **éthologie** » pour la première fois pour qualifier l'étude naturaliste du comportement des espèces. Les naturalistes considèrent les êtres vivants comme produits des pressions évolutives de leur environnement. Selon les naturalistes, tels que Darwin (1809-1882), il existe une continuité évolutive entre l'humain et l'animal. C'est de ce courant naturaliste profondément évolutionniste et comparatiste que nait l'éthologie en tant que discipline de la biologie. L'influence de Lorenz, Tinbergen et von Frisch sera fondamentale dans le développement de ce courant. En 1963, Tinbergen détaille la démarche et les questions de l'éthologie. Il distingue les questions relatives à des problèmes proximaux : la **causalité** (les facteurs de l'environnement) ; le **développement** (l'histoire de l'individu) et les questions relatives à des problèmes ultimes comme l'**évolution** (l'histoire de l'espèce) et la **fonction** des comportements (sa valeur adaptative).

***Contexte scientifique de l'étude* :**

Au cours de ce manuscrit de thèse nous nous intéresserons principalement à la question du développement des comportements, aussi appelé « **ontogenèse comportementale** ». Ce processus dynamique et complexe désigne l'ensemble des processus de développement des comportements d'un individu. Chez les animaux, humain compris, chaque individu a son propre phénotype comportemental (ensemble de caractères observables). Sa réalisation fait appel aux contraintes imposées par le génome mais également aux influences du milieu dans lequel ce programme se déroule. Pendant longtemps, une dichotomie très forte existait entre **les comportements innés** (déterminés génétiquement, ne nécessitant pas d'apprentissages préalables) et **les comportements acquis** (qui sont le résultat de l'expérience et de l'apprentissage individuel). Cette dichotomie est encore très débattue dans la communauté scientifique. Pourtant, il ne faut jamais perdre de vue que la génétique interagit avec l'expérience acquise pour déterminer le comportement. Par exemple, le développement vocal des primates non-humains est considéré comme étant sous l'emprise d'un fort déterminisme génétique (Snowdon & Hausberger, 1997) mais dont l'acquisition nécessite un apprentissage social complexe (Pistorio, Vintch, & Wang, 2006; Snowdon & Elowson, 2001; Takahashi et al., 2015). Les influences environnementales vont alors façonner les individus et ce dès la période prénatale. Pourtant, en raison de leur **inaccessibilité** et de leur développement cérébral inachevé, les embryons et fœtus étaient considérés comme immatures et incapables d'apprentissage. Ce n'est seulement qu'après le début des études sur les embryons et les fœtus que le nouveau-né n'est plus considéré comme étant une « page blanche sur laquelle il n'y a rien écrit ». Le début de l'embryologie expérimentale par Roux en 1888, les premières études comportementales chez les embryons d'animaux par Gottlieb dans les années 1960 et les premières échographies obstétricales en 1950 ont permis de comprendre que les embryons et les fœtus ne sont pas isolés de leur environnement. Ils sont en effet capables de **percevoir** et d'**apprendre** d'un grand nombre de stimulations environnementales qui auront un impact plus ou moins important sur leur développement et donc sur leur phénotype comportemental. Il est de plus possible que des **stimulations stressantes** aient des effets sur ces capacités cognitives précoces. L'objectif de cette thèse est alors d'étudier ces capacités précoces et les effets du stress sur ces dernières chez deux céphalopodes : la seiche commune *Sepia officinalis* et la seiche pharaon *Sepia pharaonis*. Ces ovipares nous donnent la possibilité d'étudier les effets du stress embryonnaire en nous affranchissant de la mère. Contrairement aux oiseaux, leurs œufs sont transparents et les embryons sont directement observables.

Chapitre 1

Introduction générale

Chapitre 1 – Introduction générale

Dans ce chapitre introductif, nous montrerons que les embryons sont capables de percevoir et de répondre à leur environnement mais aussi d'apprendre de celui-ci. Nous verrons aussi que les stimulations environnementales, lorsqu'elles sont trop fortes peuvent perturber la mise en place de certains comportements et que ce stress prénatal peut avoir un impact fort sur l'ontogenèse comportementale des jeunes.

I. Développement prénatal des systèmes sensoriels

Pendant la période prénatale, les systèmes sensoriels des Vertébrés se développent en suivant une séquence chronologique et invariante : le système somatosensoriel (tactile et vestibulaire) se développe le premier, suivi du système chimiosensoriel (olfactif et gustatif), du système auditif et finalement du système visuel (Bremner et al., 2012; Carlsen & Lickliter, 1999; Gottlieb, 1968, 1976a; Hepper, 2015; Lickliter, 2000; Spreen, Risser, & Edgell, 1995). Le degré de maturité de chaque système sensoriel à la naissance/éclosion varie selon les espèces. Chez les espèces nidicoles (ou « altrices ») telles que les rats, les souris et les pigeons, les jeunes sont relativement peu développés à la naissance/l'éclosion : les yeux sont fermés et le plumage/pelage est non développé. Leurs systèmes auditif et visuel sont peu fonctionnels pendant les premiers jours de vie postnatale. En revanche, les animaux nidifuges (ou « précoces »), tels que les cobayes, les moutons et les poules, ont des petits particulièrement développés à la naissance, capables de se déplacer et de survivre avec peu ou pas de soins parentaux. Pour ces espèces précoces, tous les systèmes sensoriels et moteurs se développent et sont matures avant la naissance (Gottlieb, 1968, 1971; Lickliter, 2000). Qu'ils soient nidicoles ou nidifuges, les systèmes sensoriels des Vertébrés commencent à se développer pendant la phase de neurulation de l'embryogenèse (Hepper, 2015; Spreen et al., 1995). Sur le plan anatomique, les systèmes sensoriels commencent à se développer simultanément. Le développement fonctionnel se déroule toutefois en suivant une séquence sans chevauchement. Dans la section suivante, nous examinerons brièvement le développement anatomique et fonctionnel des systèmes sensoriels des Vertébrés ainsi que de ceux de certains Invertébrés.

1) Le système somatosensoriel

Selon Purves et ses collaborateurs le système somatosensoriel comprend trois composantes: (1) la sensibilité externe de la peau liée au touché, à la température et/ou à la douleur ; (2) la proprioception de la position et des mouvements du corps liés aux récepteurs musculaires et articulaires et (3) la sensibilité interne ou viscérale aux changements physiologiques ou biochimiques de l'environnement interne (Purves et al., 2004).

Les études concernant les réponses embryonnaires aux stimulations vestibulaires sont très rares dans la littérature et se limitent aux études axées sur les potentiels d'action évoqués vestibulaires (Jones et al., 2000; Jones, Erway, Bergstrom, Schimenti, & Jones, 1999; Jones & Jones, 2000; Jones, Fermin, Hester, & Vellinger, 1993). Au cours de ces études aucune description comportementale n'a été réalisée. En ce qui concerne les réponses aux stimulations tactiles, que ce soit chez les espèces nidicoles ou nidifuges, la première réponse à une stimulation tactile apparaît très tôt dans le développement embryonnaire. Les réponses des embryons sont d'abord très générales (mouvement de l'ensemble du corps) et deviennent de plus en plus spécialisées (mouvement de la zone stimulée) lors du développement prénatal (poussin domestique *Gallus gallus domesticus* : Bekoff, 2001; Hamburger, 1971; Rogers, 1995 ; zebra fish *Danio rerio* : Saint-Amant & Drapeau, 1998 ; rat *Rattus norvegicus* : Bekoff, 2001; Narayanan, Fox, & Hamburger, 1971.

Chez les espèces nidifuges, le système somatosensoriel est développé plus tôt que chez les espèces nidicoles. Pour illustrer cette observation, prenons l'exemple du rat (*Rattus norvegicus*), une espèce chez laquelle des stimuli péri-oraux induisent des réponses locales (mouvements d'évitement par exemple) à partir du 16ème jour embryonnaire (69% de la gestation totale). À partir du 17ème jour (74%) les réponses deviennent de plus en plus globales (mouvement de tout le corps) et cette motilité réflexogène gagne les régions postérieures (Narayanan et al., 1971). Quatre jours avant la naissance (83%), ces mouvements généralisés cèdent la place à des réponses spécialisées (mouvement de la zone stimulée uniquement). Chez une espèce nidifuge, telle que le poussin domestique (*Gallus gallus domesticus*), la motilité réflexogène de l'embryon apparaît à partir du 7ème jour embryonnaire (31% de l'incubation totale) suite à une stimulation de la région péri-orale (Hamburger, 1971). La réponse n'est d'abord que locale, mais elle devient globale dès le 8ème jour (38%). A partir du 11ème jour (52%), ces réponses deviennent plus spécialisées (Hamburger, 1971).

2) Le système chimiosensoriel (olfactif et gustatif)

La détection des substances chimiques dans l'environnement extérieur est réalisée au moyen de trois systèmes sensoriels oraux et nasaux : le système olfactif (odeur), gustatif (saveurs) et trigéminal (détection des substances irritantes qui entrent en contact avec la peau ou les muqueuses des yeux, du nez ou de la bouche) (Bremner et al., 2012; Gilles & Anctil, 2006; Viana, 2011). Chez l'embryon, les études comportementales sont principalement axées sur l'olfaction et la gustation qui sont des sens difficilement dissociables. En effet, les odeurs/saveurs qui se retrouvent dans leur environnement immédiat vont entrainer des stimulations à la fois du système sensoriel olfactif mais aussi gustatif (par exemple chez l'humain la molécule odorante se retrouvera dans le liquide amniotique et sera capté par les papilles gustatives mais aussi par la muqueuse olfactive). Le développement de ce système sensoriel a fait l'objet de nombreuses études chez les mammifères en raison de son rôle dans l'établissement du lien mère-enfant (Bremner et al., 2012; Lecanuet et al., 1993; Lecanuet, Granier-Deferre, & Schaal, 2004; Purves et al., 2004; Schaal, 1984; Schaal, Coureaud, Marlier, & Soussignan, 2001; Schaal, Marlier, & Soussignan, 2000). Il a été démontré que les mammifères sont capables de percevoir les stimuli chimiques *in utero* grâce aux mouvements respiratoires qui renouvellent en permanence le liquide amniotique en contact avec la muqueuse olfactive de l'embryon. Les premiers mouvements respiratoires sont observés à différents moments selon les espèces. Chez les espèces nidicoles telles que les chiens (*Canis familiaris*), ces mouvements commencent au 50ème jour embryonnaire (69% de la gestation totale), alors qu'ils apparaissent beaucoup plus tôt chez le mouton (*Ovis Aries*), une espèce nidifuge (40ème jour embryonnaire ; 27% ; Dawes, Fox, Leduc, Liggins, & Richards, 1972; Hepper & Wells, 2006). Même s'ils sont à l'intérieur d'un œuf, les embryons ovipares peuvent également percevoir certains stimuli environnementaux. Chez la poule domestique, les réponses électrophysiologiques de l'épithélium olfactif apparaissent en premier dès le 13ème jour embryonnaire (62%) après une stimulation olfactive (Lalloue, Ayer-Le-Lievre, & Sicard, 2003). Cette espèce est également capable de réagir aux stimuli chimiques la veille de l'éclosion (20ème jour ; 95%). La détection des odeurs provoque une augmentation de la fréquence cardiaque, du nombre de mouvements de tête et de claquements de becs (Tolhurst & Vince, 1976).

3) Le système auditif

L'audition joue un rôle important dans le comportement de nombreux animaux. Les différents sons émis par les animaux sont extrêmement importants dans le contexte social : communication entre les individus pour indiquer la présence de prédateurs ; reconnaissance mère/jeune, territorialité, parade sexuelle... Ce système sensoriel est également mis en place très tôt dans le développement. Chez la plupart des Vertébrés (mammifères, reptiles et oiseaux), les sons sont recueillis par l'oreille externe et/ou le canal auditif et sont acheminés jusqu'au tympan. Ils sont ensuite transmis à l'oreille moyenne (1 à 3 osselets selon les espèces) puis à l'oreille interne (cochlée chez les mammifères et les oiseaux) dont le rôle est de transformer la vibration sonore en influx nerveux (Gilles & Anctil, 2006; Graven & Browne, 2008; Rosenzweig, Leiman, & Breedlove, 1998). Chez la souris, le rat et le poussin, la cochlée se développe avant la naissance et l'éclosion (Kandler & Friauf, 1993; Rubel & Fritzsch, 2002; Saunders, Coles, & Richard Gates, 1973).

De nombreux Vertébrés sont ainsi capables de percevoir et de réagir au son avant la naissance/l'éclosion. Chez les mammifères, comme chez l'humain, l'embryon est doté de toutes les structures anatomiques nécessaires à l'audition et est capable de répondre par une accélération du rythme cardiaque et une variété de mouvements du corps tels que des changements de position, des mouvements de la tête et des membres (Graven & Browne, 2008; Lecanuet et al., 1987, 1987; Lecanuet, Granier-Deferre, & Busnel, 1988; Rondal, 1999). Chez le canard musqué (*Cairina moschata*), les vocalisations spécifiques et non-spécifiques entrainent des potentiels électriques dans les noyaux cochléaires auditifs à partir du 21ème jour embryonnaire (75% de l'incubation totale ; Höchel, Pirow, & Nichelmann, 2002). Au 23ème jour (82%), la fréquence cardiaque augmente en raison de ces stimuli, démontrant une réponse physiologique. À ce moment-là, ils sont également capables de faire la distinction entre les vocalisations spécifiques et non-spécifiques, répondant uniquement aux vocalisations spécifiques par des claquements de bec (Gottlieb, 1971). De la même manière, les premières réponses cardiaques à différents sons sont apparentes à partir du 27ème jour embryonnaire (77-80% ; Höchel et al., 2002).

4) Le système visuel

Le système sensoriel visuel permet de reconnaître les congénères (parents, fratrie, partenaires sexuels…) mais aussi les prédateurs et les proies. Il est donc très important que cette modalité sensorielle soit développée, déjà mature et fonctionnelle à la naissance ou l'éclosion, en particulier chez les espèces précoces qui doivent être relativement autonomes pour survivre (Gottlieb, 1971). Chez les vertébrés, il a été démontré que le système visuel se développait au cours de la vie fœtale et devenait fonctionnel avant la naissance. Chez l'humain par exemple, une lumière peut induire des réactions chez le fœtus. Au cours d'une intervention médicale, une lumière froide introduite dans l'utérus par le tractus génital chez les femmes au moment de la 24ème semaine de grossesse (86% du total) entraîne une augmentation du rythme cardiaque fœtal (Peleg & Goldman, 1980). Même si les stimulations visuelles prénatales chez les mammifères peuvent apparaitre très limitées, la lumière externe peut cependant modifier la luminosité intra-utérine en fonction de sa puissance et en fonction de l'épaisseur des tissus maternels (Parraguez et al., 1998). Effectivement, à l'aide de capteurs lumineux intra-utérins introduits par chirurgie chez la brebis gestante, la transmission de la lumière externe *in utero* apparaît variable tout au long de la journée et augmente en fonction de l'âge gestationnel. La luminosité intra-utérine peut aller jusqu'à 4,7% de celle mesurée sur les flancs maternels (Parraguez et al., 1998). Cependant, au cours de cette expérience, aucune réponse embryonnaire n'est mentionnée.

Chez les espèces ovipares, les embryons se développent à l'extérieur de l'organisme maternel mais la lumière va traverser l'œuf et atteindre l'embryon en développement. Les oiseaux peuvent percevoir ces stimulations grâce à leur œil droit puisque l'embryon est tourné de telle sorte que seul cet œil reçoit les stimulations lumineuses passant à travers la coquille (l'œil gauche est caché par son corps ; Rogers & Workman, 1989; Rogers, 1982, 1989). De la même façon, toujours chez l'oiseau, les embryons de colin de Virginie (*Colinus virginianus*) sont capables de percevoir et de réagir à la lumière. La fréquence et l'activité cardiaques augmentent lorsqu'une lumière est appliquée sur l'œuf dès le 21ème jour d'incubation (91%) (Reynolds & Lickliter, 2002).

II. Capacité d’apprentissage embryonnaire

Nous venons de voir dans la première partie que les embryons et les fœtus peuvent percevoir et réagir aux différents stimuli environnementaux et que cette capacité est possible grâce au développement chronologique, séquentiel et invariant des systèmes sensoriels. Ces stimuli peuvent influencer le développement comportemental des individus et leurs effets persistent après la naissance. Par exemple, certaines stimulations tactiles et vestibulaires peuvent moduler le rythme d'activité des jeunes oiseaux après l'éclosion (poules domestiques; Guyomarc’h, Yris, & Fontelle, 1973). Certains stimuli visuels, tels que la lumière, peuvent influencer la latéralité visuelle des jeunes (poussin domestique : Riedstra & Groothuis, 2004; Rogers, 1989, 2012; colin de Virginie *Colinus virginianus*: Casey & Lickliter, 1998). En plus de ces effets d'exposition, nous savons maintenant que les embryons peuvent apprendre de ces stimulations prénatales. Une définition très large consiste à envisager l’apprentissage comme toute modification durable du comportement liée à une expérience sensorielle passée (Pearce, 2013). De ce fait, lorsqu’il y a perception il peut alors y avoir apprentissage. Cette seconde partie traitera des différentes capacités d'apprentissage chez l’embryon. Nous évoquerons dans un premier temps les capacités d’apprentissage prénatal simple de type habituation/deshabituation. Puis nous détaillerons les apprentissages perceptifs (sans renforcement) qui sont importants dans la mise en place des préférences alimentaires et de la reconnaissance sociale et maternelle. Enfin nous verrons que les embryons sont capables d’apprentissage associatif et qu’ils sont primordiaux dans la reconnaissance des dangers.

1) Habituation / Déshabituation

Le paradigme d’habituation/déshabituation est sans doute le plus répandu et le plus utilisé. C’est un apprentissage non-associatif simple dans lequel un organisme cesse de répondre à un stimulus non douloureux après une exposition répétée ou prolongée à celui-ci (Bouton, 2007; Thompson & Spencer, 1966). Il existe chez les organismes les plus simples (organismes unicellulaire) jusqu’aux plus complexes comme les humains (Bouton, 2007; Thompson & Spencer, 1966). Un phénomène de fatigue motrice peut être exclu en appliquant un stimulus déshabituateur à la suite de cette répétition. Si ce nouveau stimulus induit une restauration de la réponse, cela signifie que l'embryon s'est habitué au premier. Dans les études comportementales, le stimulus déshabituateur permet également de tester les capacités de discrimination. Si une nouvelle réponse est visible, cela signifie que l'individu peut alors distinguer les deux (Lecanuet, Fifer, Krasnegor, & Smotherman, 2013).

L'une des premières études portant sur ces capacités a été réalisée en 1992 par Smotherman et Robinson sur l’embryon de rat. Ces données sur l'habituation à une stimulation chimiosensorielle ont indiqué que la fréquence cardiaque et l'activité motrice peuvent varier indépendamment. Dans cette étude, une exposition à une solution odorante de citron chez l’embryon de rat induit une augmentation de l’activité motrice et une bradycardie au cours des derniers jours de la gestation (Smotherman & Robinson, 1992). Dans une seconde expérience, ils ont montré qu'une simple présentation de menthe après une série d'expositions au citron permettait de rétablir efficacement les réactions motrices embryonnaires au citron (Smotherman & Robinson, 1992). Au cours de ces expériences, nous pouvons facilement comprendre que cet apprentissage simple est nécessaire pour que les jeunes apprennent à ne pas réagir à toutes les stimulations environnementales. Une reconnaissance mais aussi une mémorisation de l’odeur est nécessaire et est mise en évidence avant la naissance ou l'éclosion.

Ce paradigme a également été utilisé pour mettre en évidence les capacités de perception et de reconnaissance. Chez les cobayes (*Cavia porcellus*), les individus ayant été exposés à des cris d’appels de poules avant la naissance ont une fréquence cardiaque stable pendant les expositions postnatales quotidiennes. À l'inverse, les individus non exposés à ces cris avant la naissance ont un rythme cardiaque qui augmente les premiers jours des tests postnatals, puis ce rythme cardiaque diminue progressivement reflétant une habituation postnatale (Vince, 1979). De même, chez l’oiseau, Colombelli-Négrel et collaborateurs ont mesuré la réponse de la fréquence cardiaque embryonnaire en utilisant un paradigme d'habituation/déshabituation avec des embryons de Mérion superbe (*Malurus cyaneus*). Leur fréquence cardiaque a diminué en réponse à la diffusion de cris d'appels de conspécifiques familiers uniquement. Cette espèce est alors capable de discrimination individuelle (Colombelli-Négrel, Hauber, & Kleindorfer, 2014).

2) Apprentissage perceptif

Apprentissage prénatal chimiosensoriel et préférences alimentaires

Chez les mammifères, les préférences olfactives et gustatives sont influencées par l'exposition prénatale à des stimuli chimiosensoriels *via* le régime alimentaire de la mère (Coureaud, Schaal, et al., 2002; Hepper & Wells, 2006; Hepper, 1996; Mennella, Jagnow, & Beauchamp, 2001). Le fœtus reçoit, *via* le liquide amniotique, des arômes dérivés consommés par la mère pendant la gestation (Schaal et al., 2000 ; Mennella et al., 2001). La consommation d'anis chez le chien domestique ou les humains au cours des 15 derniers jours de gestation (95%) influence la réponse des nouveau-nés devant ce stimulus (Hepper & Wells, 2006; Schaal et al., 2000). Les jeunes des deux espèces ont une préférence pour l'anis dès la première heure de vie. Cette préférence serait mise en place avant la première succion car le chiot s'oriente préférentiellement vers cette odeur avant le premier contact avec la mère (Hepper & Wells, 2006).

Les expériences chimiques prénatales peuvent également influencer les réponses postnatales pendant la période de sevrage. Même après cinq mois, sans perception du stimulus, l'enfant mange plus facilement le goût de carotte dans son aliment solide si sa mère buvait du jus de carotte au cours du dernier trimestre de sa grossesse (Mennella et al., 2001). Les expositions prénatales amènent donc les nourrissons à réagir favorablement à la saveur familière et facilitent ainsi la transition vers la consommation d'aliments solides. Toutefois, chez le chien domestique, une exposition au début de la période postnatale est nécessaire pour maintenir l'apprentissage prénatal dix semaines après la naissance (Hepper & Wells, 2006). Les chiots recevant uniquement des stimulations chimiques prénatales ne montrent aucune préférence pour un stimulus 10 semaines après la naissance. À l'inverse, les chiots qui ont reçu ces stimulations pendant la période périnatale (20 jours de gestation et 20 premiers jours de vie) montrent des préférences pour un stimulus dix semaines après la naissance (Hepper & Wells, 2006).

Les embryons d'oiseaux sont également capables de percevoir *in ovo* des stimuli chimiosensoriels provenant de l'environnement extérieur. Chez la poule domestique, l'exposition prénatale à une odeur normalement aversive, l'odeur de fraise, peut réduire l'aversivité de cette odeur et/ou augmenter l'attirance pour celle-ci. Ainsi, les poussins exposés à cette odeur dès le 15ème jour embryonnaire (71%) passent plus de temps à proximité et consomment plus d'eau aromatisée à la fraise (Sneddon, Hadden, & Hepper, 1998). La concentration et la période de stimulation joueront également un rôle dans le développement comportemental des poussins. Si, à partir du 12ème jour d'incubation (57%), une odeur d'orange mélangée à de la vanille se diffuse dans l'air à différentes concentrations (0,37% ou 37%), les poussins n'ont pas la même réponse postnatale à ce stimulus. Les poussins qui ont reçu la plus faible concentration au cours de la période embryonnaire passent plus de temps à manger de la nourriture portant cette odeur par rapport aux individus non exposés. À l'inverse, les poussins ayant reçu la concentration la plus élevée évitent tous les aliments présentant ce stimulus (Bertin et al., 2010). En ce qui concerne la période de stimulation, seuls les individus exposés à l'odeur pendant quatre jours à la fin de l'incubation (80%) consomment significativement plus de nourriture que les jeunes témoins non exposés. Les poussins exposés quatre jours en milieu d'incubation (61%) ne différaient pas des témoins (Bertin, Calandreau, Arnould, & Levy, 2012). Ces expériences démontrent la capacité des embryons à collecter et à mémoriser les stimuli externes. La composition olfactive/gustative de l'œuf influence également les préférences postnatales des jeunes oiseaux. Ainsi, les poules domestiques nourries avec des aliments enrichis en huile de poisson pondent des œufs parfumés à cette huile. Lors de l'incubation, les embryons sont en contact avec ces molécules lorsqu'ils consomment le vitellus. Après l'éclosion, ces poussins ingèrent significativement plus de nourriture avec cette odeur que les jeunes témoins non exposés (Aigueperse, Calandreau, & Bertin, 2013). De même, chez les amphibiens, ces stimuli chimiosensoriels influent également sur les préférences alimentaires des jeunes après l'éclosion. Hepper & Waldman (1992) ont examiné l'effet d'expériences olfactives embryonnaires sur le comportement après la naissance de deux espèces de grenouilles : *Rana temporaria* et *Rana sylvatica*. L'injection d'orange et de citral dans les œufs a induit une préférence pour ces odeurs directement après l'éclosion. Fait intéressant, les préférences ont été maintenues après la métamorphose (Hepper & Waldman, 1992). Les préférences des têtards ont également été influencées "naturellement" par les odeurs de l'eau entourant les embryons en développement.

Apprentissage prénatal auditif et reconnaissance sociale et maternelle

Chez certaines espèces de mammifères et d'oiseaux, la reconnaissance maternelle peut être auditive (Graven & Browne, 2008; Hepper, 2015; Hepper, 1996; Lecanuet et al., 1987). La voix maternelle par exemple bénéficie d'une double transmission à la fois aérienne et interne, cette dernière étant tissulaire et osseuse. Lorsque les stimuli externes traversent les tissus abdominaux maternels, ils sont atténués et modifiés mais certaines caractéristiques de la parole sont préservées. L'expérience auditive prénatale jouerait un rôle dans les préférences postnatales concernant la voix de la mère. En effet, les enfants de un à trois jours qui ont eu peu de contact avec leur mère (moins de 12 heures) sont capables de discriminer et de montrer une préférence pour la voix de la mère (DeCasper & Fifer, 1980). Inversement, ces enfants ne montrent aucune préférence pour la voix de leur père ayant la même expérience postnatale (la voix des humains est moins audible *in utero* car plus grave que la voix des femmes) (Lecanuet et al., 1987). Ce phénomène montre que l'expérience prénatale est un facteur influençant fortement les préférences postnatales. De plus, le fœtus discrimine certaines caractéristiques de la langue pendant la période prénatale. En effet, dans une expérience, DeCasper & Spence (1986) ont demandé aux femmes de lire une histoire à voix haute deux fois par jour au cours des 12 dernières semaines de grossesse (70% de la gestation totale). Deux jours après la naissance, les nourrissons manifestent une préférence pour l'histoire entendue *in utero*, même si elle est lue par une femme inconnue (DeCasper & Spence, 1986). Les nouveau-nés discriminent également le discours dans leur langue maternelle et le discours dans une autre langue, même s'il est prononcé par un seul locuteur. Ainsi, les nouveau-nés français de 4 jours émettent plus de succions non nutritives quand le discours est en français que quand il est en russe. À l'inverse, les enfants soumis exclusivement à la langue espagnole avant la naissance mais ayant vécu en France au cours des quatre premiers jours de leur vie n'ont aucune préférence entre les discours en français et en russe (Mehler et al., 1988).

Comme chez l'humain, l'expérience auditive prénatale influence les préférences auditives postnatales chez certaines espèces d'oiseaux. Chez le canard musqué, entendre leurs propres vocalisations et celles des autres membres de la couvée jusqu'à la veille de l'éclosion influence le développement des préférences auditives postnatales. Les jeunes préfèrent les appels maternels spécifiques aux appels non-spécifiques 24 h après l'éclosion. Sans ces stimuli auditifs (embryons isolés et dévocalisés avec du collodion rendant les membranes de la syrinx inflexibles), les jeunes ne manifestent pas cette préférence. Cependant, si les embryons sont dévocalisés et exposés à des appels de poule domestique (30 min par heure), leurs préférences changeront ensuite pour ces appels non-spécifiques à 48h et à 65h de la vie postnatale. Enfin, si les jeunes sont isolés (non dévocalisés) et sont exposés aux appels de poule domestique, ils préfèrent toujours les appels maternels spécifiques (Gottlieb, 1991a). L'exposition prénatale à des vocalisations non-spécifiques peut alors modifier les préférences postnatales uniquement si les jeunes ne perçoivent pas leurs propres vocalisations. Elles ont donc un impact beaucoup plus important que les vocalisations recueillies à l'extérieur de l'œuf. Il faut cependant parfois une exposition prénatale et postnatale pour inverser les préférences auditives des jeunes. Chez le colin de Virginie, les préférences pour les vocalisations spécifiques sont modifiées à la fois par une exposition prénatale et postnatale. L'exposition prénatale aux vocalisations de cailles japonaises (*Coturnix coturnix japonica*) (36h avant l'éclosion, 93%) induit une perte de préférences spécifiques car les poussins n'expriment pas de préférences entre les appels spécifiques et non spécifiques. Par contre si l'exposition prénatale est associée à une exposition postnatale (24h après l'éclosion), les poussins ont une préférence pour les appels non-spécifiques (Harshaw & Lickliter, 2010). Le type de stimulation entendu a aussi son importance dans l'apprentissage. Toujours chez le colin de Virginie, les embryons sont capables d'apprendre un cri d'appel maternel individuel même s'ils sont incubés avec des cris de contentement. Par contre, aucun apprentissage n'a lieu si ce cri d'appel est couplé avec des cris de détresse (Sleigh, Columbus, & Lickliter, 1996).

3) *Apprentissage associatif*

Apprentissage aversif

Lorsque les individus sont capables de créer des relations entre les événements et les stimuli on parle d'apprentissage associatif. Pendant longtemps, cet apprentissage n'a été étudié que chez l'adulte et chez les espèces au cerveau complexe comme les mammifères. Puis, ces études ont été réalisées chez les embryons. Les premières études, renommées dans ce domaine, ont été celles de Smotherman et collaborateurs (Smotherman, 2002; Smotherman & Robinson, 1985, 1988, 1992; Smotherman, Robinson, Ronca, Alberts, & Hepper, 1991). Ils ont montré pour la première fois que les embryons de rats sont capables de former des aversions conditionnées pendant la période prénatale. Effectivement, ils sont capables de percevoir les molécules chimiques injectées directement dans le liquide amniotique dès le 17ème jour embryonnaire (74% de la gestation totale) (Smotherman & Robinson, 1985).

Une simple présentation d'une flaveur de menthe (amenant à des sensations olfactives et gustatives) ne modifie pas le comportement de l'embryon. Par contre, la présentation d'une molécule aversive, le chlorure de lithium (LiCl), induit une diminution globale de l'activité embryonnaire. De ce fait, si la menthe présentée est couplée avec le LiCl, l'embryon modifiera son comportement deux jours après suite à la simple présentation de la menthe.

Reconnaissance des prédateurs

La reconnaissance des prédateurs augmente la survie des individus en alertant les proies sur les dangers potentiels. Les proies adoptent alors des comportements d'évitement efficaces. Cette reconnaissance des prédateurs peut être apprise tôt dans le développement individuel. Bien que généralement incapables d'éviter les prédateurs, les embryons perçoivent des stimuli environnementaux, qui peuvent potentiellement être utilisés pour déterminer les facteurs de risque susceptibles d'être présents dans leur futur environnement. Cette capacité d'apprentissage embryonnaire a été observée chez la plupart des espèces aquatiques et est très étudiée chez les amphibiens (Ferrari & Chivers, 2009a, 2009b, 2010; Ferrari, Crane, & Chivers, 2016; Ferrari, Manek, & Chivers, 2010; Golub, 2013; Mathis, Ferrari, Windel, Messier, & Chivers, 2008; Saglio & Mandrillon, 2006).

La première étude montrant explicitement ces capacités à reconnaître les prédateurs a été réalisée par Mathis et ses collaborateurs en 2008. Elle a montré que lorsque les embryons de salamandres (*Ambystoma annulatum*) étaient exposés à des indices chimiques venant des prédateurs ou à un signal d'alarme (20 têtards broyés dans 50 ml d'eau à l'aide d'un mortier et d'un pilon), les larves manifestaient ensuite un plus grand nombre de comportements anti-prédateurs tels que la recherche d'abri et une réduction de l'activité locomotrice (Mathis et al., 2008). Par la suite, d'autres études ont été inspirées par ce résultat et ont montré que la reconnaissance des prédateurs peut également être apprise et être généralisée à d'autres prédateurs similaires (Ferrari & Chivers, 2009b). Les travaux de Ferrari et collaborateurs ont montré que les embryons d'amphibiens peuvent apprendre à reconnaître certains indices chimiques avant l'éclosion en utilisant un apprentissage associatif. Les proies vont utiliser l'odeur du prédateur, des indices sur leur alimentation et/ou signal d'alarme tel que l'odeur de congénères blessé (Ferrari & Chivers, 2009a, 2009b, 2010; Ferrari et al., 2010; Garcia, Urbina, Bredeweg, & Ferrari, 2017). Ils ont montré que les embryons de grenouille des bois (*Rana sylvatica*) exposés à différentes concentrations de congénères blessés, associés à une odeur de prédateur de 15h00 à 17h00, induiraient des réponses anti-prédateurs plus élevées au cours de la même période (Ferrari & Chivers, 2010). Contrairement aux poissons chez lesquels la peau peut contenir des phéromones d'alarme (Commens & Mathis, 1999; Sorensen & Wisenden, 2015), aucune phéromone n'a été identifiée chez les amphibiens à notre connaissance. Les embryons répondent donc à un ensemble d'odeur libérés par les dommages mécaniques subis (Bairos-Novak, Mitchell, Crane, Chivers, & Ferrari, 2017).

4) Importance de ces stimulations prénatales sur l'ontogenèse comportementale

Gottlieb a identifié trois fonctions potentielles de ces premières expériences : "maintenance" ; "induction" et "facilitation" (Gottlieb, 1981, 1971, 1976a; Valsiner & Connolly, 2003). Les stimulations peuvent avoir un effet de « maintenance » permettant de conserver l'intégrité d'un système neural ou comportemental déjà complètement formé. Par exemple, une privation sensorielle au début du développement peut entraîner une atrophie du tissu neural dans le système sensoriel privé de stimuli (Gottlieb, 1976). Ces stimulations peuvent aussi avoir un effet « inducteur », orientant le développement dans une direction plutôt que dans une autre. Pour Gottlieb, c'est l'effet le plus marquant de l'expérience. Pour illustrer ce phénomène inductif nous pouvons prendre l'exemple des stimulations chimiosensorielles prénatales qui influencent les préférences alimentaires des jeunes après la naissance. Enfin les stimulations prénatales peuvent faciliter le développement comportemental. Gottlieb a décrit la facilitation comme un processus conduisant à une accélération du développement comportemental. La capacité comportementale apparaît plus tôt chez les individus stimulés que chez les individus non stimulés avant la naissance. Contrairement à l'induction, les expériences de facilitation régulent la maturation, améliorent les performances, augmentent la différenciation perceptive et les capacités d'apprentissage (Gottlieb, 1976b). Si nous prenons l'exemple du canard, la privation de stimulation auditive réduit les capacités de reconnaissance maternelle de l'éclosion (Gottlieb, 1971). En résumé, les expériences prénatales peuvent accélérer le développement, augmenter le niveau de réussite finale ou les deux.

III. Réorganisation des systèmes sensoriels

Les différents stimuli environnementaux sont perçus et traités tôt dans le développement. Ils peuvent avoir des effets différents en fonction de la modalité sensorielle stimulée et ils préparent les individus à leur future vie postnatale. Il y a donc une continuité sensorielle transnatale (Lecanuet et al., 1993). Cependant, nous allons voir dans la partie qui va suivre que ces stimulations peuvent aussi avoir des effets délétères sur la construction comportementale. Étant donné que l'établissement des différents systèmes sensoriels suit un ordre chronologique et invariant avec un certain degré de chevauchement, certains stimuli environnementaux peuvent réorganiser le développement d'autres systèmes sensoriels. Dans la littérature, Gottlieb, puis Lickliter et ses collaborateurs ont fréquemment démontré ce phénomène en utilisant une sous- ou sur-stimulation environnementale (Carlsen & Lickliter, 1999; Gottlieb, Tomlinson, & Radell, 1989; Honeycutt & Lickliter, 2001; Jaime & Lickliter, 2006; Lickliter, 1994, 2000; Lickliter & Lewkowicz, 1995; Sleigh & Lickliter, 1996, 1998).

1) Les stimulations tactiles influencent le développement auditif et visuel

Les stimulations tactiles et vestibulaires prénatales peuvent influencer le développement du système auditif et visuel chez les oiseaux. Une étude a révélé que chez les embryons de colin de Virginie, cette stimulation prénatale modifie le fonctionnement perceptif postnatal. Les juvéniles incubés en isolement physique au cours des derniers jours d'incubation (pas de retournement des œufs) n'utilisent que les indices auditifs pour reconnaitre la mère alors que les juvéniles contrôles utilisent aussi des indices visuels (Lickliter & Lewkowicz, 1995). De plus, les juvéniles incubés en isolement physique n'ont pas réussi à apprendre le cris d'appel maternel diffusé pendant la période prénatale contrairement aux juvéniles contrôles (Lickliter & Lewkowicz, 1995). Le manque de stimulations tactiles et vestibulaires peut donc avoir un impact fort et altérer la reconnaissance de la mère après l'éclosion.

De la même façon, des sur-stimulations auront un impact sur les capacités de reconnaissance maternelle. En effet, toujours chez le colin de Virginie, si les œufs sont retournés pendant 420 min durant la dernière semaine d'incubation (contre 42 minutes pour le groupe contrôle), les jeunes vont préférer utiliser des signaux auditifs plutôt que visuels pour reconnaître la mère (Carlsen & Lickliter, 1999). Les sur-stimulations tactiles et vestibulaires ne semble cependant pas affecter l'apprentissage auditif prénatal puisque les juvéniles apprennent le cris d'appel maternel diffusé avant l'éclosion (Carlsen & Lickliter, 1999). Ces résultats pris ensemble montrent que si les stimuli tactiles et vestibulaires sont trop faibles ou trop forts, ils peuvent interférer avec l'émergence du fonctionnement perceptif.

2) Les stimulations auditives influencent le développement auditif et visuel

Chez la poule domestique, l'effet des stimuli auditifs prénatals sur les réponses visuelles postnatales dépend des caractéristiques auditives. Les cris de détresse (D) ou les cris de contentement (C), diffusés au cours des dernières 24 heures d'incubation (96% de l'incubation totale) n'ont pas les mêmes effets sur les poussins. Seules les jeunes exposées au C ont une réactivité visuelle accélérée. En effet, ils se déplacent en fonction de repères visuels à 48 heures tandis que les poussins non stimulés utilisent ces indices à 72 heures de vie. Inversement, les poussins exposés au D utilisent uniquement des signaux auditifs, quel que soit leur âge (Sleigh & Lickliter, 1996). De plus, il semble que ce soit la première stimulation auditive prénatale perçue qui influence l'orientation du développement sensoriel. Les individus qui ont reçu dix minutes de D suivies de dix minutes de C (groupe D-C) n'utilisent que les signaux auditifs, alors que les jeunes du groupe C-D utilisent des indices visuels à 48 heures (Sleigh & Lickliter, 1998).

Une exposition asynchrone à différents stimuli perçus *in ovo* est également importante pour l'apprentissage des caractéristiques spécifiques. Ainsi, les embryons de colin de Virginie exposés à des vocalisations maternelles 24 heures avant l'éclosion préfèrent cet appel familier à un appel non familier (exposition auditive unimodale). Toutefois, si cette exposition prénatale est couplée à une exposition à la lumière (exposition multimodale synchrone), les poussins ne font aucune préférence entre l'appel familier et l'appel non familier. Si cette exposition multimodale devient asynchrone (retardée), ils préfèrent l'appel familier (Honeycutt & Lickliter, 2001). Toutefois, si l'exposition multimodale est à la fois synchrone et contiguë dans l'espace (même source émettrice), les poussins s'orientent préférentiellement vers des signaux audio et visuels pour reconnaitre la mère plutôt que vers des signaux auditifs ou visuels seuls. Inversement, si cette exposition n'est pas synchrone et contiguë, les poussins se déplacent vers les indices auditifs seuls sans tenir compte des indices visuels. La stimulation prénatale multimodale facilite donc la sensibilité des poussins pour traiter les informations audio-visuelles après l'éclosion uniquement si elles sont synchrones et contiguës dans l'espace (Jaime & Lickliter, 2006).

3) *Les stimulations visuelles influences le développement auditif et visuel*

Les stimulations visuelles prénatales peuvent également influencer les préférences auditives postnatales chez le colin de Virginie. Ainsi, des poussins incubés dans l'obscurité s'orientent préférentiellement vers l'appel maternel spécifique à 24h, 48h, 72h et 96h de vie, même s'ils sont associés à des indices visuels non-spécifiques (Colin écaillé - *Callipepla squamata*). Inversement, les individus incubés à la lumière s'orientent préférentiellement vers des vocalisations spécifiques (associées à des signaux visuels non-spécifiques) à 24h et 48h de vie. Ces individus ne montrent aucune préférence à 72 heures de vie et s'orientent préférentiellement vers des femelles spécifiques (associées à des vocalisations non-spécifiques) à 96 heures de vie (Lickliter, 1994). Cette expérience met en évidence l'effet des stimuli visuels prénatals sur la hiérarchie des indices utilisés dans le contexte social postnatal. Les jeunes bénéficiant d'une stimulation visuelle prénatale utilisent des indices visuels pour la reconnaissance sociale.

IV. Stress prénatal et ontogenèse comportementale

Dans la partie précédente nous avons donc vu que les embryons ne sont pas isolés d'un point de vue sensoriel mais capables de perception et d'apprentissage. Certains stimuli émanant de l'environnement immédiat des embryons sont donc traités, intégrés et sont importants pour la construction de certains comportements. Ces effets auront plus ou moins d'influence sur l'ontogenèse des jeunes si ces stimuli sont renforcés après la naissance, mais une stimulation prénatale à elle seule peut induire et influencer une grande partie du comportement postnatal. Une sur- ou sous-stimulation peut influencer durablement l'ontogenèse du jeune (Gottlieb, 1976b, 1991b; Lecanuet et al., 1987; Lickliter, 2011) et être considérées comme un stress. Dans la partie qui va suivre, nous verrons qu'une expérience prénatale négative au travers du stress maternel (femelle stressée pendant la gestation/ phase de ponte) est très largement étudié dans la littérature et engendre des effets délétères sur le développement comportemental et cognitif des jeunes après la naissance/éclosion.

1) *Conséquences physiologiques du stress*

Le stress, défini comme une « perception cognitive incontrôlable et/ou imprévisible est exprimé par une réponse physiologique et comportementale » (Koolhaas et al., 2011). Deux types de stress doivent être différenciés. D'une part, le stress aigu correspond à une stimulation négative brève et non répetée. Il induit des modifications comportementales (e.g. fuites ; freezing) et physiologiques (e.g. sécrétion de catécholamines) considérées comme adaptatives car celles-ci permettent à l'individu de réagir face à un danger potentiel (Jones, 1996 ; Valance, Boissy, Després, Constantin, & Leterrier, 2007). D'autre part, le stress chronique est répété et les modifications comportementales (e.g. stéréotypies) et physiologiques (libération d'hormones de stress comme les catécholamines et glucocorticoïdes ; Hill, 1983) perdurent dans le temps (Miller, Chen, & Zhou, 2007). Les catécholamines (noradrénaline et adrénaline notamment) sont secrétées par les glandes médullosurrénales et engendrent un ensemble de réponses physiologiques (accélération du rythme cardiaque, augmentation de la pression artérielle et du rythme respiratoire). Quant aux glucocorticoïdes (cortisol, corticostérone), ils sont secrétés par les glandes corticosurrénales et peuvent, sur le long terme, induire des conséquences néfastes pour l'organisme telles qu'une diminution des ressources énergétiques et un affaiblissement du système immunitaire (Miller et al., 2007). Comme nous pouvons le voir sur la Figure 1, le stress entraîne une activation de l'hypothalamus qui sécrète alors l'hormone CRH (corticotropin-releasing hormone). Cette hormone amène à son tour l'hypophyse à produire l'hormone ACTH (adrénocorticotropine) qui circule dans le système sanguin et atteint les glandes surrénales où elle provoque la libération de glucocorticoïdes. Ce processus forme une boucle de rétroaction négative où l'excès de glucocorticoïdes active ses récepteurs du cerveau et supprime la production de CRH. Par contre en cas de stress chronique, cette boucle ne fonctionne plus d'où une production excessive de CRH, et de glucocorticoïdes (Leonard, 2005).

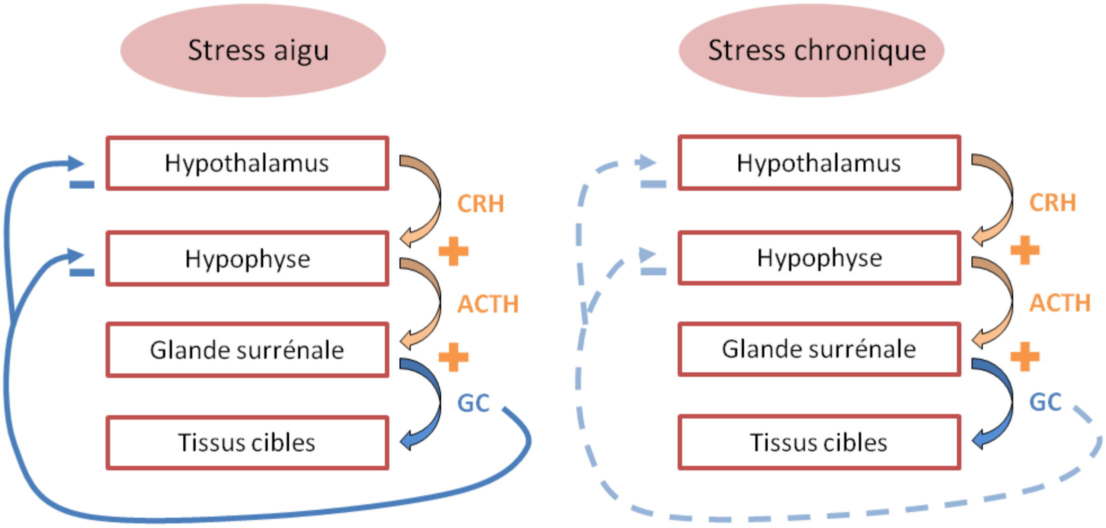

Figure 1 : Représentation schématique de l'axe hypothalamus-hypophyse-surrénal (ou axe HPA : hypothalamo-pituitary-adrenal) et de la réponse physiologique face a un stress aigu (gauche) et chronique (droite). De nombreux tissus possèdent des récepteurs aux glucocorticoïdes (e.g. hippocampe, thyroïde, gonades). CRF : corticotropin-releasing factor, ACTH : adrenocorticotropic hormone. GC : glucocorticoïdes. En cas de stress aigu, il existe un rétrocontrôle négatif où les GC en excès vont diminuer voire supprimer la production de CRH et de ACTH. Inversement, en cas de stress chronique, il y a dysfonctionnement du rétrocontrôle négatif.

2) Les effets du stress maternel

Chez les mammifères, les glucocorticoïdes maternels peuvent atteindre l'embryon par l'intermédiaire du sang et moduler leur développement comportemental et cognitif. Ce type de stress est qualifié de « stress maternel » puisque c'est la mère qui est stressée pendant la phase de gestation ou de ponte. Ces effets sont très étudiés dans la littérature car ils permettent de comprendre au mieux les effets du stress sur le développement fœtal. Effectivement, les facteurs de stress sont nombreux chez l'humain : tracas quotidiens (affaires intérieures, problèmes financiers ou relationnels...) ; événements de la vie (divorce, maladie grave, décès d'un membre de la famille ou d'un ami…) ; catastrophes naturelles ou techniques (tsunamis, ouragans, inondations, tempête de glace, tremblement de terre, Tchernobyl…) ; ou encore des événements traumatisants comme les attaques terroristes, la guerre… (Mulder et al., 2002). Les bébés étudiés dans ces études ont très souvent une naissance prématurée, un très faible poids à la naissance, une grande détresse (beaucoup de pleurs) et une déficience cognitive (Harville, Xiong, & Buekens, 2010). Afin d'éliminer au mieux les biais relatifs aux études menées sur l'humain (environnement social par exemple), des études sur les animaux ont été réalisées (généralement sur le rat : *Rattus norvegicus* et le singe rhésus : *Macaca mulatta*).

Ces expériences montrent que les jeunes issus de mères exposées à divers facteurs de stress pendant la gestation (choc électrique, immobilisation, bruits inattendus…) ont un développement moteur retardé et présentent, à l'âge adulte, une exploration réduite, une plus forte réactivité émotionnelle, des fonctions cognitives altérées (attention, apprentissage) et des modifications du comportement social et sexuel (Braastad, 1998; Clarke, Wittwer, Abbott, & Schneider, 1994; Harville et al., 2010; Mulder et al., 2002; Schneider, Roughton, Koehler, & Lubach, 1999; Teran-MacIver & Larson, 2008; Weinstock, 2008).

Les conditions de vie stressantes peuvent également affecter le développement comportemental des jeunes chez les oiseaux. La composition des œufs sera modulée par ce stress et la femelle pondeuse produira des œufs plus riches en corticostérone et en stéroïdes sexuels (testostérone, androstènedione) et aura des jeunes plus émotifs (Groothuis, Müller, von Engelhardt, Carere, & Eising, 2005; Guibert et al., 2010; Henriksen, Rettenbacher, & Groothuis, 2011; Houdelier et al., 2011). De plus, de récentes études montrent que le stress maternel (changement d'alimentation, bruit, manipulation par l'humain…) a de forts effets sur la reproduction et sur les comportements postnatals des jeunes. Il peut induire des déficits dans certains comportements comme une modulation de la réactivité émotionnelle et une hyperactivité chez l'oiseau : poule domestique : De Haas et al., 2017 ; caille japonaise : Mezrai, Houdelier, & Lumineau, en préparation) et des effets délétères sur les capacités cognitives (poule ; caille japonaise : Charrier et al., en préparation). Chez la caille japonaise, ces effets perdurent même sur la seconde génération montrant des effets épigénétiques du stress prénatal (Charrier et al., en préparation).

3) Les effets du stress embryonnaire sont peu étudiés

Les effets du stress embryonnaire (embryon stressé pendant la période prénatale) sont très peu étudiés et leurs conséquences sont encore méconnues. Généralement, ces études traitent les effets des influences prénatales que nous avons détaillées précédemment. L'analyse des effets de perturbations stressantes appliquées directement sur l'embryon apparait difficile à mettre en œuvre chez les mammifères puisque ces évènements stressants vont à la fois affecter l'embryon et la mère (Braastad, 1998; Henriksen et al., 2011). Aussi, pour étudier les effets du stress embryonnaire il est nécessaire d'étudier des espèces ovipares pour lesquelles l'environnement prénatal peut être contrôlé car l'embryon se développe à l'extérieur de l'organisme maternel.

Cette question scientifique est au cœur du **projet ANR PReSTO'Cog** traitant les « effets de stress prénatals sur le développement précoce des comportements et des capacités cognitives : une approche comparative » (projet ANR -13- BSV7- 0002 2014-2017). Ce projet, concernant l'étude du stress prénatal chez des espèces ovipares, est une collaboration entre cinq laboratoires français étudiant des modèles animaux différents :

- **La seiche commune (*Sepia officinalis*) – EthoS UMR CNRS 6552 – Caen**
- La poule domestique (*Gallus g. domesticus*) – CNA UMR INRA– Nouzilly
- La caille japonaise et la caille des blés (*Coturnix c. japonica* et *Coturnix c.*) – EthoS UMR CNRS 6552 – Rennes
- La truite arc-en-ciel (*Oncorhynchus mykiss*) – LPGP INRA UMR 1037 – Rennes
- Le poisson zèbre (*Danio rerio*) – IFREMER RBE LRH-LR – L'Houmeau

Ces espèces représentent un large éventail de groupes d'animaux : les Invertébrés (seiches) et les Vertébrés (poissons, oiseaux) ainsi que les espèces sauvages (seiches et cailles des blés) ou domestiques (poules et cailles japonaises). Tous sont ovipares et nidifuges, ce qui permet de séparer l'effet du stress maternel des effets du stress embryonnaire pendant la période prénatale et de s'affranchir de l'influence maternelle postnatale.

Ce projet a pour but de répondre à 3 grandes questions :

1) Le stress prénatal a-t-il des effets bénéfiques ou délétères sur les comportements adaptatifs des juvéniles ?
2) Les stress embryonnaires induisent-ils les mêmes effets chez le jeune que le stress maternel ?
3) La nature du stress embryonnaire (naturel ou artificiel) modifie-t-elle ces effets ?

Les premiers résultats ont montré que contrairement au stress maternel, les effets du stress embryonnaire sont plus modérés. Le **stress naturel** (odeur ou vocalisation de prédateur, phéromone d'alarme…) peut influencer la réactivité émotionnelle (caille japonaise : Mezrai et al., en préparation ; poisson zèbre ; truite arc-en-ciel) ou induire des déficits cognitifs (caille japonaise ; poisson zèbre ; truite arc-en-ciel). De la même façon, le **stress artificiel** (bruit métallique, lumière forte…) vont moduler le comportement prédateur (seiche : O'Brien et al., 2017), la réactivité émotionnelle (caille japonaise : Mezrai et al., en préparation ; poule ; poisson zèbre), la motivation sociale (caille japonaise : Mezrai et al., en préparation) et induire des déficits cognitifs (caille japonaise et truite arc-en-ciel).

V. Présentation de la thèse

Notre étude est rattachée au projet ANR PReSTO'Cog et a pour objectif d'étudier les effets du stress embryonnaire sur les capacités d'apprentissage périnatales chez la seiche. Chez ce modèle, la précédente étude menée par O'Brien et ses collaborateurs n'a montré que peu d'effet du stress embryonnaire sur les comportements des jeunes après l'éclosion (des odeurs de prédateur ne vont pas moduler les comportements des jeunes mais un stress lumineux va perturber le camouflage : O'Brien, Jozet-alves, et al., 2017).

L'objectif de ce travail vise donc à analyser **l'effet du stress embryonnaire sur les capacités cognitives périnatales des seiches**. Étant donné que cette question n'a jamais été abordée auparavant dans la littérature, il faut cependant effectuer un travail en amont et savoir si les embryons de seiches sont capables de percevoir des stimulations environnementales avant l'éclosion et s'ils sont capables d'apprendre.

La thèse est alors organisée en six chapitres. Dans le **chapitre 2**, nous présenterons notre méthodologie générale après nos modèles d'études *Sepia officinalis* et *Sepia pharaonis.* Nous verrons que ces deux espèces sont très semblables mais que leur développement embryonnaire se fait dans des conditions visuelles différentes (*Sepia officinalis* se développe dans un œuf noir alors que *Sepia pharaonis* se développe dans un œuf transparent). Leurs capacités de perception et de réponse embryonnaire seront analysées dans le **chapitre 3** et nous verrons ainsi si la transparence de la capsule de l'œuf peut avoir un impact dans leurs réponses. Le **chapitre 4** mettra en évidence leurs capacités d'apprentissage. Les effets du stress prénatal seront testés dans le **chapitre 5**. Enfin nos résultats seront discutés dans le **chapitre 6**.

Chapitre 2

Méthodologie générale

Chapitre 2 – Méthodologie générale

I. Modèle biologique étudié

1) Généralités

Les céphalopodes

Les céphalopodes forment une classe de l'embranchement des mollusques et sont exclusivement marins. Cette classe comprend environ 700 espèces différentes et est divisée en deux sous-classes : les **Nautiloidea** (ayant une coquille externe et deux paires de branchies – exemple du Nautile *Nautilus pompilius*) et les **Coleoidea** (qui ont une coquille interne et une seule paire de branchies). Au sein de ce dernier, on distingue les octopodes qui ont 8 bras (e.g. la pieuvre *Octopus vulgaris*) et les calmars et seiches ayant 10 bras dont une paire de tentacules (e.g. le calmar *Loligo vulgaris* et la seiche *Sepia officinalis* respectivement) (cf. Figure 2).

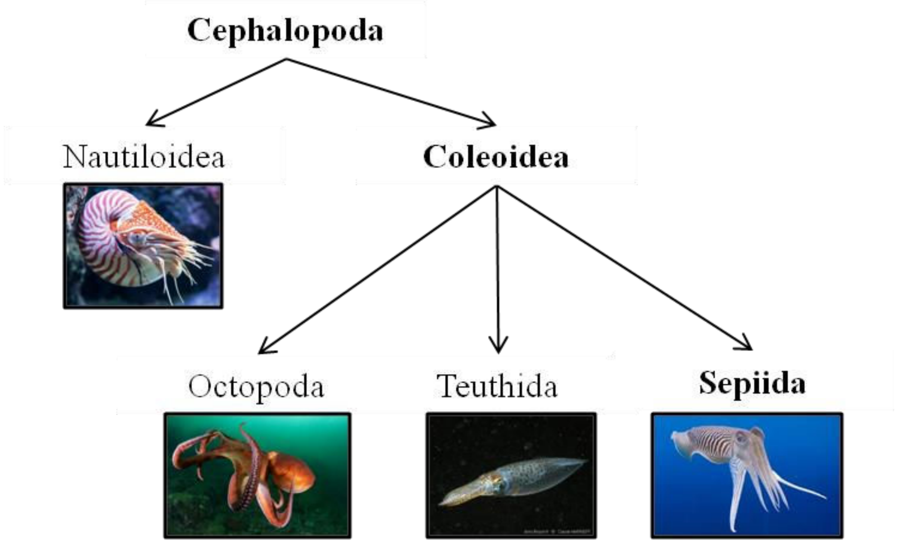

Figure 2 : Classification phylogénétique simplifiée des céphalopodes.

Les seiches (sous-classe des *Coleoidea* ; ordre de *Sepiida* ; genre *Sepia*) regroupent plus de 90 espèces, parmis lesquelles la seiche commune *Sepia officinalis* et la seiche pharaon *Sepia pharaonis* (Hanlon & Messenger, 1998). Bien que ces deux espèces soient très semblables morphologiquement (cf. Figure 3), chez *Sepia officinalis* la zone striée de la coquille ne dépasse pas la moitié de sa longueur et les massues tentaculaires portent des rangées de 5 à 6 ventouses alors que chez *Sepia pharaonis* la zone striée de la coquille atteint les trois quarts de sa longueur et les massues tentaculaires portent des rangées de 8 ventouses.

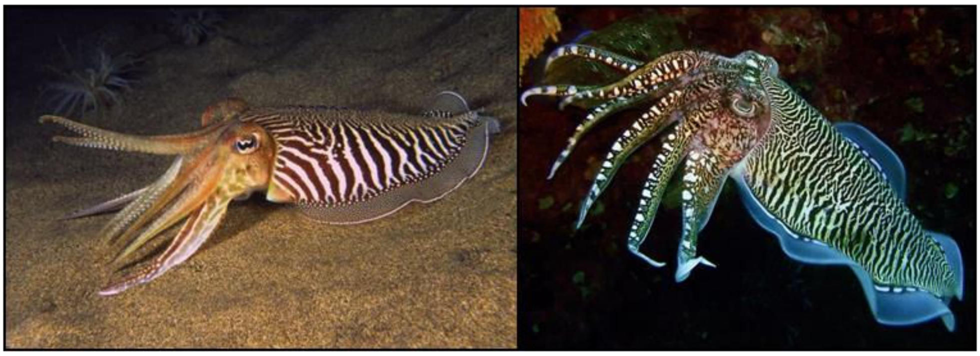

Figure 3 : Photos de *Sepia officinalis* (gauche) et *Sepia pharaonis* (droite).

Ces deux espèces ne partagent pas non plus le même habitat. *Sepia officinalis* vit en atlantique Nord-Est, du Nord de l'Europe jusqu'aux côtes de l'Afrique du Nord-Ouest, ainsi que dans la mer Méditerranée (cf. Figure 4 ; Gras, 2013). *Sepia pharaonis* est quant à elle largement distribuée de l'Afrique de l'est à l'océan Pacifique Ouest (cf. Figure 5 ; Lee, Lin, Chiao, & Lu, 2016).

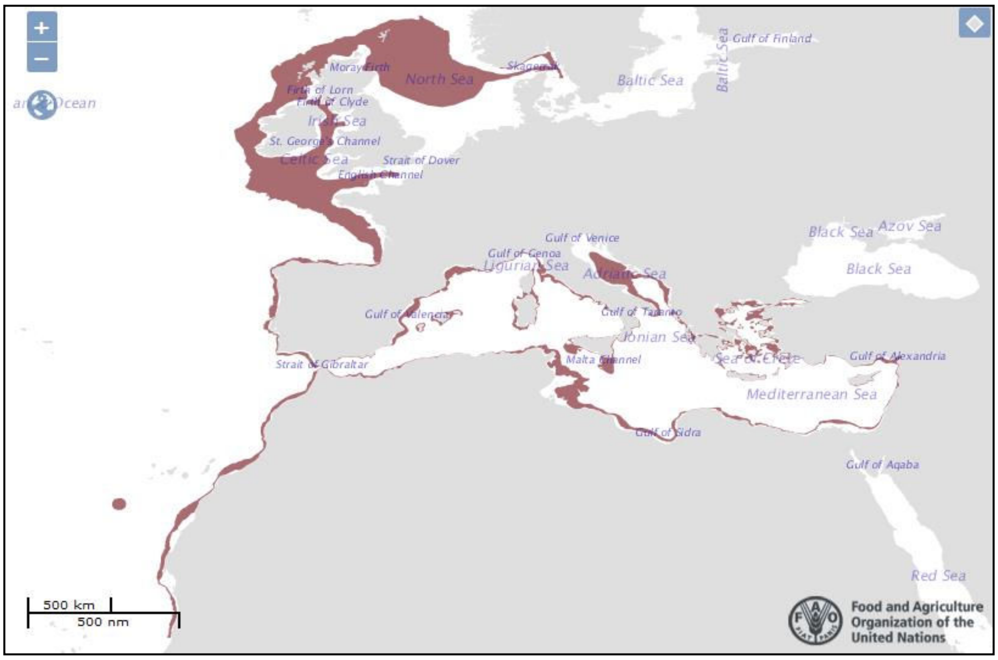

Figure 4 : Aire de distribution de la seiche *Sepia officinalis* en rouge (FAO, 2018).

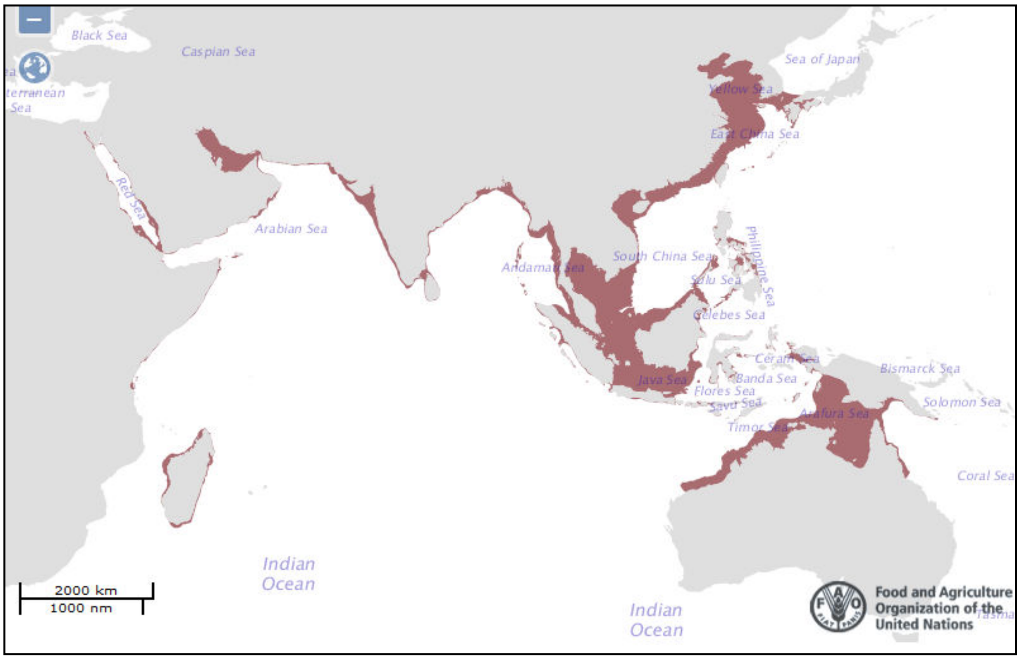

Figure 5 : Aire de répartition de *Sepia pharaonis* en rouge (FAO 2018).

Régime alimentaire et prédateurs

Sepia officinalis et *Sepia pharaonis* se nourrissent quasiment exclusivement de proies vivantes et mobiles telles que des crevettes, des crabes ou des poissons. Elles vont détecter les proies en utilisant la modalité visuelle (Messenger, 1968) puis vont s'orienter, se positionner et capturer celles-ci. Il existe deux méthodes de capture dépendant du type de la proie (cf. Figure 6). Pour les grosses proies peu mobiles (e.g. crabes), les seiches utilisent une méthode de « **coiffage** » où elles vont effectuer un bond rapide et coiffer la proie de ses huit bras. Généralement elles vont contourner les crabes et les capturer par la face postérieure et ainsi éviter les pinces qui représentent une réelle menace (Boycott, 1958; Duval, Chichery, & Chichery, 1984; Messenger, 1968). Pour les plus petites proies très mobiles (e.g. crevettes et poissons), elles vont utiliser une méthode d'« **éjection des tentacules** » où elles vont lancer rapidement leurs deux massues tentaculaires pourvues de ventouses sur la proie qui est ensuite ramenée entre les bras jusqu'à la cavité buccale (Messenger, 1968).

Figure 6 : Photographies illustrant les deux méthodes de capture des proies chez la seiche. A gauche, la méthode de coiffage (© Ellenbogen) ; à droite la méthode d'éjection des tentacules (© Larry).

Stratégies de défense

Les seiches sont des proies potentielles pour une large variété de Vertébrés. Les jeunes seiches, de par leur plus petite taille, sont beaucoup plus soumises aux prédateurs. Nous y trouvons les mammifères marins (e.g. les dauphins), certains requins (e.g. la petite roussette *Scyliorhinus canicula*) ainsi que des poissons carnivores (e.g. le bar commun *Dicentrarchus labrax* en Europe ; le poisson ballon *Arothron hispidus* en Asie). Face à ses prédateurs, les seiches présentent 2 stratégies défensives :

1) Les défenses primaires, qui ne seront pas décrites ici, ont pour fonction de diminuer les chances d'être détectées par un prédateur potentiel (stratégie cryptique : camouflage et ensablement)
2) Les défenses secondaires qui entrent en jeu lorsque les seiches ont été détectées par le prédateur : fuite et jet d'encre.

Le jet d'encre est un comportement caractéristique adopté par presque tous les céphalopodes coléoïdes. C'est une défense visible qui est dispersée sous de multiples formes. La forme la plus répandue est la production d'un « pseudomorphe ». Il a généralement la taille d'un céphalopode et sert probablement à retenir l'attention visuelle du prédateur pendant que celui-ci s'échappe (Derby, 2014; Hanlon & Messenger, 2018). L'encre est composée de sécrétions provenant de deux glandes : (1) la glande de la poche à encre produit une encre noire contenant de la mélanine ; (2) la glande dans l'entonnoir produit le mucus. L'encre de seiche est composée de mélanine mais aussi de catécholamines, DOPA et dopamine (qui sont des monoamines dérivées de la tyrosine), d'acides aminées tels que la taurine mais aussi de certains métaux comme du cadmium, du cuivre et du plomb (Derby, 2014; Madaras, Gerber, Peddie, & Kokkinn, 2010; Prota et al., 1981).

En plus d'être un moyen de dissuasion directe des prédateurs (effets interspécifiques), l'encre serait aussi un signal d'alarme pour les conspécifiques (effet intraspécifiques) (Derby, 2014). Ce type de défense fonctionne indirectement, non pas en agissant sur les prédateurs eux-mêmes, mais plutôt en signal d'alarme pour les conspécifiques. Chez le calmar *Loligo opalescens* une exposition à de l'encre peut entraîner une éjection d'encre chez l'individu testé ainsi qu'un changement dans l'apparence des individus (camouflage ; Gilly & Lucero, 1992; Lucero, Farrington, & Gilly, 1994). De plus, chez le calmar *Loligo opalescens,* la dopamine à des concentrations biologiquement pertinentes est suffisante pour entraîner une éjection d'encre (Gilly & Lucero, 1992; Lucero, Farrington, & Gilly, 1994). L'encre est éjectée par les individus pour fuir et faire diversion mais aussi lors de sa capture. Cette éjection est soit volontaire (observation personnelle de *S. pharaonis* crachant de l'encre avant sa capture) soit provoquée par le prédateur (cf. Figure 7 ; Derby, 2014; Finn, Tregenza, & Norman, 2009).

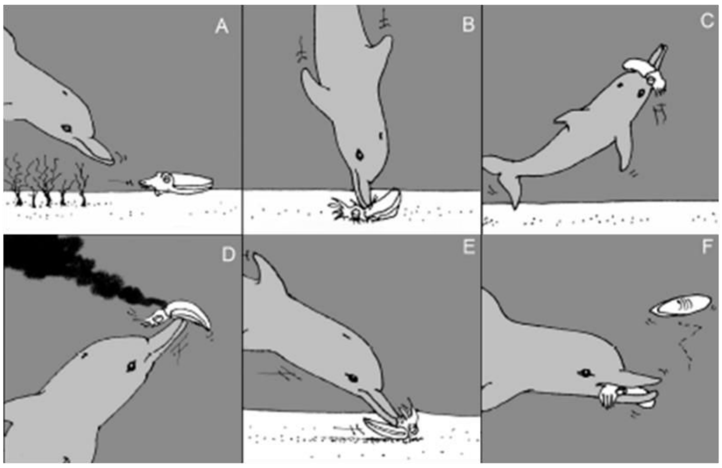

Figure 7 : Manipulation de *Sepia apama* par le dauphin *Tursiops aduncus*. Dans cette séquence, la proie est: (A) chassée du substrat, (B) tuée, (C) portée à la surface, (D) frappée contre le substrat pour libérer l'encre, (E) trainée sur le substrat pour enlever la peau et l'os de seiche, et (F) consommée (Finn et al., 2009).

Reproduction

Au début du printemps (vers le début du mois de mai), *Sepia officinalis* migrent de sa zone d'hivernage que sont les eaux profondes du large vers la côte où les températures sont plus élevées et la nourriture abondante (Boletzky, 1983; Boucaud-Camou & Boismery, 1991). C'est dans ces eaux peu profondes que les animaux plus âgés (2 ans en général) se reproduisent jusqu'en juin/juillet. Quant à *Sepia pharaonis*, la reproduction débute en février/mars (Chembian & Mathew, 2011) et de la même façon, les adultes migrent vers des zones moins profondes, plus riches en proies et plus chaudes pour la reproduction. Ces deux espèces sont sémelpares, les adultes meurent juste après la reproduction et la ponte.

Les femelles des deux espèces peuvent pondre jusqu'à 2000 œufs. Chez *Sepia officinalis* ils sont fixés en grappe sur un substrat solide (algues, casiers, filets…) alors que chez *Sepia pharaonis* ils sont pondus en grappe sous des roches et substrat plat. La majorité des seiches du genre *Sepia*, pondent des œufs transparents blancs ou légèrement teintés de jaune (exemple de *Sepia pharaonis* mais aussi de *Sepia apama*, *Sepia latimanus*, *Sepia lycidas*, *Sepia esculenta et Sepia acuelata).* La seiche *Sepia officinalis* va quant à elle pondre des œufs noirs recouverts d'encre (cf. Figure 8). Il est probable que l'encre joue le rôle d'écran visuel contre les prédateurs (Bertram & Burger, 1981; Derby, 2014; Derby, Tottempudi, Love-Chezem, & Wolfe, 2013). Cet écran visuel peut également être observé chez certaines espèces où les œufs sont recouverts de sable « collé » directement sur la capsule (cf. Figure 8 ; e.g. *Sepia lycidas*).

Figure 8 : Photographies des œufs en grappes de *Sepia officinalis* à gauche, de *Sepia pharaonis* au centre (© Mezrai) et de *Sepia lycidas* à droite (© Nori).

2) *Développement embryonnaire*

L'œuf des espèces du genre *Sepia* est caractérisé par la présence de plusieurs enveloppes protégeant l'embryon du milieu extérieur (cf. Figure 9-A). La capsule de l'œuf est formée des sécrétions des glandes de l'appareil génital femelle. L'ovocyte mature traverse la glande de l'oviducte et est recouvert d'une première sécrétion formant la première couche interne de l'œuf. Il est ensuite libéré à l'intérieur de la cavité du manteau puis il est intégré aux glandes nidamentaires et aux sécrétions provenant de la poche à encre chez *Sepia officinalis* pour former la couche externe (Boletzky, 2003). La capsule de l'œuf est une structure complexe associant des polysaccharides, des protéines, des peptides, des bactéries et de la mélanine. Cet ensemble représente la seule barrière physique et chimique entre l'embryon et son environnement. Les peptides présents dans la capsule des œufs de *Sepia officinalis* auraient un potentiel antibactérien notamment contre les souches bactériennes *E. coli* et *B. megaterium* (Cornet, 2015; Cornet et al., 2015; Duval, 2008).

Les œufs sont de types « télolécithes » comportant des réserves nutritives contenues dans un sac vitellin qui s'étend entre les bras de l'embryon (Boletzky, Andouche, & Bonnaud-Ponticelli, 2016). Ce vitellus est consommé tout au long de l'embryogenèse. Tout au long du développement embryonnaire les œufs vont grossir. La capsule de l'œuf subit de nombreux changements structuraux car le volume du liquide périvitellin (PVF) augmente progressivement et entraine une distension de l'enveloppe devenant plus translucide les derniers stades (cf. Figure 9-B).

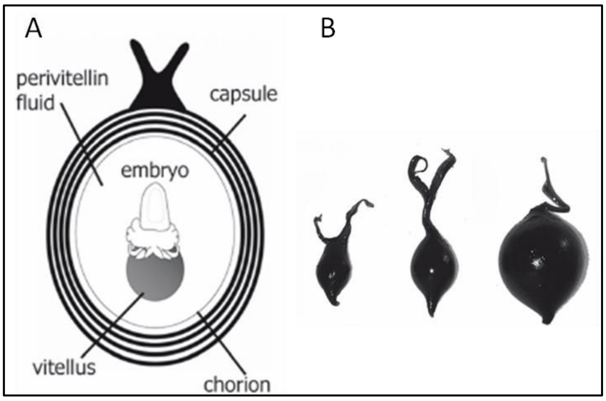

Figure 9 : A - Schéma d'un œuf de seiche montrant l'embryon et son vitellus, la capsule, le liquide périvitellin et le chorion. B – photographie d'œufs de seiche au début, milieu et fin d'incubation (Boletzky et al., 2016).

Chez Sepia officinalis

Le développement embryonnaire de la seiche *Sepia officinalis* est divisé en trois grandes périodes : la segmentation ; la gastrulation et l'organogenèse (Boletzky et al., 2016; Lemaire, 1970). La durée de l'embryogenèse dépend de la température et dure 31 jours à 21.4°C et 87 jours à 15°C (Lemaire, 1970).

a) La segmentation (du stade 1 au stade 9)

Au cours de la segmentation la cellule mère va se diviser jusqu'au stade blastula sans augmentation du volume de l'embryon. Comme chez tous les œufs télolécithes, la segmentation du disque germinatif est partielle. Chez *Sepia officinalis*, le premier sillon de segmentation à lieu 2 à 10 heures après la ponte (Lemaire, 1970). Il est méridien et divise le disque germinatif de façon symétrique. Les plans de divisions suivants sont inégaux et les divisions qui en résultent se traduisent par la formation d'éléments de tailles différentes. La segmentation est de type « méroblastique » (inégale et discoïdale) et produit un disque de blastomères (le blastoderme) à l'origine de l'embryon (chez d'autres céphalopodes la segmentation est en spirale). A la fin de la segmentation (stade de blastula), nous pouvons observer deux zones sur le pôle animal : une zone centrale composée de blastomères et une zone périphérique constituée de blastocônes à l'origine du syncytium vitellin (cf. Figure 10).

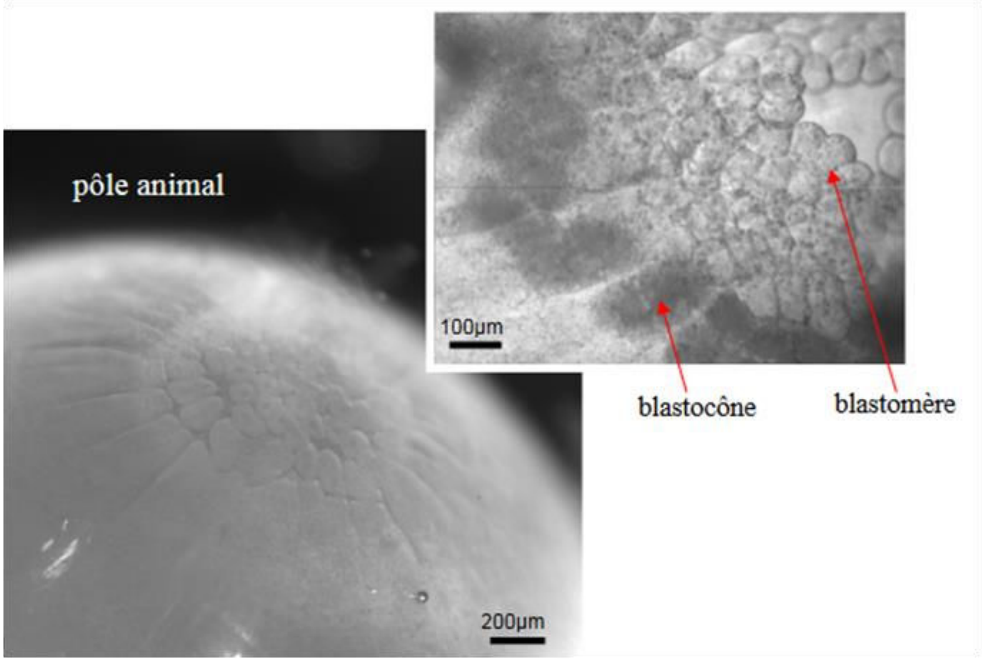

Figure 10 : Stade 9 de l'embryon de seiche *Sepia officinalis* (fin de segmentation). Nous pouvons voir au pôle animal le blastoderme à l'origine de l'embryon (photo de gauche). Il est composé de blastocônes et de blastomères (photo de droite) (Navet 2010).

b) <u>La gastrulation (stade 10 à 13)</u>

Au cours de la gastrulation, le blastoderme est transformé en structure multicouche comprenant trois feuillets : l'ectoderme, le mésoderme et l'endoderme. Toutes les parties de l'animal adulte dérivent de ces trois feuillets. La gastrulation commence par la mise en place de l'endomésoderme et se termine au stade 13 quand le vitellus est totalement recouvert par le syncytium et l'ectoderme extra-embryonnaire (épibolie terminée). Selon Lemaire (1970), la gastrulation se termine au stade 17 mais l'étude de Boletzky et collaborateurs (2016) a montré qu'il existe une phase de différenciation des organes (phase plane) juste après la gastrulation (Boletzky et al., 2016; Lemaire, 1970).

c) <u>L'organogenèse (stade 14 à 30)</u>

<u>Phase plane : stade 14 à 18</u>

Cette phase a été décrite par Boletzky et collaborateur en 2016. Elle correspond à une phase de différentiation des organes. L'épibolie s'achève entre les stades 14 et 15. Nous commençons à observer les rudiments des yeux dans la partie centrale et le sac vitellin est complètement formé à partir du vitellus, du syncytium vitellin et des éléments du mésoderme et ectoderme extra-embryonnaire. Puis, au stade 15 les branchies et les bourgeons des bras commencent à se former. Ainsi que l’entonnoir qui commence à apparaitre. Au stade 16, la bouche, certains bras, les rudiments du manteau, l’anus et les nageoires commencent à se former. Enfin, au stade 17 et 18 l’embryon est constitué d’un disque, appelé blastodisque circulaire, reposant sur une large masse vitelline au niveau du pôle animal (cf. Figure 11).

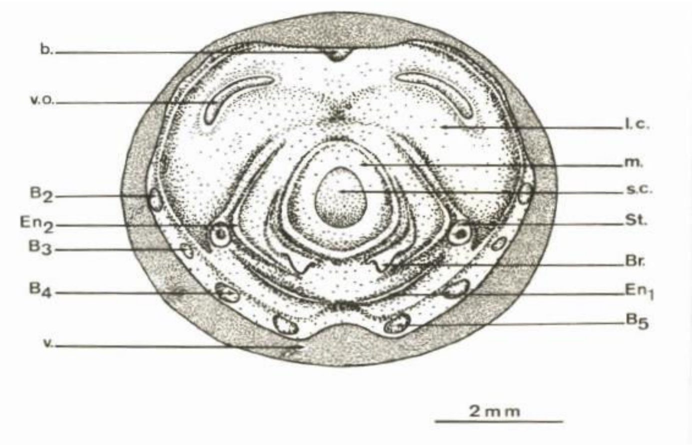

Figure 11 : Schéma représentant l'œuf de *Sepia officinalis* au stade 17. Nous pouvons voir la future bouche (b) ; la vésicule optique primaire (v.o) ; les ébauches de l’entonnoir (En1 et En2) ; les ébauches des bras (B2, B3, B4, B5) ; les ébauches des branchies (br) ; le vitellus (v) ; les lobes céphaliques (l.c) ; le manteau (m) ; le sac coquillier (s.c) et les statocytes (st) (Lemaire 1970).

<u>Phase d’extension : stade 19 à 22</u>

Durant cette phase, l’embryon et les différentes parties qui le composent se développent rapidement et sont de plus en plus nettes. Les différentes structures de son corps commencent à prendre du relief. L’embryon est alors visible et se dresse au-dessus du niveau du sac vitellin (cf. Figure 12). Au stade 20, les vésicules oculaires sont complètement fermées. De plus à ce stade les statocystes (voir partie suivante) sont invaginés et se ferment tout en restant en communication avec l'extérieur par un pore fin qui sera finalement totalement contracté (Boletzky et al., 2016). A la fin du stade 22, l’embryon commence à se développer dans l'axe antéropostérieur.

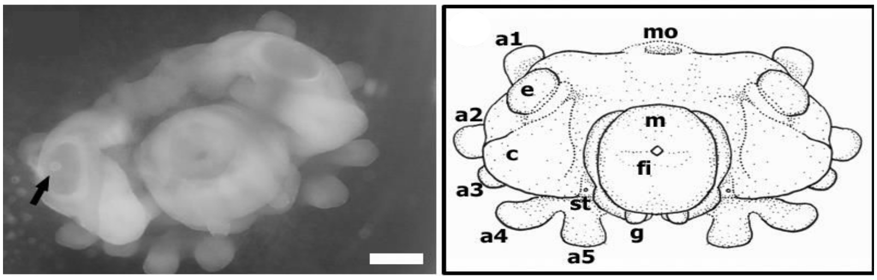

Figure 12 : Photographie (gauche) et sa représentation schématique (droite) d’un embryon de *Sepia officinalis* au stade 21(Boletzky et al., 2016). Nous pouvons voir le cristallin qui commence à se former (flèche sur la photo de gauche). a1, a2, a3, a4 et a5 : bras 1 à 5 ; c : joue ; e : œil ; fi : nageoire ; g : branchie ; m : manteau ; mo : bouche ; st : statocyste. L’échelle représente 500µm.

<u>Phase de croissance : stade 23 à 30</u>

Au cours de cette phase le volume de l'embryon va augmenter. En plus d'une maturation morphologique (les embryons ressemblent de plus en plus aux juvéniles) il va y avoir une maturation fonctionnelle des différents organes. L'un des éléments les plus saillants lorsque l'on observe les embryons durant cette période est la coloration la rétine (cf. Figure 13). Au stade 24 ils deviennent oranges puis rouges au stade 26 et enfin marrons au stade 28 (Boletzky et al., 2016; Lemaire, 1970).

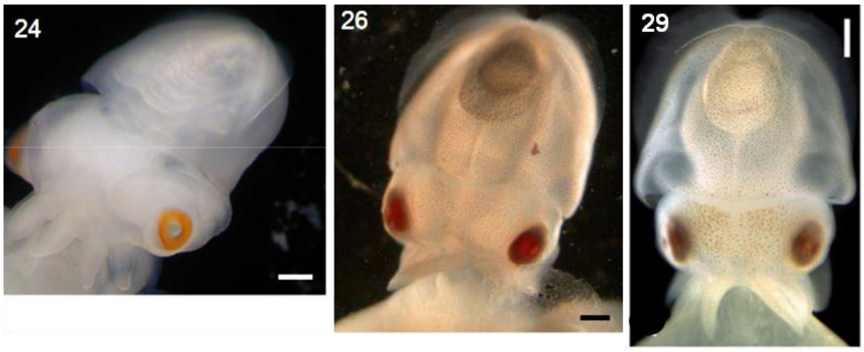

Figure 13 : Embryons de Sepia officinalis au stade 24, 26 et 29. L'échelle représente 500µm (Andouche, 2013; Navet, 2010).

Contrairement aux autres céphalopodes, les seiches nouveau-nées présentent un développement direct car il n'y a pas de stade larvaire *ex ovo*. Les jeunes ressemblent morphologiquement aux adultes et adoptent tout de suite le mode de vie necto-benthique des adultes (cf. Figure 14). Cela signifie qu'elle vit à proximité du fond marin (benthos) mais qu'elle se déplace également au sein de la colonne d'eau (necton).

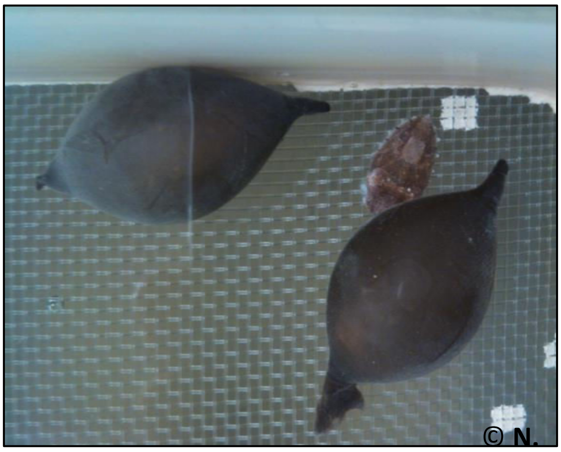

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Figure 14 : Photographie d'une seiche juste après l'éclosion à côté de deux œufs fécondés au stade 30 (© Mezrai).

Chez Sepia pharaonis

Le développement embryonnaire de la seiche *Sepia pharaonis* est divisé en 30 stades répartis dans trois grandes étapes : la segmentation ; la gastrulation et l'organogenèse. La durée de l'embryogenèse dure au environ 28 jours entre 18°C et 25°C (Lee et al., 2016). Tout au long de ce manuscrit nous allons utiliser la table de développement embryonnaire décrite en détail par Lee et ses collaborateurs en 2016.

a) La segmentation (du stade 1 au stade 9)

Comme *Sepia officinalis*, la segmentation à lieu entre les stades 1 et 9 et se déroule exactement de la même manière (Lee et al., 2016). Le premier sillon de segmentation à lieu environ 10 heures après la ponte, il est méridien et divise le disque germinatif de façon symétrique. Les plans de divisions suivants sont inégaux comme chez *Sepia officinalis*. A la fin de la segmentation nous observons également une zone centrale composée de blastomères et une zone périphérique constituée de blastocônes (cf. Figure 15).

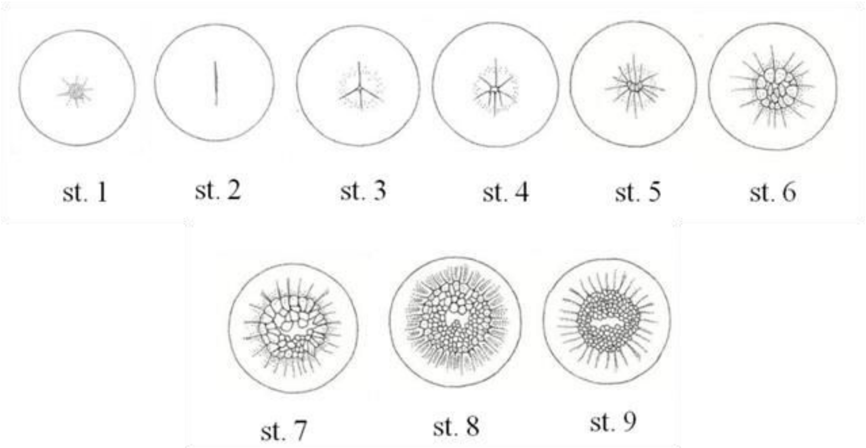

Figure 15 : Représentation schématique de la segmentation au cours du développement embryonnaire de *Sepia pharaonis* (Lee et al., 2016).

b) La gastrulation (stade 10 à 15)

La gastrulation de *Sepia pharaonis* se déroule comme chez *Sepia officinalis* et se termine à la formation du blastoderme. Les blastocônes radiaux ont progressivement disparu (stade 11) et que le blastoderme est complet (stade 12). Comme pour *Sepia officinalis*, la fin de la gastrulation à lieu quand l'épibolie est terminée (le vitellus est recouvert par le blastoderme ; cf. Figure 16).

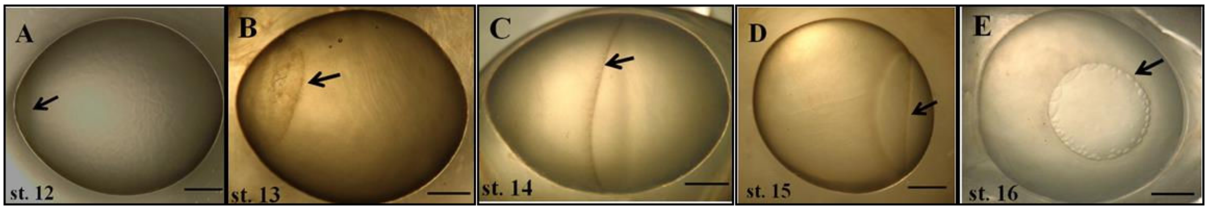

Figure 16 : Photographies d'un œuf de *Sepia pharaonis* pendant la gastrulation. La flèche nous montre le blastoderme qui s'étend du pôle animal (A) jusqu'au pôle végétale (E). l'échelle représente 1mm (Lee et al., 2016).

c) *L'organogenèse (stade 16 à 30)*

L'organogenèse de *Sepia pharaonis* suit le même développement que celui de *Sepia officinalis*. Dès le début (stade 16), nous apercevons les rudiments du sac coquillé, des sacs optiques primaires, des bras et tentacules au niveau du pôle animal. Puis les différents organes commencent à se mettre en place progressivement. Au stade 19, nous voyons très clairement l'embryon au-dessus du sac vitellin et au stade 20 nous pouvons voir les bras en développement (cf. Figure 17).

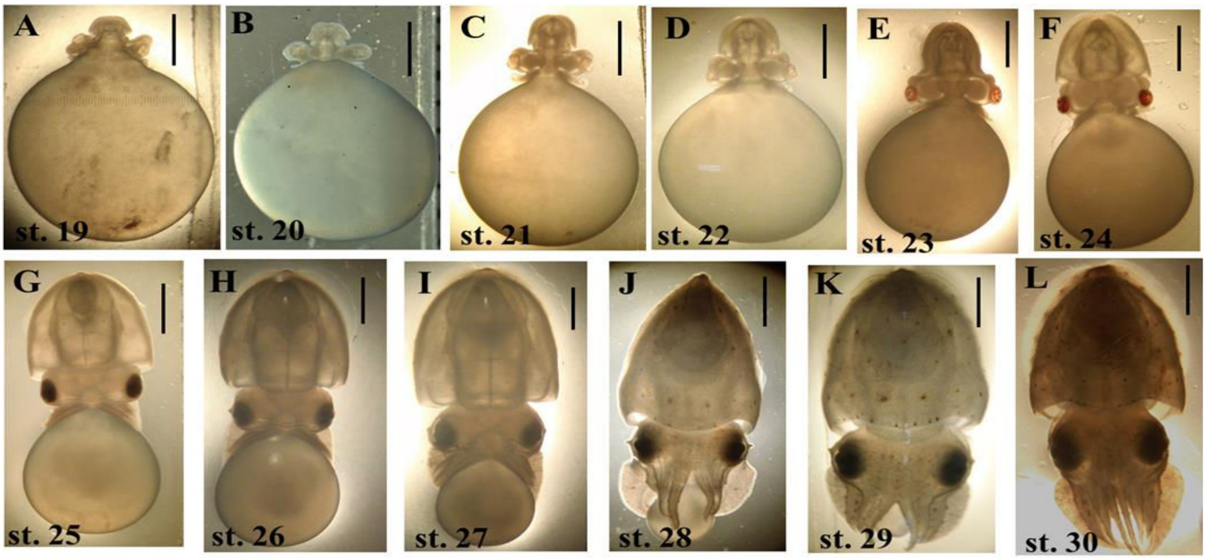

Figure 17 : Photographies d'un œuf de *Sepia pharaonis* pendant l'organogenèse. Nous observons très l'embryon au-dessus du sac vitellin dès le stade 19 (A). Le corps de l'embryon grossit progressivement alors que le sac vitellin externe diminue significativement en taille tout au long de cette phase. L'échelle représente 2mm (Lee et al., 2016).

Comme chez *Sepia officinalis*, la rétine de *Sepia pharaonis* se colorent progressivement. Ils deviennent oranges au stade 23 (cf. Figure 17-E) puis rouges au stade 24 (cf. Figure 17-F) et enfin marrons au stade 25 (cf. Figure 17-G). La coloration des yeux de *Sepia pharaonis* est alors beaucoup plus rapide que chez *Sepia officinalis* (qui s'étend du stade 24 à 28).

3) Les organes des sens

Perception tactile et vestibulaire

➢ *Les statocystes :*

Les statocystes des céphalopodes sont des petits organes situés en dessous du cerveau (cf. Figure 18). Ils donnent des informations sur la gravité et sur l'accélération nécessaire au maintien de l'orientation (Hanlon & Messenger, 2018; O'Brien, Mezrai, Darmaillacq, & Dickel, 2017). Ils jouent également un rôle dans la détection d'infrasons (Hanlon & Messenger, 2018). Lorsqu'ils sont détruits par chirurgie cela engendre des perturbations dans la nage des individus (*Octopus vulgaris* : Boycott, 1960; *Sepia officinalis :* Messenger, 1970).

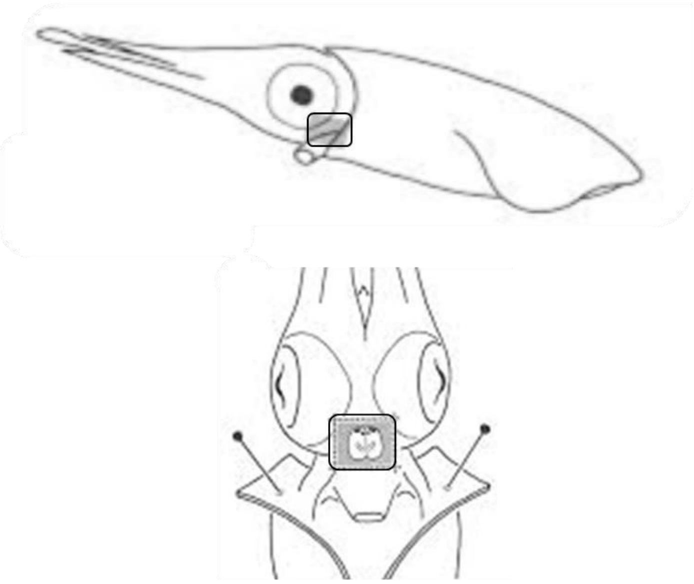

Figure 18 : Représentation schématique représentant l'emplacement des statocystes chez le calmar (d'après Budelmann, 1990). Ils sont placés dans le cartilage sous le cerveau des céphalopodes (zone noire encadrée). Dessin du haut : dessin en vue latérale ; dessin du bas : dessin en vue ventrale.

➢ *Ligne latérale analogue :*

L'épiderme de la tête et des bras de *Sepia officinalis* comportent 10 lignes de cellules ciliées qui répondent aux déplacements d'eau locaux. Ce système de mécanoréception, observé pour la première fois en 1928 par Naef est analogue à la ligne latérale que l'on retrouve chez les poissons et certains amphibiens aquatiques (Budelmann & Bleckmann, 1988). Cette ligne épidermique est déjà présente chez les nouveau-nés et les cellules ciliées sont capables de répondre à des mouvements d'eau ayant une fréquence sinusoïdale allant de 0.5 à 40 Hz (cf. Figure 19 ; Bleckmann, Budelmann, & Bullock, 1991; Hanlon & Messenger, 2018). Selon Budelmann et ses collaborateurs ce système serait suffisamment sensible pour permettre à une seiche de détecter un poisson de 1 m de long à une distance de 30 m (Budelmann, Riese, & Bleckmann, 1991). Des expériences comportementales menées par la même équipe ont montré que les seiches sont capables d'attraper des petites crevettes dans l'obscurité totale laissant supposer que cette ligne latérale analogue aurait un rôle dans la détection des proies (Budelmann et al., 1991).

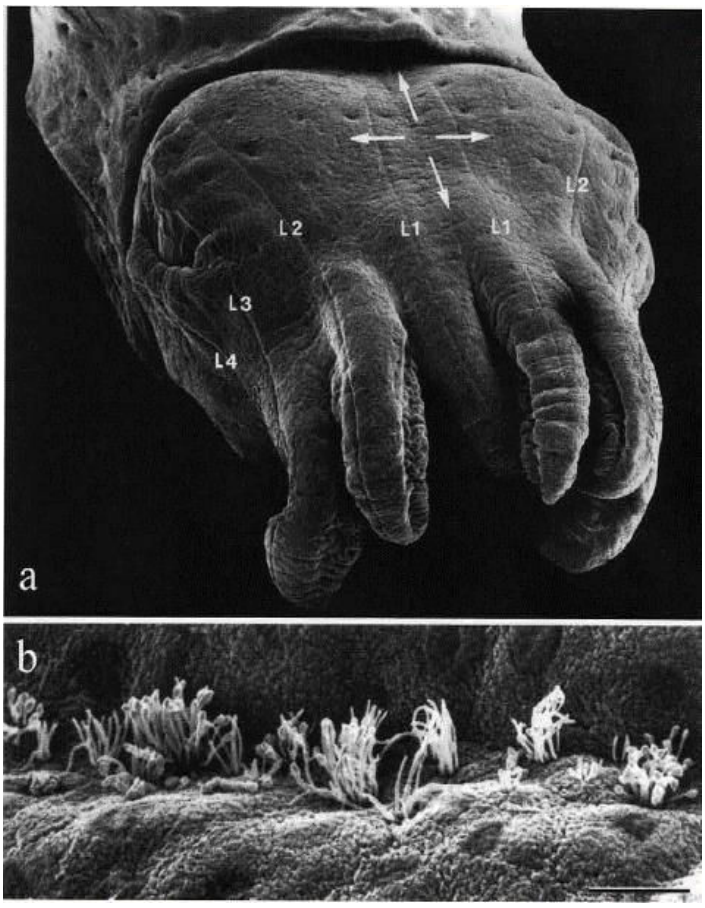

Figure 19 : Microscopie électronique à balayage d'une seiche juvénile (*Sepia officinalis*) et de sa ligne latérale analogue. Les rainures sur la tête et sur le long de la surface dorsale des bras (L1 à L4) contient des cellules ciliées (b) qui sont polarisées dans le sens des flèches représentées en (a). L'échelle représente 10 µm. (Budelmann, Schipp & Boletzky 1997 in Hanlon & Messenger, 2018).

- *Récepteurs tactiles :*

Tous les céphalopodes semblent être sensibles au sens du touché. Les études se sont pourtant plus focalisées sur la pieuvre commune *Octopus vulgaris* qui présentent des mécanorécepteurs sur les ventouses, le manteau et les nageoires (Gray, 1960; Graziadei & Gagne, 1976). Chaque ventouse contiendrait au moins 600 récepteurs tactiles. Chez la seiche, les mécanorécepteurs ont été décrits sur les nageoires et sur les ventouses (Graziadei, 1964; Kier, Messenger, & Miyan, 1985).

Perception olfactive et gustative (chémoréception)

L'olfaction est un sens chimique à distance alors que la gustation nécessite un contact direct avec l'item (alimentaire par exemple).

- *Gustation*

On sait que les poulpes et les seiches ont des chimiorécepteurs sur les lèvres et sur les ventouses (Graziadei, 1964a, 1964c, 1965). Les rebords des ventouses en particulier contiennent un grand nombre de cellules ciliées. Chez les pieuvres, il y a environ 10 000 de ces cellules primaires sur chaque ventouses. Étant donné qu'il y a 200 ventouses sur chaque bras, il y a environ 16 millions de cellules chez une pieuvre adulte. Chez les seiches, il n'y a qu'une centaine de cellules par ventouses et beaucoup moins de ventouses sur les bras (Graziadei, 1964c). Cette différence souligne les différents styles de vie des deux groupes : les pieuvres utilisent leurs bras pour détecter les aliments, tandis que les seiches utilisent leurs bras principalement pour tenir leurs proies capturées après une attaque visuelle (Hanlon & Messenger, 2018).

- *Olfaction*

Les céphalopodes ont aussi la capacité de détecter certains stimuli chimiques environnementaux grâce à un organe olfactif situé sous la peau derrière chaque œil (cf. Figure 20 ; Cosmo & Polese, 2017; Gleadall & Shashar, 2004; Nixon & Young, 2003; Scaros, Croll, & Baratte, 2018). Les pieuvres peuvent par exemple détecter des odeurs (odeur de crustacés par exemple) uniquement grâce à ce sens chimique (le nerf optique des individus ayant été sectionné afin de les rendre aveugles ; Chase & Wells, 1986). Même avec de très faibles concentrations, les individus testés se dirigent vers la source émettrice (chimiotaxie). Les seiches aussi sont capables de percevoir et de répondre à différentes odeurs. Une étude menée par Boal et Golden (1998) a montré que le rythme ventilatoire des seiches (indiqué par les mouvements de l'entonnoir et des plis collaires) augmentait de manière significative après l'exposition à de l'encre de seiche, de l'eau contenant de la nourriture, de l'eau contenant un congénère, de l'eau de mer non familière et de l'eau contenant des tortues (prédateurs potentiels). Cette expérience souligne les capacités de perception chez les jeunes seiches. Cependant, étant donné que les individus répondent à toutes les odeurs il est impossible de savoir s'ils les discriminent.

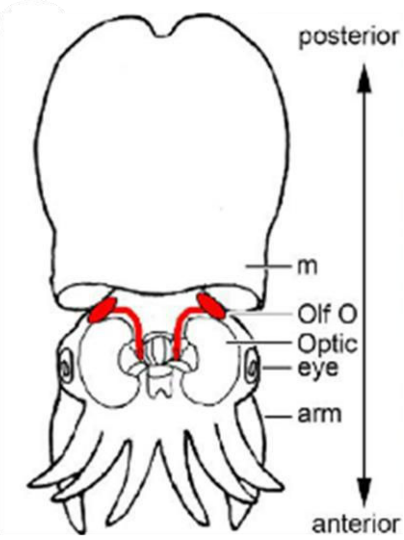

Figure 20 : Représentation schématique d'un embryon de seiche au stade 29 montrant la localisation du système olfactif (en rouge). L'organe olfactif (Olf O en rouge) est relié au lobe olfactif dans le cerveau par le nerf olfactif (en rouge) (Scaros et al., 2018).

Perception visuelle

La vision est le sens le plus développé chez la seiche. D'un point de vue structural les yeux sont semblables à ceux des Vertébrés et comportent une cornée, un iris, une pupille, un cristallin et une rétine (cf. Figure 21).

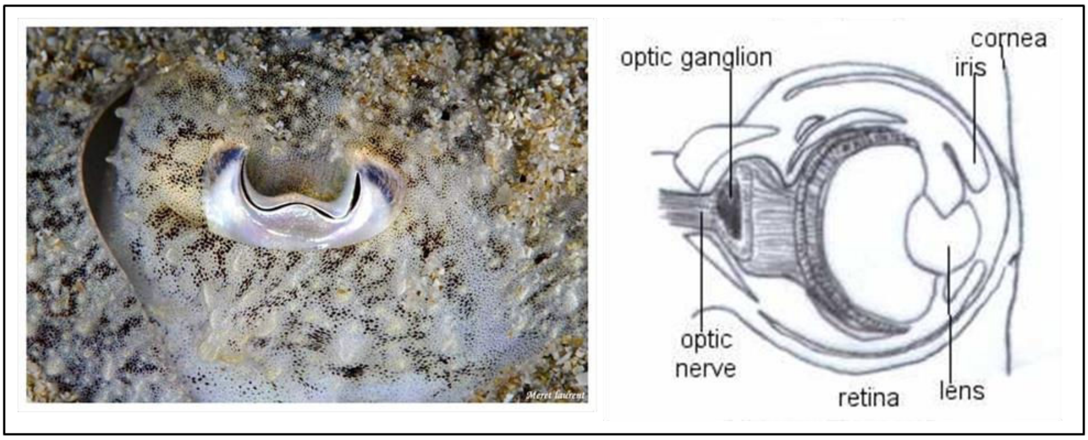

Figure 21 : Photographie d'un œil de seiche adulte à gauche (© Meret). Représentation schématique d'une coupe horizontale de l'œil montrant le ganglion optique, le nerf optique, l'iris, la cornée, la rétine et le cristallin.

Les yeux sont en position latérale ce qui permet d'avoir un champ visuel de quasiment 360° (Messenger, 1968). La vision est principalement monoculaire et il y a une vision antérieure binoculaire jouant un rôle important au moment de la capture des proies : les muscles extraoculaires permettant la convergence des yeux (Douglas, Williamson, & Wagner, 2005). En dépit de la forte ressemblance avec les Vertébrés, les yeux des seiches ne comportent ni cônes ni bâtonnets. Les récepteurs visuels sont des longues cellules (400µm) très fines (5µm de diamètre) portant des microvillosités contenant la rhodopsine, le pigment visuel unique des seiches (Sugawara, Katagiri, & Tomita, 1971). Ainsi, les seiches ne seraient pas capables de voir les couleurs étant donné qu'il faut au moins deux pigments pour voir en couleur (Mäthger, Barbosa, Miner, & Hanlon, 2006; Tansley, 1965).

La pupille des seiches change de taille très rapidement en fonction de l'intensité lumineuse et possède une forme en W (due à la contraction de la paupière : Douglas, Williamson, & Wagner, 2005). En plus de cette sensibilité aux différences d'intensités lumineuses, la seiche est sensible au plan de polarisation de la lumière (Cartron, 2012; Mäthger, Denton, Marshall, & Hanlon, 2009; Shashar, Rutledge, & Cronin, 1996). Lorsque la lumière traverse la surface de mer, une partie des rayons sont réfléchis et réfractés. Ce phénomène va alors induire une polarisation linéaire partielle de la lumière (cf. Figure 22). Les seiches sont capables de percevoir la lumière polarisée ce qui lui permet de la distinguer sur les écailles des poissons ou sur la cuticule des crustacés. La détection des proies sera ainsi plus aisé puisque cela va créer un contraste entre la proie et son environnement (Cartron, 2012; Shashar, Hagan, Boal, & Hanlon, 2000).

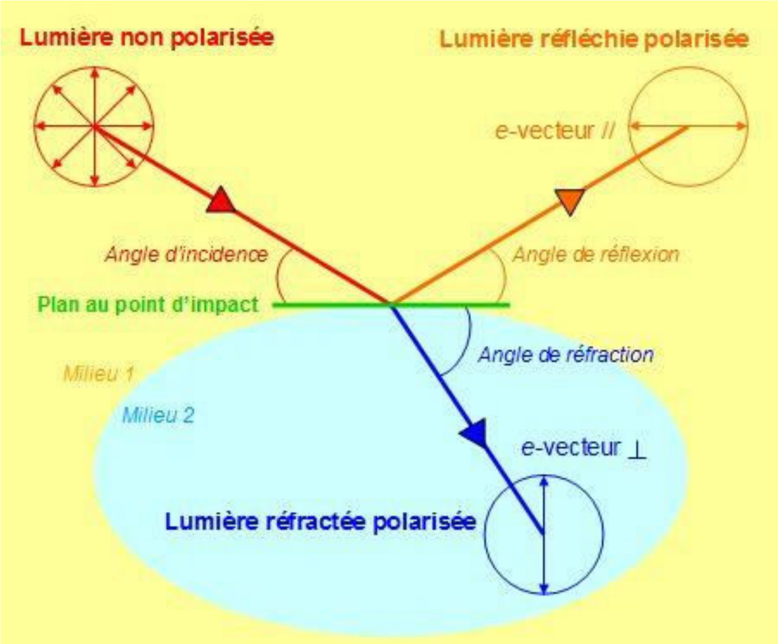

Figure 22 : Schéma des phénomènes de réflexion (en orange) et réfraction (en bleu) d'une lumière non polarisée (en rouge) lors du passage d'un milieu de propagation 1 (jaune) à 2 (bleu clair). L'angle d'incidence et de réflexion sont équivalents mais différents de l'angle de réfraction. Le e-vecteur de la lumière réfléchie est parallèle au plan au point d'impact (en vert) tandis que le e-vecteur de la lumière réfractée est perpendiculaire à celui-ci (Leila Cartron, 2012).

Capacité de perception prénatale chez la seiche

Les embryons de seiche sont protégés de l'environnement extérieur par la capsule de l'œuf mais ils n'en sont pas entièrement isolés. Au début du développement de *Sepia officinalis*, la couche d'encre dans la membrane de l'œuf absorbe la lumière et empêche la plupart des informations visuelles de pénétrer (Paulij, Herman, Roozen, & Denucé, 1991). De plus, les œufs peuvent être pondus à une profondeur où il ne reste que 10% de l'intensité lumineuse en surface (Bloor, Attrill, & Jackson, 2013). En dépit de ces limitations, la membrane devient translucide en raison de la dilatation des œufs et la rétine commence à se développer à partir du stade 20 (Lemaire, 1970). Par conséquent, la réaction à une stimulation lumineuse est observé au stade 25 (Romagny et al., 2012). De même, les embryons sont capables de percevoir les signaux chimiques présents dans l’eau qui diffusent à travers la membrane de l'œuf et les stimuli tactiles générés par les mouvements dans l'environnement extérieur au stade 23 (Romagny et al., 2012). Ainsi, la période d’organogenèse du développement embryonnaire est caractérisée par une accumulation progressive de la quantité d’informations sensorielles pénétrant dans l’œuf. Ces résultats ont été obtenus chez *Sepia officinalis* mais à notre connaissance, aucune étude n’a encore été réalisée chez *Sepia pharaonis*.

4) Capacité d’apprentissage chez la seiche

Habituation

Chez *Sepia officinalis*, la translucidité partielle des œufs au stade avancé permet d’observer les mouvements du manteau de l’embryon à l’intérieur et élargit la gamme de stimuli pouvant être testés visuellement. En réponse à un nouveau stimulus visuel, tactile ou chimiosensoriel, les embryons réduiront la respiration et mouvements de manteau. La reprise ultérieure d'une augmentation du mouvement du manteau après une exposition répétée ou chronique au stimulus indique une habituation. Cela a été démontré chez des embryons au stade final (30) soumis à des expositions répétées à la lumière vive (Romagny et al., 2012).

Empreinte et apprentissage perceptif

L'empreinte est une forme d'apprentissage simple caractérisée par l'établissement d'une préférence indélébile et persistante pour un objet au cours d'une période sensible limitée, généralement au début du développement (Bolhuis, 1991; Lorenz, 1937; Sluckin, 2017). Contrairement à l'apprentissage perceptif, l'empreinte peut être définie par 5 critères : 1) il n'y a pas de renforcement ; 2) l'empreinte se met en place pendant une période sensible ; 3) elle est indélébile ; 4) cette préférence sera généralisée à d'autres objets partageant des caractéristiques similaires ; 5) l'empreinte a des conséquences sur les comportements ultérieurs de la vie (Sluckin, 2017).

L'empreinte alimentaire a été démontrée chez *Sepia officinalis*. Selon Wells (1958) et d'autres auteurs (Darmaillacq, Chichery, & Dickel, 2006; Darmaillacq, Chichery, Shashar, & Dickel, 2006), les nouveau-nés ont une préférence « innée » pour les crevettes ou pour les objets en forme de crevettes. Darmaillacq et al. (2004a; 2006a; 2006b) ont démontré que cette préférence pouvait être annulée par une exposition chimique et/ou visuelle aux crabes peu de temps après l'éclosion. Cette préférence induite répondait aux critères de l'empreinte : elle durait au moins trois jours, persistait après la consommation d'une crevette et n'était induite que pendant une courte période sensible au début de la vie de la seiche (Darmaillacq, Chichery, & Dickel, 2006; Darmaillacq, Chichery, Poirier, & Dickel, 2004; Darmaillacq, Chichery, Shashar, et al., 2006). Il est intéressant de noter que cette période sensible d'induction des préférences des proies commence avant l'éclosion : les embryons exposés visuellement aux crabes pendant au moins une semaine avant l'éclosion ont une préférences pour les crabes (Darmaillacq, Lesimple, & Dickel, 2008).

En plus de la capacité de distinguer différents types de crustacés décapodes, la préférence des proies pourrait également être induite sur la base du contraste de luminosité : alors que la seiche naïve préfère le crabe noir au crabe blanc comme premier repas, les seiches exposées aux crabes blancs préfèrent ces derniers comme premier repas (Guibé, Poirel, Houdé, & Dickel, 2012). De plus, les seiches exposées aux crabes blancs avant ou après la naissance préfèrent les crabes noirs aux crevettes, ce qui indique que *Sepia officinalis* généralise les caractéristiques d'une préférence apprise à l'alternative la plus proche si l'élément préféré n'est pas disponible (Guibé, Boal, & Dickel, 2010).

Enfin, outre la démonstration de l'apprentissage prénatal des préférences alimentaires, il a été démontré que l'exposition à d'autres objets écologiquement importants dans l'environnement d'incubation peut avoir une incidence sur le comportement futur. On a constaté que les nouveau-nés naïfs préféraient spontanément les abris sombres. L'exposition prénatale dans des abris blancs a éliminé cette préférence, ce qui a permis à la seiche de se cacher également sous un abri noir ou blanc (Guibé & Dickel, 2011).

Apprentissage associatif et mémoire

Chez les seiches, ce phénomène a été mis en évidence pour la première fois chez les adultes et les subadultes en utilisant un paradigme d'aversion pour le goût (Darmaillacq, Dickel, Chichery, Agin, & Chichery, 2004). Au cours de cette étude, 81% des seiches préféraient les crabes 1 à 3 jours après avoir attaqué une crevette recouverte d’un produit chimique désagréable (quinine).

L'apprentissage associatif a également été démontré chez des seiches juvéniles en utilisant un paradigme appelé le test « prawn in a tube » (PIT). Dans ce test, une crevette est placée dans un tube transparent et est offerte aux seiches. À cause du tube, les seiches peuvent voir la crevette mais ne peuvent pas la capturer. Après plusieurs attaques infructueuses sur la crevette inaccessible dans le tube, les seiches adultes sont capables de se souvenir de l'association pendant plusieurs minutes (Messenger, 1973; Wells, 1958, 1962). Si on leur présente une crevette dans un tube entre 20 et 60 minutes après avoir appris, ils attaquent à nouveau comme s'ils n'avaient jamais appris la tâche. Mais si on leur présente une crevette une heure ou plus après avoir appris, ils se rappelaient à nouveau de ne pas attaquer (Messenger, 1973). Cette tendance résulterait de processus séparés de la mémoire à court terme (STM) et à long terme (LTM) (Dickel, Chichery, & Chichery, 1998). Contrairement aux adultes, les seiches âgées de moins de 8 jours continuent d’attaquer pendant plusieurs heures la crevette inaccessible, ce qui montre qu’elles ne sont pas en mesure de faire une association entre la présence du tube et un manque de récompense (Agin, Poirier, Chichery, Dickel, & Chichery, 2006; Dickel et al., 1998). Après cet âge, les seiches présentent une STM pleinement opérationnel (Dickel et al., 1998). En revanche, leur LTM sera à maturité vers 90 jours (Dickel, Chichery, & Chichery, 2001).

II. Méthodologie générale

1) Animaux utilisés

Sepia officinalis

Les études réalisées avec *Sepia officinalis* ont été faites au Centre de Recherches en Environnement Côtier à Luc sur mer (CREC – Normandie – France). Tous les œufs étudiés ont été pondus au large de Luc-sur-mer sur des cordes attachées au fond de la mer ou bien sur des casiers à seiche. Ils ont été maintenus dans des grands bacs de 1000 litres (168cm de diamètre, 61.5cm de hauteur), en circuit d'eau naturelle semi ouvert, sous une photopériode LD 12 :12 et à une température de 15 ± 2°C. Les œufs ont tous été détachés de la grappe afin d'assurer une meilleure oxygénation et ils ont été placés dans des passoires flottantes (cf. Figure 23). Une semaine avant le début de chaque expérience, les œufs ont été installés dans des bacs de 65 litres (80x60x40cm) sous les mêmes conditions mais à une température de 18 ± 2°C. Ce changement de condition a été effectué pour avoir un développement optimal des embryons (Lemaire, 1970).

Figure 23 : Passoires comportant des œufs de *Sepia officinalis* (© Mezrai).

Chaque matin, les nouveau-nés ont été récupérés et installés dans de nouveaux contenants (par groupe ou individuellement en fonction de l'expérimentation en cours). Puis, ils ont été nourris quotidiennement à partir du $4^{ème}$ jour avec des crevettes ou des crabes de petites tailles. Les seiches mangent rarement avant le $4^{ème}$ jour puisqu'elles ont encore des réserves internes de vitellus. Une fois les expériences comportementales terminées, toutes les seiches ont été libérées en mer sur la plage de Luc-sur-mer.

Sepia pharaonis

Les études menées chez cette espèce ont été réalisées au cours de séjours à Taiwan (2016 par Lorenzo Arduini ; 2017 par moi-même ; 2018 par Iris Lemercier et Melvyn Martin). Tous les œufs étudiés proviennent de la station marine de recherche « Aquaticlch Biotech Company Ltd. Aquaculture » (Yilan – Taiwan). Ils sont issus de reproduction entre des adultes de 2 ans (4 femelles et 2 mâles) pêchés et élevés dans cette zone semi-naturelle (première génération). Les œufs ont ensuite été transférés à l' « Institute of Systems Neuroscience & Department of Life Science » (National Tsing Hua University – Hsinchu – Taiwan). Durant le trajet, les œufs étaient maintenus dans de grands contenants de 30x50x30cm remplis d'eau de mer naturelle. Une pompe à air a été installée dans ces contenants afin d'éviter un stress hypoxique chez les embryons. Une fois amenés à l'institut, les œufs ont été maintenus en eau de mer naturelle en circuit semi-ouvert, à une température de 25±2°C et une photopériode LD 12 :12. Chaque œuf a été séparé individuellement des grappes et incubé dans un contenant en flottant dans les cuves de 300L (cf. Figure 24; maximum de 20 œufs par panier de 15 x 20 x 3 cm). De la même façon que pour *Sepia officinalis*, chaque matin, les nouveau-nés ont été récupérés et installés dans de nouveaux contenants (par groupe ou individuellement en fonction de l'expérimentation en cours). Puis, ils ont été nourris quotidiennement à partir du 4ème jour avec des crevettes. Enfin tous les juvéniles ont été donnés et élevés à la station marine de Yilan (cf. Figure 25).

Figure 24 : Photographie des œufs de *Sepia pharaonis* avant (gauche) et après leur séparation (© Mezrai).

Figure 25 : Photographie de seiches nouveau-nées (*Sepia pharaonis* ; © Mezrai).

2) Variables mesurées et méthodes utilisées

Mesure du rythme ventilatoire au cours des tests de perception et d'apprentissage

Variable mesurée : le rythme ventilatoire

Le rythme ventilatoire (RV) est un paramètre physiologique qui a déjà été utilisé chez les seiches juvéniles et adultes (Boal & Golden, 1999; Boal & Ni, 1996). Il peut être observé directement en vue ventrale à partir du mouvement de ventilation de l'entonnoir avec les changements de pression d'inhalation et d'expiration ou bien en vue dorsale à partir des mouvements rythmiques des plis collaires au niveau du manteau. A l'instar des études menée par Boal et ses collaborateurs, le RV a été mesuré chez les juvéniles (**chapitre 5**) mais aussi pour la première fois chez les embryons (**chapitre 3 et 4**). Il nous a alors fallu mettre au point un protocole afin de pouvoir observer ce paramètre au cours de la période prénatale. Chez les embryons, Romagny et al. (2012) ont mesuré la fréquence de contraction du manteau au cours de leur étude. Mais cette variable n'est observable que chez les embryons en cas de très fortes stimulations. Nous ne pouvions pas utiliser cette variable étant donné que nous souhaitions tester des stimuli d'intensité variable (e.g. : odeur de proies ; lumière forte…) chez les embryons et chez les juvéniles. De plus, la mesure du RV s'est avérée être une variable détectable très tôt dans le développement des embryons. Au cours de nos expériences, la mesure du RV a été réalisée 1 minute avant (rythme de base après une acclimatation de 5 min) et 1 minute après chaque stimulation (réponse à la stimulation).

Dispositif expérimental :

Le premier dispositif expérimental testé est représenté sur la Figure 26. L'embryon était d'abord placé dans un contenant en plastique transparent de 150 ml installé sous un microscope binoculaire équipé d'un éclairage incident à LED (Jeulin® 571265). Deux bouteilles d'eau étaient placées en hauteur de la loupe binoculaire et étaient reliées au contenant de l'embryon par des tuyaux de 0,5cm de diamètre. Ces bouteilles pouvaient alimenter ou non le contenant de l'embryon grâce à un robinet installé sur le tuyau. La première bouteille était remplie d'eau de mer provenant du bac des embryons et alimentait le contenant de l'embryon pendant la période d'acclimatation. Puis, pendant la phase de stimulation le robinet de la première bouteille était fermé et le robinet de la deuxième bouteille (comportant l'odeur testée par exemple) était ouvert afin d'alimenter le cristallisoir. La caméra vidéo du microscope a été connectée à un ordinateur, permettant l'observation et le comptage direct du RV.

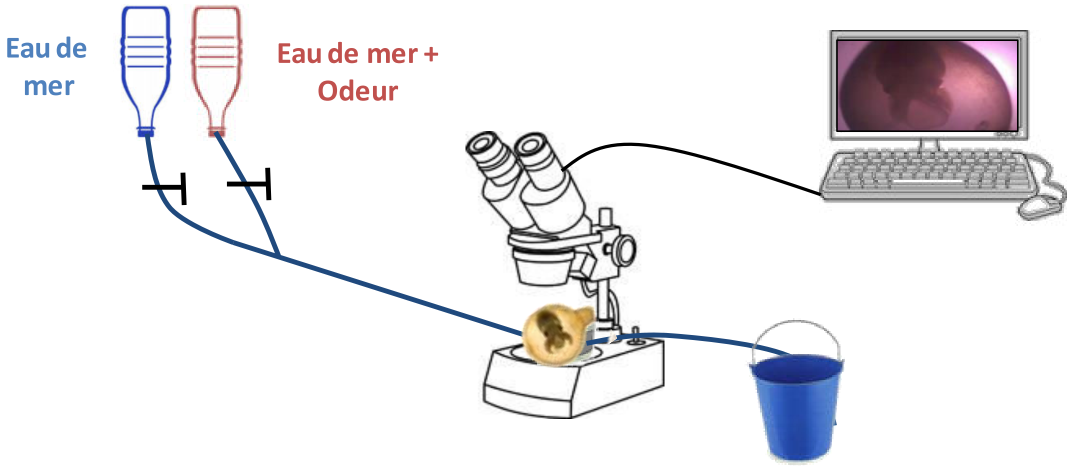

Figure 26 : Schéma du premier dispositif testé pour observer le RV des embryons. L'œuf est placé dans un contenant sous une loupe binoculaire reliée à un ordinateur. Deux bouteilles pouvaient alimenter ou non le cristallisoir de l'embryon grâce à un robinet installé sur le tuyau. La première bouteille était remplit d'eau de mer provenant du bac des embryons et alimentait le cristallisoir pendant la période d'acclimatation. Pendant la phase de stimulation le robinet de la première bouteille était fermé et le robinet de la deuxième bouteille (comportant l'odeur testée) était ouvert afin d'alimenter le cristallisoir.

Outre certains problèmes techniques (eau de mer inondant les loupes binoculaires, mouvement de l'œuf pendant l'expérience, biais induit par la manipulation des bouteilles d'eau, difficulté dans le réglage des débits d'eau…) ce dispositif ne nous permettait pas de savoir avec exactitude quand l'odeur testée arrivait à proximité de l'œuf. Nous avons alors utilisé un autre dispositif beaucoup plus simple et pouvant être répliqué en limitant les biais. Ce dispositif est représenté sur la Figure 28. A l'exception de l'article 2 (où le dispositif utilisé n'est pas le même ; voir chapitre 4-I), tous les œufs testés ont été placés dans un cristallisoir de 100 ml (chapitre 3) ou 150 ml (chapitre 4 et 5) installé sous le microscope binoculaire (*Sepia officinalis :* Jeulin® 571265 ; *Sepia pharaonis* : loupe binoculaire numérique Saekodive AL-18LED-3C6AA). Pour tester les stimulations chimiosensorielles, 3 ml d'odeur sont déposés à 1cm de l'œuf avec une pipette. Pour tester les stimulations visuelles, les embryons sont placés dans un contenant de 50 ml totalement étanche et les stimuli visuels sont présentés face à l'embryon. Comme nous pouvons le voir sur la Figure 27, les embryons ne manquent pas d'oxygène dans le bécher de 50ml puisqu'il reste plus de 95% au bout de 10 min dans ce dernier (même s'il contient un juvénile, un embryon, un embryon et un crabe ou s'il est vide).

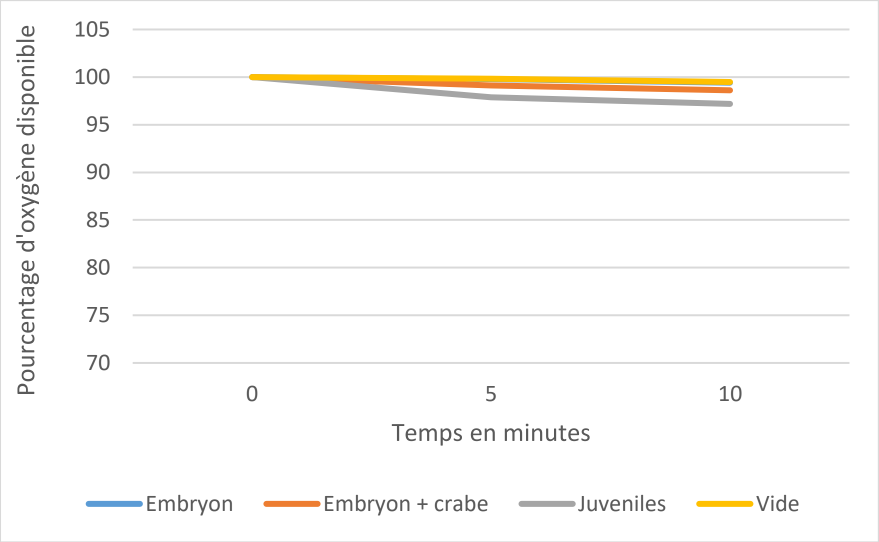

Figure 27 : Pourcentage d'oxygène disponible dans le bécher de 50ml pendant 10 minutes. Le bécher contient un embryon (bleu) ; un embryon et un crabe (orange) ; un juvénile (gris) ou vide (jaune).

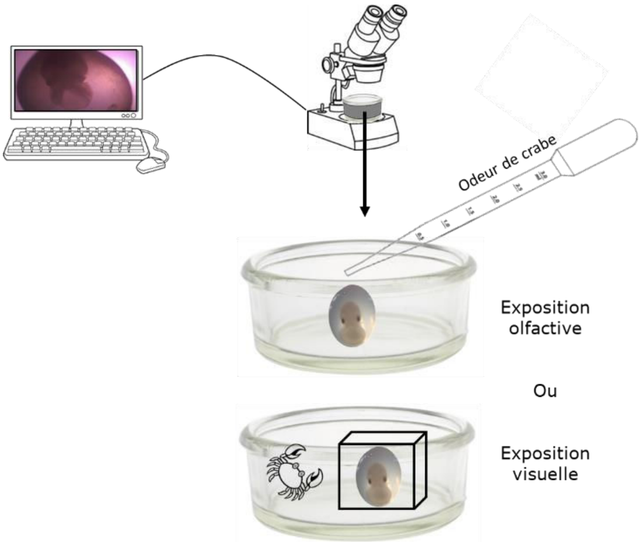

Figure 28 : Schéma du dispositif utilisé pour mesurer le RV des embryons. L'œuf est placé dans un cristallisoir de 100 ou 150 ml sous une loupe binoculaire reliée à un ordinateur.

Empreinte alimentaire et choix postnatal alimentaire

Afin de tester les effets du stress sur les capacités d'apprentissage simple chez les embryons de seiche, nous avons utilisé un protocole d'empreinte alimentaire décrit par Darmaillacq et collaborateurs en 2008 (**chapitre 5**). Pour ce faire, les œufs des deux espèces ont été placés pendant au moins une semaine dans un dispositif avec des proies (des crabes pour *Sepia officinalis* et des gammares pour *Sepia pharaonis*). Puis, sept jours après l'éclosion, les juvéniles de chaque groupe ont été soumis à un test de discrimination à double choix entre deux proies (2 crabes/gammares et 2 crevettes). Le dispositif utilisé était une arène rectangulaire en PVC noir se terminant en 3 compartiments adjacents séparés (cf. Figure 29). Le verre transparent utilisé dans la procédure entre les proies et les seiches n'affectait pas la polarisation de la lumière. Le positionnement des proies a été modifié de manière aléatoire pour éliminer la possibilité d'un choix basé sur la position et la latéralité des proies. Les seiches sont d'abord placées dans le dispositif pendant une période d'acclimatation de 10 min (les proies sont cachées par une plaque noire en plexiglass). Puis, cette plaque est retirée et pendant 15 minutes la seiche pouvait choisir entre les différentes proies et nous notons le premier choix et la latence de choix des jeunes.

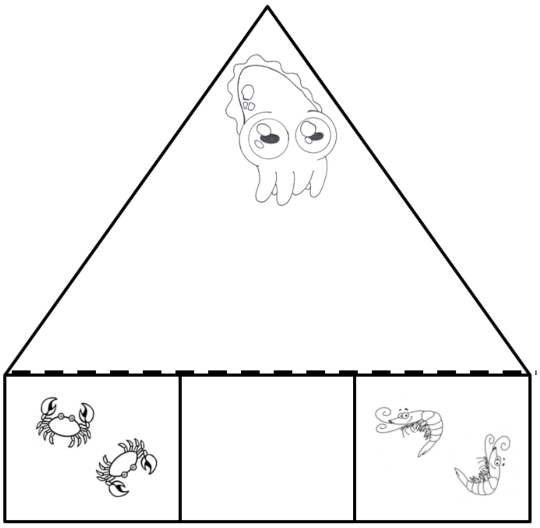

Figure 29 : Représentation schématique de la discrimination de choix bidirectionnelle. La seiche pouvait choisir entre les crabes et les crevettes. Les proies ont été placées au hasard à droite et à gauche pour éviter les biais de latéralité. Les proies ont été isolées de la seiche par un verre transparent (ligne pointillée).

3) Note éthique

Depuis le 1^er^ janvier 2013, l'utilisation des céphalopodes en expérimentation animale est soumise à la réglementation européenne concernant le bien-être animal (directive 2010/63/UE). Même si les embryons de seiches n'entrent pas dans le cadre de cette directive, une saisine a été déposée et validée par le comité d'éthique local (Cenomexa #54). Par ailleurs, les structures d'élevage et d'expérimentation ont également reçu l'agrément des services vétérinaires compétents (A14384001). De même, à Taiwan, l'ensemble du protocole expérimental ainsi que le maintien des animaux ont été validé par le Comité national de protection et d'utilisation des animaux de l'Université Tsing Hua (Protocole IACUC n°10510). Dans un souci de bien-être, nous avons suivi les directives publiées sur le soin et le bien-être des céphalopodes afin d'éviter le stress chez les animaux (Fiorito et al., 2015).

Chapitre 3

Perception et réponse embryonnaire chez la seiche

Chapitre 3 - Perception et réponse embryonnaire chez la seiche

Contexte du chapitre 3 :

Comme nous l'avons vu dans le **chapitre 1** les embryons peuvent percevoir un grand nombre de stimulations environnementales *in ovo* ou *in utero* pouvant avoir un effet sur leurs comportements après l'éclosion. L'objectif de ce **chapitre 3** est de déterminer si les embryons de seiche peuvent percevoir certaines stimulations environnementales et de savoir comment ils y répondent. Pour cela, au cours de la **première partie** de ce chapitre, le rythme ventilatoire (RV) des embryons de seiche de *Sepia officinalis* a été mesuré avant et après chaque exposition à des proies et des prédateurs (des éléments essentiels pour la survie des jeunes dès leur éclosion) mais aussi à de l'encre de seiche et du broyat d'embryon (des potentiels signaux d'alarme). Puis, au cours de la **seconde partie** de ce chapitre, nous avons cherché à savoir à partir de quand les systèmes chimiosensoriels et visuels des embryons de *Sepia officinalis* et *Sepia pharaonis* sont fonctionnels. Pour cela, nous avons exposé les embryons (du stade 21/22 au stade 30) à des odeurs de prédateur et de la lumière forte.

Ce chapitre s'organise de la façon suivante :

I - Etude préliminaire : Description de la réponse embryonnaire chez *Sepia officinalis* face à des stimulations environnementales.

II- Article (1) **- Mezrai, N.,** Dickel, L., Chiao, C.-C., & Darmaillacq, A.-S., submitted in *developmental psychobiology*. A difference in timing for the onset of visual and chemosensory systems during embryonic development in two closely related cuttlefish species.

I. Etude préliminaire : description de la réponse embryonnaire face à des stimulations environnementales

Afin de commencer nos investigations sur les capacités de perception et de réponse chez l'embryon de seiche, nous nous sommes d'abord intéressés à des éléments essentiels pour la survie des jeunes : l'alimentation et la détection de dangers. Dans la littérature il a déjà été montré que les embryons de seiches sont capables de percevoir des proies la dernière semaine avant l'éclosion des seiches (Darmaillacq et al. 2018) mais aussi des odeurs de prédateurs (Romagny 2012). Nous avons alors voulu caractériser la réponse du RV des embryons face à ces items. De plus, les embryons ont été exposés à un signal d'alarme : de l'encre de seiche (Derby, 2014) et des odeurs de congénères blessés (un signal d'alarme chez certains Vertébrés ; Ferrari & Chivers, 2009a, 2009b; Ferrari et al., 2010; Garcia et al., 2017).

Introduction

La seiche est un prédateur qui chasse à vue (Hanlon & Messenger, 2018; Messenger, 1968) et certains céphalopodes peuvent utiliser des indices chimiosensoriels pour s'orienter vers des sources alimentaires ou pour reconnaitre les proies (Boal & Golden, 1999; Chase & Wells, 1986). Les analyses des contenus stomacaux rapportent que les jeunes seiches se nourrissent principalement de petits crustacés (e.g. crevettes, mysis et amphipodes ; Blanc, Du Sel, & Daguzan, 1998; Boletzky, 1983). Ces différentes proies sont présentes autour des œufs dans l'environnement et nous avons des preuves indirectes que les embryons les perçoivent. En effet, les embryons exposés à des crabes pendant au moins une semaine avant l'éclosion préfèrent les crabes aux crevettes comme premier repas (proies pourtant préférées par les seiches naïves ; Darmaillacq, Lesimple, & Dickel, 2008). En plus de devoir trouver des proies, les seiches, surtout lorsqu'elles sont jeunes, sont soumises à une forte pression de prédation. Dans leur environnement naturel, les prédateurs de *Sepia officinalis* sont les poissons carnivores tels que les bars communs (*Dicentrarchus labrax)* mais aussi les roussettes (*Scyliorhinus canicula*). Les odeurs de ces prédateurs peuvent être perçues très tôt au cours de la période prénatale (à partir du stade 23 ; Romagny et al. 2012). De même, les jeunes doivent être en mesure de reconnaitre leur signal d'alarme afin d'échapper au mieux aux prédateurs et ainsi augmenter leur chance de survie (Derby, 2014). Cependant nous ignorons si les embryons sont capables de percevoir et de répondre à leur signal d'alarme, l'encre de seiche, et nous ignorons si à l'instar des Vertébrés, l'odeur de congénères blessés peut être un signal d'alarme chez la seiche. Au cours de cette étude préliminaire, le RV des embryons sera mesuré avant et après une exposition à :

1. des proies : odeur ou vision de crabes ou de crevettes
2. des odeurs de prédateurs et de non-prédateur
3. de l'encre de seiche et de l'odeur de congénères blessés (broyat)

Matériel et méthodes

Les proies :

Tous les embryons testés proviennent d'œufs pondus au large de Luc-sur-mer (Calvados, Normandie) comme décrit dans le **Chapitre 2-II**. Les crevettes grises européennes (*Crangon crangon*) et les crabes verts (*Carcinus maenas*) ont également été pêchés sur la plage de Luc-sur-mer. Ils ont été stockés dans deux bacs d'eau de mer différents de 1000 litres (168cm de diamètre ; 61,5cm de hauteur), en circuit d'eau semi ouvert, sous une photopériode LD 12 : 12 et une température de 15 ± 2°C. Nous avons utilisé la modalité visuelle seule (présentation de deux crevettes ou deux crabes) ou la modalité olfactive seule (présentation d'odeur de crevettes ou de crabes). Pour tester la modalité visuelle les embryons de seiche ont été exposés à deux crevettes ou a deux crabes (3±1 mm de longueur pour les crevettes et 5 ; 8 ; 10 ou 20 mm pour les crabes). Pour tester la modalité olfactive, 3ml d'eau du bac de vie des crevettes ou des crabes ont été prélevés et présentés aux embryons. Pour des raisons d'organisation méthodologique, la modalité olfactive a été testée chez les embryons au stade 28 et la modalité visuelle au stade 29.

Les prédateurs :

Les prédateurs et non-prédateurs utilisés étaient tous issus de l'élevage au CREC. En ce qui concerne les prédateurs, les bars communs (N=10 ; *Dicentrarchus labrax)* et les roussettes (N=8 ; *Scyliorhinus canicula*) étaient maintenus dans des bacs de 1000 litres, en circuit d'eau semi ouvert, sous une photopériode LD 12 : 12 et une température de 15 ± 2°C. Quant aux non-prédateurs (poissons herbivores), les mulets (N=2 ; *Chelon labrosus*) étaient maintenus dans des bacs de 65 litres sous les mêmes conditions. Les prédateurs étaient nourris quotidiennement avec du colin surgelé et les non-prédateurs avec des granules végétales (Tetra® Aliment Complet Pleco Veggie Wafers). Pour des raisons d'organisation méthodologique les embryons ont été testés au stade 27.

Signal d'alarme :

L'encre présentée aux embryons a été obtenue en diluant de l'encre de seiche fraichement éjectée par deux juvéniles de deux mois. Pour ce faire nous les avons placés dans un cristallisoir de 150 ml et nous les avons poursuivis avec une épuisette. Une fois le cristallisoir complètement noir (seiches non visibles) nous avons prélevé 3ml que nous avons dilué dans 150 ml d'eau de mer. L'encre testée devenait alors transparente. L'odeur de congénère blessés à quant à elle été obtenue par broyage d'embryons de seiche selon la méthode employée par Ferrari et ses collaborateurs (Ferrari & Chivers, 2009a, 2009b, 2010; Ferrari, Crane, & Chivers, 2016; Ferrari, Manek, & Chivers, 2010; Golub, 2013; Mathis, Ferrari, Windel, Messier, & Chivers, 2008; Saglio & Mandrillon, 2006). Deux embryons (stade 29) ont été retirés délicatement de leur œuf puis mis à mort par décapitation. L'ensemble a ensuite été placé dans 9ml d'eau de mer puis broyé au pilon dans mortier en porcelaine. 3 ml de ce liquide transparent a été prélevé à la pipette et présenté à chaque embryon. Pour ces expériences, les embryons ont tous été testés au stade 29.

Résultats

Exposition à des crevettes grises européennes (Crangon crangon)

Lorsque l'on expose les embryons à 3ml d'odeur de crevette, le RV reste stable avant et pendant la stimulation (cf. Figure 30; test de Wilcoxon : N=11 ; z=-0,736 ; p=0,4615). Par contre si cette exposition est visuelle (2 crevettes), le RV des embryons augmente significativement pendant la stimulation (cf. Figure 30; test de Wilcoxon : N=8 ; z=-2,388 ; p=0,0169).

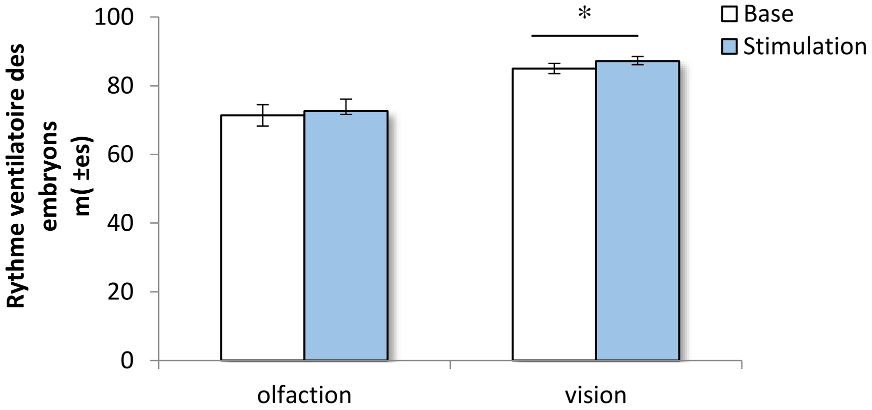

Figure 30 : Rythme ventilatoire des embryons de *Sepia officinalis* avant (rythme de base en blanc) et pendant une exposition (stimulation en bleu) à une odeur et à la vue de crevettes grises (*Crangon crangon*). Test de Wilcoxon * : p < 0,05.

Exposition à des crabes verts (Carcinus maenas)

Lorsque les embryons de *Sepia officinalis* sont exposés à une odeur de crabe, le rythme ventilatoire ne change pas (cf. Figure 31; test de Wilcoxon : n=8 ; z=-0,962 ; p=0,3363). Par contre, si les embryons de seiche sont exposés aux crabes (modalité visuelle uniquement) leur RV augmente significativement uniquement s'ils sont de petites tailles (cf. Figure 32; 5mm : test de Wilcoxon : N=14 ; z=-3,054 ; p=0,023). Si leur taille est de 8 mm, le RV a tendance à augmenter (cf. Figure 32; test de Wilcoxon : p=0,0545 z=-1,923 n=16) mais si leur taille dépasse 10 mm le RV reste stable avant et après l'exposition aux crabes (cf. Figure 32; test de Wilcoxon : 10mm : n=14 ; z=-0,114 ; p=0,9093 ; 20mm : n=24 ; z=-0,716 ; p=0,4738).

Suite à ce résultat, une brève expérience complémentaire a été réalisée. Les crabes ont été donnés à des seiches naïves de 4 jours et leur comportement prédateur a été observé. Les jeunes seiches capturent tous les crabes de 5 mm et seule la moitié essayent et capturent les crabes de 8 mm. Aucune seiche n'essaye de capturer les crabes de 10 et de 20 mm.

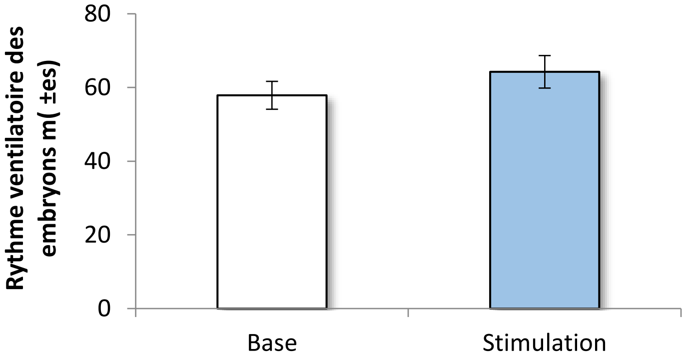

Figure 31 : Rythme ventilatoire des embryons de *Sepia officinalis* avant (rythme de base en blanc) et pendant une exposition olfactive (stimulation en bleu) à des crabes verts (*Carcinus maenas*). Test de Wilcoxon $p \geq 0,05$.

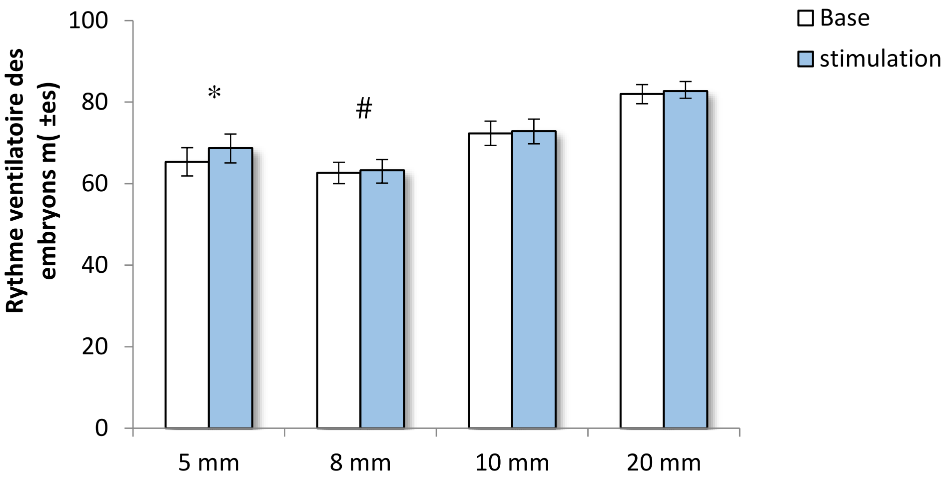

Figure 32 : Rythme ventilatoire des embryons de *Sepia officinalis* avant (rythme de base en blanc) et pendant une exposition visuelle (stimulation en bleu) à des crabes verts (*Carcinus maenas*). Test de Wilcoxon * : p < 0,05. # : 0,05 ≤ p < 0,07.

Exposition à des odeurs de prédateur

Lorsque les embryons de *Sepia officinalis* sont exposés à une odeur de mulet, le rythme ventilatoire ne change pas (cf. Figure 33; test de Wilcoxon : n=8 ; z=-1,476 ; p=0,1400). Par contre, le RV augmente significativement si les embryons de seiche sont exposés aux prédateurs (cf. Figure 33; test de Wilcoxon : Bars : n=8 ; z=-2,524 ; p=0,0116 ; Roussettes : n=8 ; z=-2,527 ; p=0,0115).

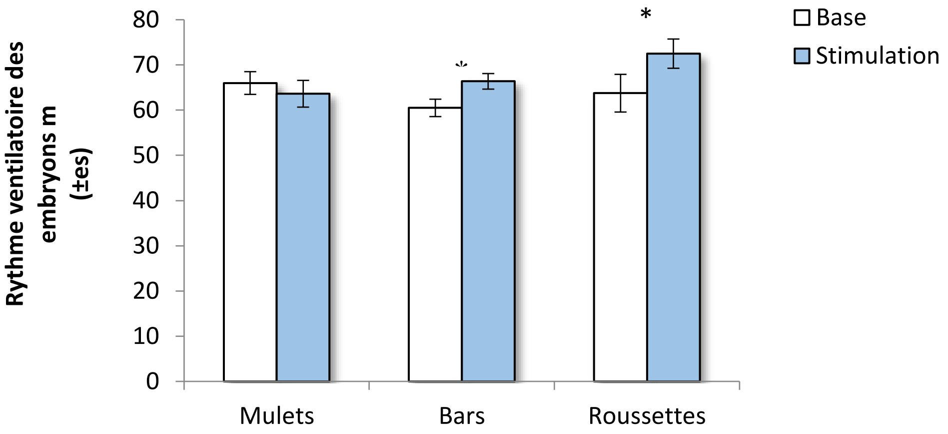

Figure 33 : Rythme ventilatoire des embryons de *Sepia officinalis* avant (rythme de base en blanc) et pendant une exposition à l'odeur des prédateurs et non-prédateurs (stimulation en bleu). Test de Wilcoxon * : p < 0,05.

Exposition à des signaux d'alarme

Lorsque les embryons de *Sepia officinalis* sont exposés à de l'encre de seiche, le rythme ventilatoire diminue significativement (cf. Figure 34; test de Wilcoxon : n=9 ; z=-2,252 ; p=0,0244). Par contre, le RV reste stable si les embryons de seiche sont exposés au broyat d'embryon ou à de l'eau de mer (cf. Figure 34; test de Wilcoxon : broyat : n= 10 ; z=-1,327 ; p=0,1846 ; eau de mer : n= 11 ; z=-1,547 ; p=0,1219).

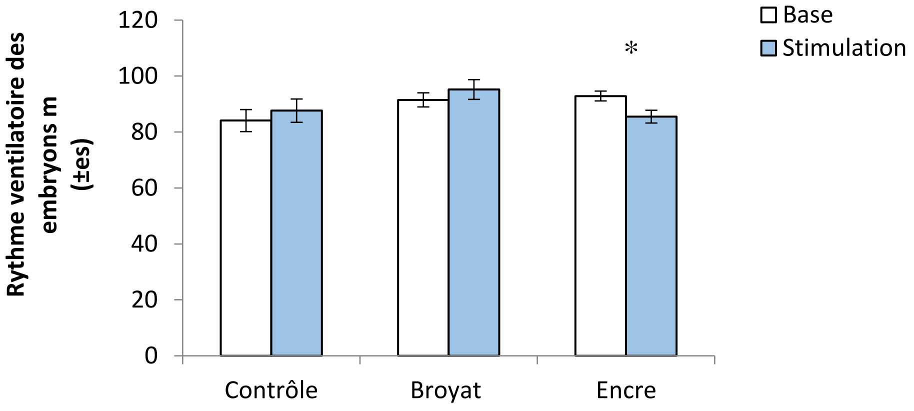

Figure 34 : Rythme ventilatoire des embryons de *Sepia officinalis* avant (rythme de base en blanc) et pendant une exposition à l'odeur des prédateurs et non-prédateurs (stimulation en orange). Test de Wilcoxon * : p < 0,05.

Discussion & perspectives

Au cours de cette étude préliminaire menée chez *Sepia officinalis*, différents stimuli ont été présentés. Pour la première fois chez l'embryon, leur réponse a été étudiée en mesurant le rythme ventilatoire (RV). Dans un premier temps les embryons ont été exposés à des proies et une réponse a été observée uniquement en cas d'exposition visuelle. En effet, lorsque les embryons sont exposés à des petites crevettes ou à des petits crabes, le RV augmente significativement. Par contre si les proies sont trop grosses les embryons ne répondent pas. Ce résultat est en accord avec le comportement prédateur de la seiche qui utilise principalement la vision (Hanlon & Messenger, 2018; Messenger, 1968). Il aurait été intéressant d'exposer les embryons de seiche à de très gros crabes, qui seraient alors considérés comme des prédateurs (Langridge, Broom, & Osorio, 2007). Dans un second temps, les embryons ont été exposés à des odeurs de poissons piscivores (bars commun et roussettes) et de poissons herbivores (mulets). Le RV augmente significativement lorsque les embryons sont exposés aux prédateurs uniquement (face à l'odeur de non-prédateur le RV reste stable). Ce résultat indique probablement une capacité de reconnaissance. Enfin, les embryons ont été exposés à de l'encre de seiche, un signal d'alarme chez les céphalopodes adultes et à du broyat de congénères. Contrairement au broyat qui n'engendre aucune réponse de la part des embryons (ce même résultat a été observé chez *Sepia pharaonis* ; Arduini unpublished data), l'encre de seiche induit une diminution significative du RV. Plusieurs réponses ont alors été observées chez les embryons. D'une part une augmentation du RV et d'autre part une diminution. A l'instar des juvéniles ayant un RV qui augmente face à des proies, des conspécifiques familiers et des non-familiers (Boal & Ni, 1996), l'augmentation du RV peut être interprétée comme un phénomène d'attention visuelle ou olfactive face à des proies ou des prédateurs. La diminution du RV peut quant à elle être interprétée comme un comportement de « freezing » décrit chez les mammifères. Ce comportement, mis en évidence chez l'adulte, serait une réponse adaptative permettant aux seiches de ne pas se faire détecter par leurs prédateurs (Bedore, Kajiura, & Johnsen, 2015). Dans leur étude, Bedore et ses collaborateurs rapportent un nouveau mécanisme cryptique chez *Sepia officinalis* dans lequel les signaux bioélectriques sont réduits *via* un comportement de « freezing » suite à une exposition de prédateur (requin marteau *Sphyrna tiburo*).

II. A difference in timing for the onset of visual and chemosensory systems during embryonic development in two closely related cuttlefish species.

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ABSTRACT:

The embryos, whether they develop in the uterus or in the egg, perceive different environmental stimuli thanks to their already or almost mature sensory system. In cuttlefish, the embryonic development of *Sepia officinalis* and *Sepia pharaonis* is very similar but there is a difference in the transparency of the egg capsule. The eggs of *S. officinalis* are tinted with black ink, which provides visual protection from predators. Conversely, those of *S. pharaonis* are totally translucent. The aim of this study is to test the visual and chemosensory perception abilities of these two cuttlefish embryos. Their early capacities are measured by observation of the ventilation rate (VR) before and after light and predator odour stimulation. Our results show that *S. pharaonis* responds to light at stage 22 and *S. officinalis* at stage 24. Conversely, *S. pharaonis* responds to predator odour at stage 23 and *S. officinalis* at stage 22. These results reveal that both species are able to respond to light and olfactory stimuli before hatching but do not have the same developmental schedule. Neither are the responses of the two cuttlefish exactly the same. In *S. officinalis*, VR always increases after light and odour stimulation. In *S. pharaonis*, VR increases after light stimulation before stage 25 but decreases after stage 25, following the predator odour stimulation. This result could reveal an ability to recognize stimuli at stage 25. The decrease could be identified as freezing-like behaviour which, due to the transparency of the egg capsule, would be more adaptive than an increase in ventilation, since the embryos are visible from the outside.

Keywords:

Sepia pharaonis –*Sepia officinalis* – light exposure – predator odour – Ventilation rate

Introduction

The sensory system development of individuals starts during the prenatal period. The vestibular and tactile systems are the first to develop, followed by the chemosensory systems (difficult dissociation of olfactory and gustatory systems), the auditory system and finally the visual system (Gottlieb, 1976); these sensory systems develop with a certain degree of overlapping. In altricial species, in which the young are very immature at birth or hatching, vision is not yet fully developed and mature (Gottlieb, 1971a ; Gottlieb, 1971b). On the contrary, in precocial species, in which the young are relatively mature and mobile right from hatching, all the sensory systems are mature at birth or hatching (Gottlieb, 1971a ; Gottlieb, 1971b). Whether they develop inside the mother (in the uterus) or in an egg, embryos are therefore able to perceive different environmental stimuli. Embryos can learn from prenatal stimulations, which are indeed crucial for the survival of the young. For example, in mammals, the amniotic fluid contains many odours and sapid substances, transmitted via maternal nutrition, which promote greater acceptance and even preference for foods with those same flavours (Beauchamp & Mennella, 2009; Mennella et al., 2001). In birds, auditory stimulation is also important for their behavioural construction. Juveniles can recognize their congeners, mother and future sexual partner if they perceive the corresponding vocalisation during the prenatal period (Colombelli-Négrel et al., 2012; Gottlieb, 1981; Gottlieb, 1991; Harshaw & Lickliter, 2010; Sleigh & Lickliter, 1996). Even though some prenatal stimulations may impair sensory system development (Carlsen & Lickliter, 1999; Gottlieb et al., 1989; Hogan & Bolhuis, 2009; Honeycutt & Lickliter, 2001, 2001; Jaime & Lickliter, 2006; Lickliter, 1994, 2000; Lickliter & Lewkowicz, 1995; Sleigh & Lickliter, 1998; Sleigh & Lickliter, 1996), others may actually enhance sensory system development. In one such experiment for example, during the last three days of incubation, the domestic hen embryo is positioned with its right eye receiving light stimulation through the shell, with the left eye hidden by its body (Rogers & Workman, 1989; Rogers, 1989, 2012). Due to the decussation of the majority of the retinal fibers of the avian visual system in the optic chiasma, light perceived by the right eye mainly stimulates growth of the left visual system. This asymmetric stimulation then generates the lateralization of certain vision-based behaviors (Andrew, Johnston, Robins, & Rogers, 2004; Casey & Lickliter, 1998; Rogers & Workman, 1989; Rogers, 1989, 2012).

Working on oviparous species is an advantage. Embryos develop entirely within the egg, being externalized from the mother and thereby affording the possibility of experimental manipulation in the prenatal environment. This allows easy access to the embryos for prenatal observation and manipulation. However, unlike birds, cephalopods offer another advantage: the transparency of the egg allows embryo observation without any invasive manipulation. Studies showed indirect evidence of embryonic induction in cuttlefish (food imprinting in *Sepia officinalis:* (Darmaillacq, Chichery, & Dickel, 2006; Darmaillacq et al., 2008; Guibé et al., 2010, 2012). Furthermore, there is direct evidence that cuttlefish embryos are also capable of both associative and non-associative learning (*Sepia officinalis*, Romagny et al., 2012; *Sepia pharaonis,* Mezrai, Arduini, Dickel, Chiao, & Darmaillacq, submitted).

During the following study, we will examine visual and olfactory perception in two cuttlefish species: *Sepia officinalis* and *Sepia pharaonis*. These two cephalopods are very closely related but live in different oceans (European and Asian respectively). In both species the embryo develops over 30 stages, the end of stage 30 corresponding to hatching (Lee et al., 2016; Lemaire, 1970) but with a very different transparency of the capsule. *S. officinalis* eggs are black (covered with maternal ink) whereas *S. pharaonis* eggs are translucent (see Figure 1). Because the capsule impregnated with melanin (ink) is supposed to play a major role in protecting the embryo from light, the amount of light reaching the embryo of each species is then potentially different and may well have an impact on the onset of sensory system development. In order to verify this hypothesis, embryonic response to light and predator odour will be tested at different stages. Unlike Romagny et al (2012), ventilation rate (VR) was used as a behavioural measure rather than mantle contractions. In addition to mantle contractions, decreased ventilation and bradycardia can be observed in cuttlefish after sudden visual or chemical stimulation (King & Adamo, 2006). Unlike heart rate, VR is easily and directly observable in cuttlefish. VR can also be used to monitor more subtle responses to low intensity stimuli (Boal & Ni, 1996). In embryos, VR can be easily observed under a microscope, either by noting the rhythmic motion of the collar flaps circulating oxygenated water to the gills, or by the movement of the funnel in response to pressure changes resulting from respiratory movements (inhalation and exhalation).

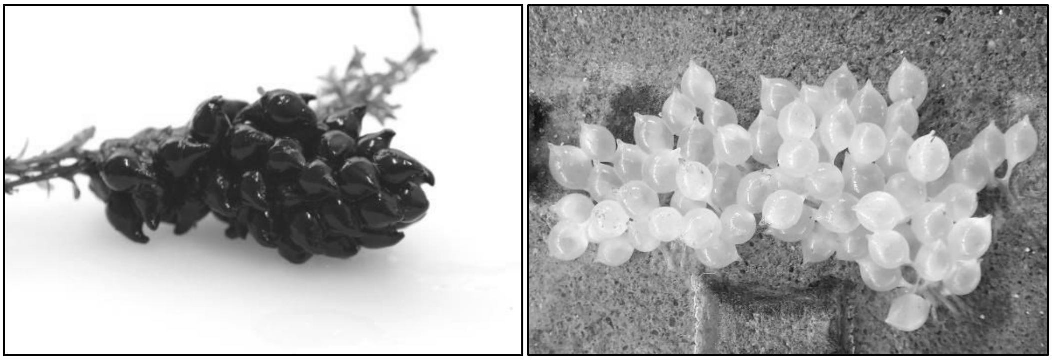

Figure 1: S. officinalis egg cluster covered with ink (A) and S. pharaonis egg cluster totally transparent (B). Image credit: Nawel Mezrai.

EXPERIMENT 1 – RESPONSE TO LIGHT AND PREDATOR ODOUR

Methods

Biological model used

Sepia pharaonis

Experiments were conducted from March to May 2017. All eggs were laid in Academia Sinica Marine Research Station or Aquaticlch Biotech Company Ltd. aquaculture (Yilan, Taiwan). They were transferred to the Institute of Systems Neuroscience & Department of Life Science (National Tsing Hua University, Taiwan). Once the eggs had been taken to the institute, they were maintained in tanks at a temperature of 25 ± 2°C until they reached the appropriate developmental stage for each experiment. All developmental stages mentioned below refer to the *Sepia pharaonis* embryonic development table (Lee et al., 2016). Experiments started on embryos at stage 21. Prior to this stage, embryos are in a vertical position (head up) and then, with the reduction of the yolk, they gradually move to a horizontal position (eyes downwards). In *S. pharaonis* eye retina becomes orange at stage 22, red at stage 23 and brown at stage 25 (Lee et al., 2016).

Sepia officinalis

Experiments were conducted from June to August 2017. All eggs were laid in the wild (eggs laid on ropes attached to the seafloor) and transported in oxygenated seawater to the CREC Marine Station (Centre de Recherches en Environnement Côtier; Luc-sur-Mer, France). Once the eggs had been taken to the CREC, they were maintained in tanks at a temperature of 18 ± 2°C until they reached the appropriate developmental stage for each experiment. All developmental stages mentioned below refer to the *S. officinalis* embryonic development table (Lemaire, 1970).

In this study only the clearest eggs were used as we wanted to avoid modifying the capsule of the egg in order to test embryonic olfactory perception ability. In this species, some eggs are sometimes without or with very little colour. This can be observed in the last eggs laid in the batch or can result from a defect of ink production in the females, which thus concerns the whole batch. This lack of pigmentation does not interfere with the development of the embryo (Boletzky, 1983; Lemaire, 1970). Experiments were started on embryos at stage 22. Likewise, in *S. pharaonis*, the ventilation rate is hard to measure before stage 22 because of the vertical position (head up). The eyes become orange at stage 24, red at stage 26 and brown at stage 28 (Lemaire, 1970).

Visual stimulation

Eight naive embryos were tested one by one at each embryonic stage (from 21 to 30 for *S. pharaonis* and from 22 to 30 for *S. officinalis*). They were placed individually in a 100 ml glass container, under a binocular microscope equipped with a cold LED illumination (Loupe binoculaire numérique Jeulin®; Ref: 571265). The light was buffered with a red filter to limit embryo stress (personal observation). A video camera connected to the microscope was then connected to a computer, allowing observation and direct counting of the ventilation rate. After a 5-min acclimatisation period, an additional light was lit 10 cm above the egg for 3 sec (3 Watt LED light; model: Saekodive AL-18LED-3C6AA). The VR was measured 1 min before and 1 min after the light stimulation.

Olfactory stimulation

The same device and the same number of eggs were used at each embryonic stage (from 21 to 30 for *S. pharaonis* and from 22 to 30 for *S. officinalis*). After a 5-min acclimatisation period, 3 ml of water containing the odour of a predator was introduced very slowly (over about 3 sec) into the container and close to the egg. The VR was measured 1 min before and 1 min after the introduction of the odour. The predator odour was obtained by collecting seawater directly from the predator aquarium. For *S. officinalis*, the predators used were Seabass (*Dicentrarchus labrax*) and the Lesser-spotted dogfish (*Scyliorhinus caniculata*), fed on vertebrate food (frozen fish and shrimps *ad libitum*). For *S. pharaonis*, the predator used was the White-spotted puffer (*Arothron hispidus*), fed on vertebrate food (shrimps *ad libitum*).

Statistics

Given the sample size, nonparametric statistical methods were used to analyse data. Mean ventilation rates during acclimatisation and stimulation periods were compared using a Wilcoxon test (R © 3.2.0). The α level for all analyses was 0.05. For the graphical representation, each histogram bar represents the index, calculated as follows:

$I = VR_{\text{stimulation}} – VR_{\text{acclimatization}}$.

This index shows whether the RV increases or decreases as a result of light or olfactory stimulation. If the RV increases we observe positive data values and if the RV decreases, the data values will be negative.

Results

The ventilation rate (VR) of each embryo was measured before stimulation ($VR_{\text{acclimatization}}$) and after stimulation ($VR_{\text{stimulation}}$). By using the index I ($I = VR_{\text{stimulation}} – VR_{\text{acclimatization}}$), we were then able to see whether the RV decreased or increased as a result of this stimulation.

Visual stimulation

In *S. pharaonis*, the embryonic VR did not change at stage 21 after light stimulation but did increase from stages 22 to 29 after light stimulation. At stage 30 the ventilation rate tended to increase after light stimulation (see figure 2: S21 (z= -1.105; p=0.2693); S22 (z=-2.120; p=0.0340); S23 (z=-2.805; p=0.0050); S24 (z=-2.527; p=0.0115); S25 (z=-2.383; p=0.0172); S26 (z=-2.527; p=0.0115); S27 (z=-2.536; p=0.0112); S28 (z=-2.243; 0.0249); S29 (z=-2.173; p=0.0298); S30 (z=-1.829; p=0.0673)).

In *S. officinalis*, the VR of the embryos did not change at stages 21, 22 or 30 after light stimulation but did increase from stages 24 to 29 after light stimulation (see figure 3: S22 (z=-1.000; p=0.3173); S23 (z=-1.633; p=0.1025); S24 (z=-2.414; p=0.0158); S25 (z=-2.316; p=0.0206); S26 (z=-2.371; p=0.0178); S27 (z=-2.521; p=0.0117); S28 (z=-2.380; p=0.0173); S29 (z=-2.371; p=0.0178); S30 (z=-0.994 ; p=0.3204)).

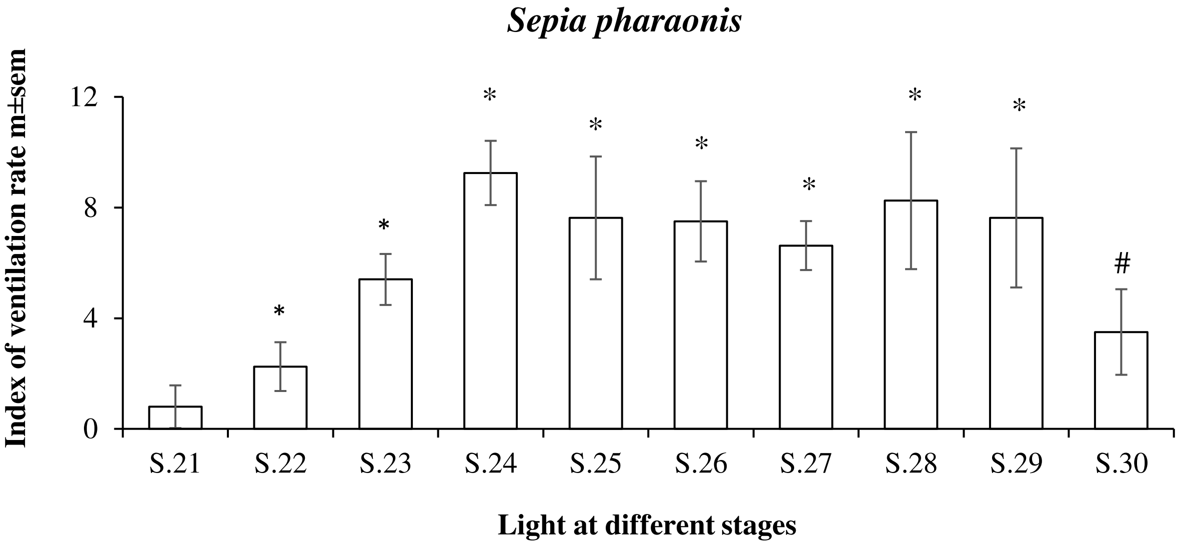

*Figure 2: Index of ventilation rate mean±sem in Sepia pharaonis at different embryonic stages after light stimulation. Wilcoxon test: * p <0.05; # 0.05≤p≤0.07.*

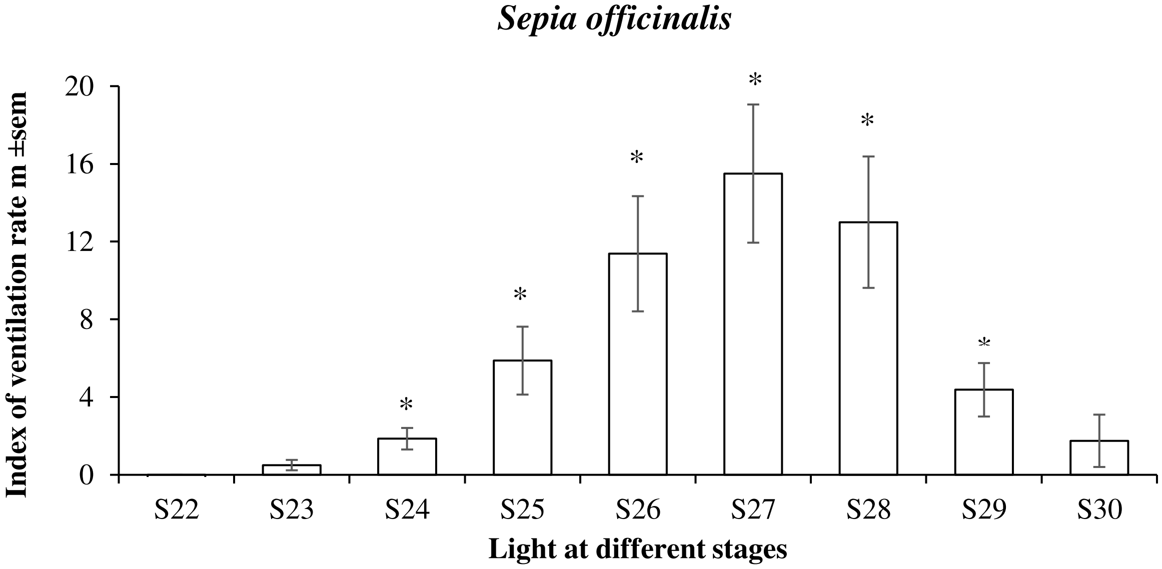

*Figure 3: Index of ventilation rate mean±sem in Sepia officinalis at different embryonic stages after light stimulation. Wilcoxon test: * means a significant increase (p <0.05) in RV following luminous stimulation.*

Olfactory stimulation

In *S. pharaonis*, the VR of the embryos did not change at stages 21 and 22 during olfactory stimulation. Subsequently, it increased from stages 23 to 24 and then decreased from stages 25 to 30 during continued olfactory stimulation (see figure 4: S21 (z=-1.00; p=0.3173); S22 (z=-1.693; p=0.0905); S23 (z=-2.322; p=0.0202); S24 (z=-2.527; p=0.0115); S25 (z=-2.103; p=0.0355); S26 (z=-2.371; p=0.0178); S27 (-2.375; p=0.0176); S28 (-2.527; p=0.0115); S29 (z=-2.379; p=0.0173); S30 (z=-2.371; p=0.0178).

In *Sepia officinalis*, the VR of the embryos increased from stages 22 to 29 during olfactory stimulation, but not at stage 30, during continued olfactory stimulation (see figure 5: S22 (z=-2.546; p=0.0109); S23 (z=-2.428; p=0.0152); S24 (z=-2.047; p=0.0407); S25 (z=-2.207; p=0.0273); S26 (z=-2.527; p=0.0115); S27 (z=-2.527; p=0.0115); S28 (z=-2.555; p=0.0106); S29 (z=-1.973; p=0.0486); S30 (z=-0.921; p=0.3573).

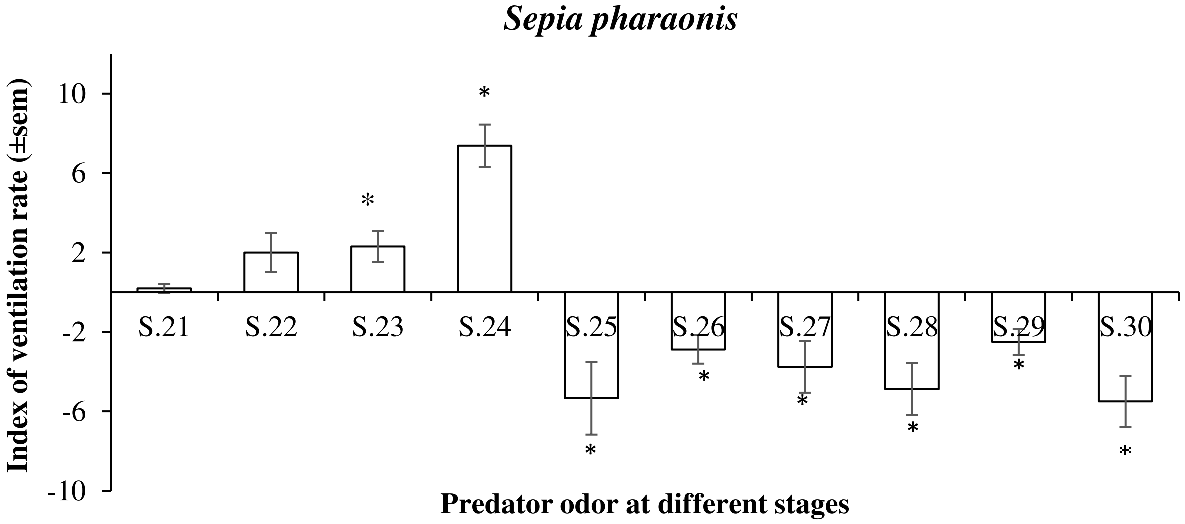

*Figure 4: Index of ventilation rate mean±sem of Sepia pharaonis at different embryonic stages after chemical stimulation. Positive values correspond to an increase of VR and negative ones to a decrease of VR. Wilcoxon test: * p <0.05*

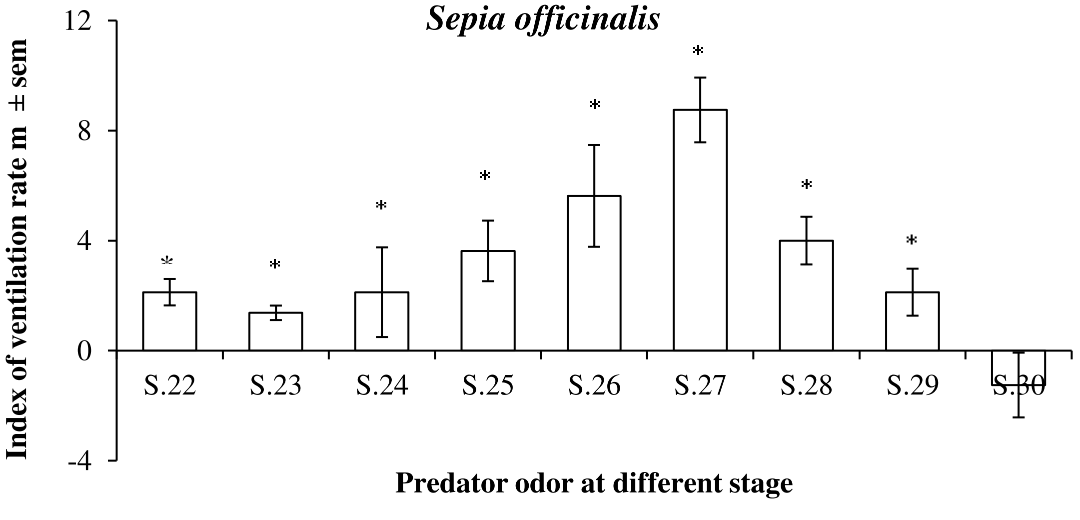

*Figure 5: Index of ventilation rate mean±sem of Sepia officinalis at different embryonic stages after chemical stimulation. Positive values correspond to an increase of VR and negative ones to a decrease of VR. Wilcoxon test: * p <0.05.*

Summary

Prenatal exposure to light and predator odour did not induce the same response in the two embryo types, but varied according to the species and embryonic stage. After exposure to light, *S. pharaonis* VR increased from stages 22 to 29 and *S. officinalis* VR increased from stages 24 to 29. Visual perception seems to occur earlier in *S. pharaonis* than in *S. officinalis.* Conversely, chemical perception seems to occur earlier in *S. officinalis* than in *S. pharaonis.* Indeed, after chemical exposure (predator odour), *S. officinalis* VR increased from stages 22 to 29 and *S. pharaonis* VR increased from stages 23 to 24, and then decreased from stages 25 to 30. In *S. pharaonis*, it is possible that the VR increase reflected perceptual abilities, and the VR decrease denoted recognition abilities. Hence, in experiment 2 we exposed embryos to seawater and non-predator odour in order to better understand these responses.

EXPERIMENT 2 – COMPLEMENTARY CONTROL EXPERIMENTS

Experiment 1 showed that *S. officinalis* started to respond to predator odour at stage 22. Likewise, *S. pharaonis* showed response to predator odour at stage 23 (increase of VR), but VR decreased from stages 25 to 30. A further study was undertaken to ensure that the above results were due to exposure to predator odour rather than to the additional handling or some other procedural factor. We thus exposed embryos to both a non-predator odour and seawater. These experiments were designed to determine whether embryos respond to seawater presentation (water introduced into the device can move the egg and thus be detected by embryos) or if they respond on perception of a threatening odour (predator odour) and/or a non-threatening odour (non-predator odour). The subjects, treatment, procedure and analysis were identical to those reported for experiment 1, with the sole exception that the predator odour during the test phase of the experiment was replaced either by seawater (no odour) or non-predator odour.

Methods

Sepia pharaonis

Eight naive embryos were tested at stages 23 and 25 with seawater (control group) or with Clownfish odour (*Amphiprion ocellaris;* non-predator group). We chose these two test periods after analysing the results of experiment 1. Indeed, *S. pharaonis* started to respond to predator odour at stage 23 (increase of VR) and at stage 25 we observed a change of response (decrease of VR).

For the control group seawater was obtained by collecting sea water in the tank without the presence of animals (empty tank). For the non-predator group Clownfish odour was obtained by collecting seawater in the Clownfish tank. Clownfish were fed *ad libitum* on standard Clownfish food (Omega 海水魚苗餌料 marine fish fry feed - Chuan Kuan Enterprise Co., Ltd.).

Sepia officinalis

Eight naive embryos were tested at stages 24 and 27 with seawater (control group) or with Grey mullet odour (*Chelon labrosus;* non-predator group). *S. officinalis* responded to predator odour from stages 22 to 30 (increase of VR) but for technical reasons stage 22 could not be exploited. For the control group, seawater was obtained by collecting seawater in the tank without animals (empty tank), and for the non-predator group Grey mullet odour was obtained by collecting seawater in the Grey mullet tank. Grey mullet were fed *ad libitum* on herbivorous food (Tetra® Aliment Complet Pleco Veggie Wafers).

Results

The ventilation rate (VR) of each embryo was measured before stimulation ($VR_{\text{acclimatization}}$) and after stimulation ($VR_{\text{stimulation}}$). By using the index I ($I = VR_{\text{stimulation}} - VR_{\text{acclimatization}}$), we could then see if the RV decreased or increased as a result of this stimulation.

In *S. pharaonis*, embryonic VR increased at stage 23 and did not change at stage 25 during non-predator odour exposure (see figure 6: S23 (z= -2.524; p=0.0116); S25 (z= -1.134; p=0.2568). Embryonic VR did not change at stages 23 and 25 during seawater exposure (see figure 6: S23 (z= -0.211; p=0.8330); S25 (z= -0.378; p=0.7055)).

In *S. officinalis*, embryonic VR did not change at stages 24 and 27 during non-predator odour exposure (see figure 7: S24 (z= -0.632; p=0.5276); S27 (z= -1.476; p=0.1400). Embryonic VR did not change at stages 24 and 27 during seawater exposure (see figure 6: S24 (z= -0.566; p=0.5716); S27 (z= -0.315; p=0.7525)).

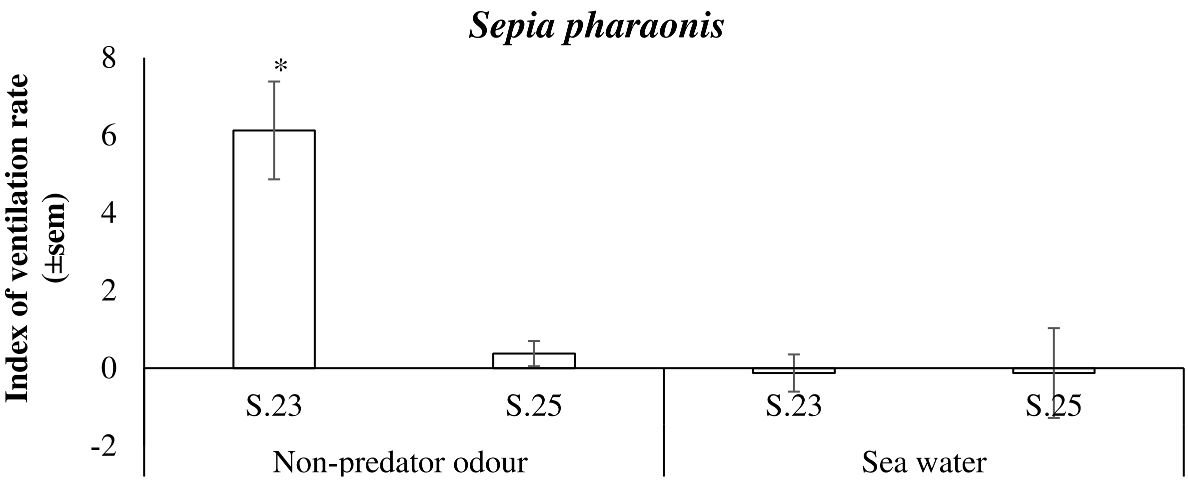

*Figure 6: Index of ventilation rate mean±sem of Sepia pharaonis at stages 23 and 25 after chemical stimulation (non-predator odour or seawater). Positive values correspond to an increase of VR and negative ones to a decrease of VR. Wilcoxon test: * p <0.05.*

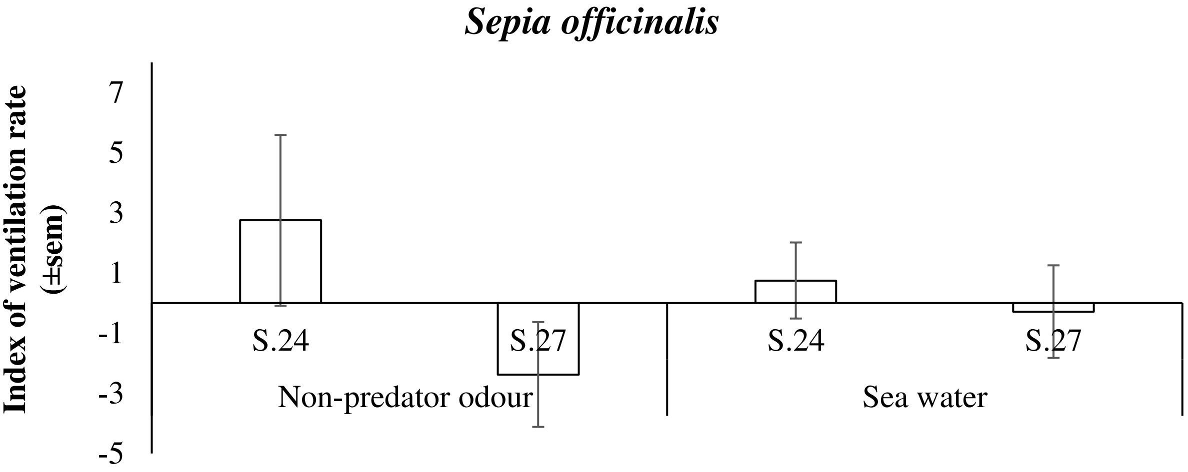

Figure 7: Index of ventilation rate mean±sem of Sepia officinalis at stages 23 and 25 after chemical stimulation (non-predator odour or seawater). Positive values correspond to an increase of VR and negative ones to a decrease of VR. Wilcoxon test: $p \geq 0.05$.

Summary

Exposure to blank seawater did not induce any response in either *S. pharaonis* or *S. officinalis* embryos. Neither did the movements of the egg caused by the introduction of water induce any response in the embryos. In the same way, the introduction of non-predator odour did not induce any response in *S. officinalis*. This species responds to threatening odours only during the prenatal period, as we observed it in Experiment 1. However, in *S. pharaonis* we observed responses differing according to the embryonic stage. Embryos responded to the non-predator odour at stage 23 but not at stage 25.

General discussion

In this study, embryonic responses to light and olfactory stimulation were tested in two cuttlefish species: *S. pharaonis* and *S. officinalis*. *S. pharaonis* responded to light as from stage 22 and to olfactory stimulation as from stage 23. Conversely, *S. officinalis* responded to light as from stage 24 and to chemosensory stimulation as from stage 22. The sensory system development is therefore not the same in these two species.

In order to compare the two cuttlefish species, we used the embryonic stage schedule described for *S. pharaonis* (Lee et al., 2016) and *S. officinalis* (Lemaire, 1970). These developmental tables do not use the same morphological criteria for each stage despite very similar development in the two species. It is therefore necessary to use a criterion other than the embryonic stage. One solution is to use a simple univocal morphological criterion: eye colour. Indeed, for both species, the eyes are first white, becoming orange, red, and then brown (Lee et al., 2016; Lemaire, 1970). In this study *S. pharaonis* embryos were seen to respond to olfactory stimuli when their eyes were orange and to light stimuli when their eyes remained white. Conversely, *S. officinalis* embryos responded to olfactory stimuli when their eyes remained white and to luminous stimuli when their eyes became orange. *S. pharaonis* has earlier vision than *S. officinalis* but *S. officinalis* has earlier olfaction than *S. pharaonis*. These results can be explained by different ecological demands between the two species. *S. pharaonis* eggs are transparent and laid near the seabed under rocks or coral (Gabr, Hanlon, Hanafy, & El-Etreby, 1998), and being able to see earlier could be an important advantage in the recognition of prey and predators. Conversely, *S. officinalis* eggs are dark and laid in shallower water on algae or other vertical supports (Boletzky, 1983). It could therefore be important for them to learn olfactory cues at an earlier stage. We can also imagine an alternative hypothesis. As the eggs of *S. pharaonis* are transparent, with light passing through the capsule, there is thus greater stimulation of the visual system. As in birds, these early stimulations could favour visual system development and change olfactory system development (Carlsen & Lickliter, 1999; Gottlieb et al., 1989; Honeycutt & Lickliter, 2001; Jaime & Lickliter, 2006; Lickliter, 1994, 2000; Lickliter & Lewkowicz, 1995; Sleigh & Lickliter, 1998; Sleigh & Lickliter, 1996). However, some preliminary studies show that incubation in the dark does not change the developmental programme of either *S. pharaonis* or *S. officinalis,* which would consequently be more evolutionary than developmental.

Another major result of this study is the difference of response to predator odour between the two species. In *S. officinalis,* VR increased as from stage 22 (white eyes) whereas in *S. pharaonis,* VR increased only at stages 23 and 24 (orange and red eyes) and decreased from stage 25 (brown eyes). One hypothesis is that *S. pharaonis* first perceives odours at stages 23 and 24 but discriminates and recognizes the predator odour only later (at stage 25). The decrease of VR can be compared to an attention response, as in children (Richards & Casey, 1991), but also to a freezing-like behaviour, which is an adaptive response preventing adult cuttlefish from being detected by predators (Bedore et al., 2015). In their study, Bedore and collaborators report a novel cryptic mechanism in *S. officinalis,* in which bioelectric cues are reduced *via* a freezing behaviour in response to a predator stimulus (Bonnethead sharks, *Sphyrna tiburo;* Bedore et al., 2015). This hypothesis is supported by the fact that *S. pharaonis* responded to a non-predator odour (Clownfish) at stage 23 (VR increase, they perceive odour) but not at stage 25 (VR did not change, they recognize odour). In *S. officinalis*, the VR increased at each stage if exposed to predator odour, but did not change when exposed to a non-predator odour (Grey mullet). Furthermore, the egg capsule of *S. officinalis* being black, the VR decrease in this species may not necessarily be adaptive, contrary to *S. pharaonis* (transparent capsule, with embryos visible from the outside).

The last point of interest in our results is the disappearance of all embryo response at the late embryonic stage in both species: stage 30, which is the last stage before hatching. The embryos lie in a dorsal position and the external yolk reserves are nearly consumed (Lee et al., 2016; Lemaire, 1970; O'Brien, Mezrai, et al., 2017). In the squid (*Loligo vulgaris*), it was shown that the perivitelline fluid contains a natural tranquilliser which comes into action at the end of embryonic development. It prevents premature hatching and reduces detection of the embryos by predators, thus affording optimal conditions for the survival of well-developed juveniles (Boletzky, 2003; Marthy, Hauser, & Scholl, 1976; Weischer & Marthy, 1983). Whether this tranquilliser is also present in cuttlefish eggs is not yet known, but it could explain why embryos no longer respond to predator odour during the last stages of development.

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Bilan du chapitre 3 :

A cours l’étude préliminaire présentée dans la **première partie** de ce chapitre 3, différents stimuli visuels et olfactifs ont été présentés aux embryons de *Sepia officinalis* : des proies et leur odeur ; des odeurs de prédateurs et de non-prédateurs ; des odeurs de congénères broyés et de l’encre de seiche. Les résultats indiquent que les embryons de cette espèce répondent aux proies et aux odeurs de prédateur par une augmentation du RV (stimulation visuelle uniquement). De plus, ils répondent à l’encre par une diminution du RV. Enfin, aucune réponse aux non-prédateurs et aux odeurs de congénères broyés n’a été observée. La réponse des embryons est alors différente en fonction des stimuli présentés. Une augmentation du RV mettrait en évidence un phénomène d’attention face à des proies et des prédateurs ; une diminution indiquerait un comportement semblable au « freezing » face à de l’encre, un signal d’alarme chez la seiche.

L’article présenté dans la **deuxième partie** de ce chapitre 3 visait à décrire le calendrier de développement des systèmes chimiosensoriel et visuel chez les seiches *Sepia officinalis* et *Sepia pharaonis*. Ces deux espèces diffèrent dans la transparence de la capsule de l’œuf. Les œufs de *Sepia officinalis* sont noirs, recouverts d’encre alors que les œufs de *Sepia pharaonis* sont transparents. Des stimulations stressantes lumineuses (lumière forte) et chimiosensoriels (odeur de prédateur) ont été appliquées du stade 21 au stade 30 chez *S. pharaonis* et du stade 22 au stade 30 chez *S. officinalis*. En ce qui concerne les stimulations lumineuses, le RV de *Sepia officinalis* augmente du stade 24 au stade 29 et celui de *Sepia pharaonis* augmente du stade 22 au stade 29. Le système visuel de *Sepia pharaonis* serait alors mature plus tôt que *Sepia officinalis*. Concernant les stimulations olfactives, le RV de *Sepia officinalis* augmente du stade 22 au stade 29 alors que celui de *Sepia pharaonis* augmente aux stades 23 et 24 puis diminue du stade 25 au stade 30. Le système chimiosensoriel de *Sepia officinalis* serait alors mature plus tôt que *Sepia pharaonis*. Les deux espèces sont alors capables de réagir aux stimuli lumineux et olfactifs avant l'éclosion, mais elles n'ont pas le même calendrier de développement. La transparence de la capsule aurait un effet sur la réponse embryonnaire, lorsque celle-ci est transparente, le RV de l’embryon va diminuer. Ce comportement est semblable au « freezing » et indique une potentielle reconnaissance de l’odeur menaçante.

Chapitre 4

Apprentissage embryonnaire chez la seiche

Chapitre 4 – Apprentissage embryonnaire chez la seiche

Contexte du chapitre 4 :

Nous venons de voir dans le **chapitre 3** que les embryons de seiche peuvent percevoir et répondre à différents stimuli *in ovo*. Chez *Sepia officinalis*, le RV change significativement lorsque les embryons sont exposés à des **proies** (augmentation du RV), à de la **lumière** (augmentation du RV) mais aussi à de l'odeur de **prédateur** (augmentation du RV) et à de l'**encre** de seiche (diminution du RV). Par contre, aucun changement n'a été observé lorsque les embryons sont exposés à de l'odeur de non-prédateur ni à de l'odeur de congénères blessés. Chez *Sepia pharaonis*, le RV des embryons change significativement lorsque les embryons sont exposés à de la **lumière** (augmentation du RV) et à de l'odeur de **prédateur** (augmentation puis diminution du RV en fonction des stades embryonnaires). Ces capacités de perception et de réponse embryonnaire sont possibles puisque les systèmes chimiosensoriel et visuel des deux espèces sont fonctionnels avant l'éclosion.

Lorsqu'un individu perçoit des informations émanant de son environnement il est aussi capable d'apprendre d'elles (voir **chapitre 1**). L'objectif de ce **chapitre 4** est alors de tester et ainsi mettre en évidence les capacités d'apprentissages directs chez les embryons de seiches (l'empreinte, un apprentissage embryonnaire « indirect », car mis en évidence après l'éclosion, a déjà été mis au point chez *Sepia officinalis* par Darmaillacq et ses collaborateurs). Pour ce faire nous avons mis au point un apprentissage associatif (conditionnement classique) chez *Sepia pharaonis* et chez *Sepia officinalis*. Dans la **première partie**, les embryons de *Sepia pharaonis* ont été exposés à de l'encre de seiche couplée à la présence d'un non-prédateur (modalité visuelle ou olfactive). Dans la **deuxième partie**, les embryons de *Sepia officinalis* ont quant à eux été exposés à une odeur de prédateur couplée à une odeur de cannelle (odeur neutre pour les embryons).

Ce chapitre s'organise alors de la façon suivante :

I - Article (2) **- Mezrai, N.,** Arduini, L., Dickel, L., Chiao, C.-C., & Darmaillacq, A.S., submitted in *Learning & Behavior*. Awareness of danger inside the egg? Evidence of innate and learned predator recognition in cuttlefish embryo.

II - Etude complémentaire : Apprentissage associatif chez *Sepia officinalis*.

I. Awareness of danger inside the egg. Evidence of innate and learned predator recognition in cuttlefish embryos.

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ABSTRACT:

Predation is one of the greatest selective pressures exerted on prey organisms. Many studies have shown the existence of innate anti-predator responses, mostly in the early developmental stages of juvenile vertebrates. Learning to recognise predators is another possible defensive resource, but such a method inevitably involves a high death risk. There is now increasing evidence that prenatal learning exists in animals but few studies have explicitly tested this sort of embryonic apprenticeship. In our experiments, *Sepia pharaonis* cuttlefish embryos were seen to respond to the presence or odour of predator fishes but not to non-predators. Interestingly, embryos can learn to associate a non-threatening stimulus with an alarm signal: cuttlefish ink. After several paired exposures, they respond to a harmless fish as if it were dangerous. Our results demonstrate both innate and acquired predator recognition in cephalopods, the embryo response being a decreased ventilation rate. Such a response is adaptive, especially in the translucent egg, since it results in reduced movement and hence lowers the risk of detection; this freezing-like behaviour may also reduce the bioelectric field, thus lessening the shark predation risk. Our results are the first report of associative learning in invertebrate embryos, and enable us to advance that a cuttlefish embryo can possess both genetic predator-avoidance skills as well as the cognitive requisites enabling it to learn and memorise new threats prior to hatching. The combination of these behavioural mechanisms is an impressive example of the early adaptability of cephalopod molluscs. Such behavioural plasticity gives the newly hatched sepia a huge selective advantage when dealing with either known or unfamiliar threats.

Keywords

Prenatal learning – predator recognition – *Sepia pharaonis* - Ventilation rate

Introduction

From the first moments of life, an individual must be able to protect itself from predators as well as find food. To ensure survival juveniles must be able to recognize predators at a very early stage in order to avoid them. Predator recognition is based on a strong innate component. In mammals, birds, amphibians, reptiles, fishes or snails, animals that are preyed on must use chemical, visual and/or auditory cues to identify their predators (Amo, López, & Martín, 2005; Balderas-Valdivia, Ramírez-Bautista, & Carpenter, 2005; Barreto, Luchiari, & Marcondes, 2003; M. M. Brown, Kreiter, Maple, & Sinnott, 1992; Dalesman, Rundle, Coleman, & Cotton, 2006; Dalesman, Rundle, & Cotton, 2007; Fendt, 2006; Griffiths, Schley, Sharp, Dennis, & Roman, 1998; Hartman & Abrahams, 2000; Hawkins, Magurran, & Armstrong, 2004; Hirsch & Bolles, 1980; Saunders, Ong, & Cuthbert, 2013). Certain prey species, however, need to learn to recognise and thus avoid their predators. Acquired predator recognition has been shown in a diverse range of taxa: birds (Curio, Ernst, & Vieth, 1978); mammals (Kindermann, Siemers, & Fendt, 2009); fishes (Chivers & Smith, 1998; Kelley & Magurran, 2003; Mathis & Smith, 1993); amphibians (Chivers & Smith, 1998; Epp & Gabor, 2008; Ferrari, Manek, & Chivers, 2010; Mathis, Ferrari, Windel, Messier, & Chivers, 2008; Mirza, Ferrari, Kiesecker, & Chivers, 2006; Wisenden, 2003; Woody & Mathis, 1998) and invertebrates (Aizaki & Yusa, 2010; Ferrari, Messier, & Chivers, 2008; Rochette, Arsenault, Justome, & Himmelman, 1998; Wisenden, 2003; Wisenden, Chivers, & Smith, 1997; Wisenden & Millard, 2001). One mode of learning is through the pairing of cues linking a predator with an alarm signal (classical conditioning). Indeed, in a marine environment the usual way for prey to detect and identify predators is by recognising olfactory and visual information (Brown & Smith, 1998; Hartman & Abrahams, 2000; Kats & Dill, 1998; Miklósi, Pongrácz, & Csányi, 1997; Utne-Palm, 2001).

Predator recognition can be learned even in the early stages of development. Within the protective egg-case embryos are able to perceive environmental stimuli which identify risk factors likely to be present in their post-hatching environment. This embryonic learning ability has been extensively studied in amphibians (Ferrari & Chivers, 2009a, 2009b, 2010; Ferrari, Crane, & Chivers, 2016; Ferrari et al., 2010; Golub, 2013; Mathis et al., 2008; Saglio & Mandrillon, 2006). The first study explicitly showing this ability to recognize predators was conducted by Mathis et al. (2008). It demonstrated that when salamander eggs (*Ambystoma annulatum*) were exposed to chemical predatory cues, larvae showed anti-predatory behaviors such as shelter-seeking and reduced locomotor activity (Mathis et al., 2008). Subsequently, further studies have shown that predator recognition can also be learned and generalised to other similar predators (Ferrari & Chivers, 2009b). By observing post-hatching responses, Ferrari and colleagues have shown that amphibian embryos can learn to recognise chemical cues before hatching by using associative identification cues concerning predators or their diet and/or alarm signals such as the smell of injured congeners (Ferrari & Chivers, 2009b, 2009a, 2010; Ferrari et al., 2010; Garcia, Urbina, Bredeweg, & Ferrari, 2017).

Cuttlefish are oviparous Cephalopod Molluscs. Embryos develop in soft elastic egg-cases and juveniles do not receive direct parental care since adult males die soon after mating and females after egg-laying (Boyle, 1987; Lee, Lin, Chiao, & Lu, 2016). Romagny and collaborators (2012) showed that the different sensory systems in *Sepia officinalis* are functional before hatching: they observed mantle contractions after tactile, olfactory and light stimulations. Furthermore, other studies have highlighted indirect prenatal learning (Darmaillacq, Lesimple, & Dickel, 2008; Darmaillacq, Mezrai, O'Brien, & Dickel, 2017; Guibé, Poirel, Houdé, & Dickel, 2012). Indeed, cuttlefish embryos that have been exposed to small crabs before hatching prefer crabs to their innately preferred shrimp prey (Darmaillacq et al., 2008). Likewise, cuttlefish that innately prefer black crabs will preferentially select white crabs following embryonic exposure to them (Guibé et al., 2012). Unlike *Sepia officinalis* in which the egg case is darkened by maternal ink, in the pharaoh cuttlefish (*Sepia pharaonis*) eggs are totally transparent. This allows direct observation of embryo response to external stimuli, whether chemosensory or visual, and thus demonstrates embryonic learning abilities without modification of the egg capsule.

The aim of this study is to see whether *Sepia pharaonis* embryos have an innate recognition capacity for predators or need to learn such survival tactics. In order to test their innate visual and chemical recognition capabilities embryos will be exposed to predatory and non-predatory cues. To test their learned visual and chemical recognition capabilities, a classical conditioning procedure will be used, involving the pairing of a neutral stimulus (the sight or odour of a non-predatory fish) with an alarm signal: cuttlefish ink, which can be a relevant warning signal (Derby, 2014). The ink is composed of secretions from two glands: (1) the ink-bag gland that produces melanin-tinted black ink; (2) the mucus-producing gland in the funnel. Cuttlefish ink is composed not only of melanin, but also of catecholamines, DOPA and dopamine (both monoamines derived from tyrosine), amino acids such as taurine, as well as certain metals such as cadmium, copper and lead (Derby, 2014; Madaras, Gerber, Peddie, & Kokkinn, 2010; Prota et al., 1981). Cephalopod ink would thus be a twofold defense strategy against predators: (1) ink as a direct predator deterrent (interspecific effects); (2) ink as an alarm cue for conspecifics (intraspecific effect) (Derby, 2014; Hanlon & Messenger, 2018). This second type of defense plays an indirect role against predation because it signals a danger to conspecifics. We can forward the hypothesis that embryos use chemical cues for predator recognition, as do many vertebrate and some invertebrate species (i.e. amphibians), but also visual cues due to the characteristics of the egg case. Likewise, we maintain that embryos can learn about a new danger through chemosensory and visual cues by associative learning. Unlike Romagny et al (2012), ventilation rate (VR) was used as a behavioural measure rather than mantle contractions because VR can be used to monitor more subtle responses to low intensity stimuli (Boal & Ni, 1996). Indeed, in addition to mantle contractions, decreased ventilation and bradycardia can be observed in cuttlefish after sudden visual or chemical stimulation (King & Adamo, 2006). Unlike heart rate, VR is easily and directly observable in cuttlefish under the microscope, either by noting the rhythmic motion of the collar flaps circulating oxygenated water to the gills, or by the movement of the funnel in response to pressure changes resulting from respiratory movements (inhalation and exhalation).

Materials and Methods

1) Biological model used

Experimental model

The model species used in the study is the pharaoh cuttlefish (*Sepia pharaonis*). The pharaoh cuttlefish is one of the most important aquaculture species of cephalopod and, in the wild, is widely distributed from the east African coast to the west Pacific Ocean (Anderson et al., 2010). Adults (4 females and 2 males) were fished and reared in a semi-natural area in Academia Sinica Marine Research Station or Aquaticlch Biotech Company Ltd. aquaculture (Yilan, Taiwan). All the eggs studied were laid in the same location (first generation) and transferred before organogenesis to the Institute of Systems Neuroscience & Department of Life Science (National Tsing Hua University, Taiwan). The transfer was made to large containers (30x50x30cm) filled with natural seawater. A bubbler pump was installed to ensure a constant oxygen supply in the containers. In the institute, eggs were maintained in natural sea water with constant renewal, at a temperature of 25 ± 2°C and on a 12:12 h light:dark cycle. The eggs were separated individually from the clusters and incubated in a plastic basket floating in the culture tank (20 eggs maximum per basket of 15x20x3cm). The volume of each tank was 300L.

Embryonic development

The developmental schedule differed for each of the eggs since they are laid singly, and the spawning period may last for several days. It took 22-24 days to complete embryonic development at a water temperature between 22-25°C (Lee et al., 2016). On the basis of morphological characteristics, 30 stages were observed during the embryonic development of *S. pharaonis:* cleavage from stages 1 to 9; blastulation and gastrulation from stages 10 to15 and organogenesis from stages 16 to 30 (Lee et al., 2016). During embryogenesis the sensory systems start to develop and become functional (Romagny, Darmaillacq, Guibé, Bellanger, & Dickel, 2012) and embryos are able to recognise and respond to chemical cues (Mezrai, Chiao, Dickel, & Darmaillacq, submitted). Indeed, embryos respond to predator odour (Narrow-lined Puffers: *Arothron manilensis*) but not to non-predator odour (Clownfish: *Amphiprion percula*) as from stage 25 (Mezrai et al., submitted).

2) Chemical and visual stimuli

Different chemical or visual stimulations were presented to the cuttlefish embryos: predatory fish, non-predatory fish and cuttlefish ink.

1) Fishes: the predator used was the Narrow-lined Puffer (*Arothron manilensis*). Two groups of Puffers were used: the first group was fed daily on standard food (defrozen shrimps). The embryos of the second group were given one cuttlefish egg per day. The non-predator used in was the Clownfish (*Amphiprion percula*), and this group was fed *ad libitum* on standard herbivorous aquarium food. All fishes were of comparable size (4 to 6 cm) and displayed similar swimming activity in the experimental tank (size: 20x60x30cm).
2) Ink: ink was obtained by submitting one-week-old cuttlefish (placed in a 300 mL glass container) to stress by approaching a net to provoke an ink-ejection response until the container became saturated with ink (i.e. the water was totally black and the cuttlefish invisible); the cuttlefish were then returned to their home-tank.

This procedure was repeated on each of the following experimental days. All fishes and cuttlefish used were maintained in natural seawater (25 ± 2°C), with constant renewal and adequate oxygenation (bubbler pump installed in each aquarium) and on a 12:12 h light:dark cycle.

3) Protocol and experimental apparatus

All experiments were conducted in a totally opaque 36x22x25cm tank in order to isolate the embryos from any external visual interference (cf. figure 1). Embryonic behaviour was recorded with an underwater camera (Olympus Stylus Tough TG-4). Each cuttlefish egg (stage 25) was placed on the bottom and in the centre of the tank for 5 min (acclimation phase) and on a plastic stand to prevent it from rolling. Then, the olfactory or visual stimuli were presented to the embryo (stimulation phase).

For the chemical stimulation, the fish aquarium (predator or non-predator) was placed next to the embryo tank and connected to a water pump. During the stimulation phase, the pump was turned on so that the fish odour (predator or non-predator) arrived close to the embryo (80 mL/min). For the ink-conditioning phase, a 3mL ink sample (cf. above) was added to 150 mL of blank seawater and mixed until the solution came more or less translucent, although despite the mixing a light grey colouration may persist. A 3 mL sample of this solution was then presented to the embryo; since the tank was dark, the embryo was probably not able to see the ink (see Boal & Golden 1999).

For the visual stimulation, the fish was placed directly in the embryo’s tank. The embryo was placed in a transparent glass container (6x4x4cm) to protect it from the fish and avoid chemical exposure to the predator odour. For the ink conditioning 3mL of black ink was presented close to the embryo.

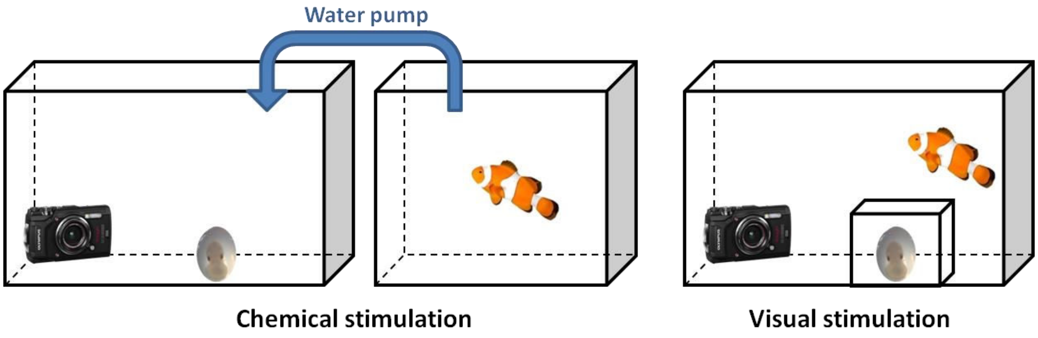

Figure 1: Schematic representation of the experimental device used. The cuttlefish egg is placed on the bottom in the centre of the tank. The camera is positioned in front of the embryo in order to record its

responses. On the left; Chemical stimulation test: the fish is in another tank connected to the experimental tank via a water pump. On the right; Visual stimulation test: the fish is placed in the embryo tank but the embryo is enclosed in a transparent glass container.

Innate recognition test

a) Innate chemical recognition test

Experimental stimuli:

1) Blank seawater ("**C**", control condition); n=9

2) Clownfish odour ("**NP**", non-predator condition); n=8

3) Puffer odour when fed with shrimps ("$\mathbf{P_{shrimp}}$", predator condition); n=6

4) Puffer odour when fed with cuttlefish embryos ("$\mathbf{P_{embryo}}$", predator condition); n=6

5) Cuttlefish ink ("**I**", ink condition); n=17.

b) Innate visual recognition test

Experimental conditions:

1) Clownfish ("**NP**", non-predator condition); n=8

2) Puffer ("**P**", predator condition); n=10

3) Black cuttlefish ink ("**I**", ink condition); n=12

Activity during both the last minute of the acclimation period and the first minute under experimental conditions (stimulation time) was recorded. Data collection was carried out by manually counting the ventilation rate (VR) at one-minute intervals. Preliminary studies showed that embryos responded immediately when exposed to stimulation: during acclimation VR did not change but it did change during the stimulation phase. The observer was blind to the treatments involved.

Learned recognition test

a) Conditioning phase

A classical conditioning procedure was used. The Clownfish (NP; non-predator) was used as a conditional stimulus (CS) and cuttlefish ink (I) was used as an unconditional stimulus (US).

Each embryo was exposed to the CS coupled with the US once a day for 30 min over a period of 4 days. The stimuli used in this experiment were obtained according to the same procedure as in the innate recognition tests described above.

Two groups were tested for chemical recognition:

1) The first group, experimental group "**NP+I**" (n=12), included embryos exposed to a Clownfish odour (Non-Predator) paired with cuttlefish ink odour.
2) The second group, control group **"NP"** (n=12), included embryos exposed to a Clownfish odour alone (Non-Predator).

Two groups were tested for visual recognition:

1) The first group, experimental group "**NP+I**" (n=10), included embryos exposed to a **N**on-**P**redator (Clownfish) paired with cuttlefish **I**nk clouds.
2) The second group, control group "**NP**" (n=12), included embryos exposed to a **N**on-**P**redator alone (Clownfish).

b) Testing phase

On day 5, all embryos were tested with the odour or sight of a Clownfish alone. Data collection was carried out by manually counting the VR one minute before and after the stimulation phase. The observer was blind to the treatments involved.

4) Statistical analyses

Given the sample size, nonparametric statistical methods are used to analyse data. Mean ventilation rates during acclimation period and stimulation phase are compared using a Wilcoxon test (R©3.2.0). The α level for all analyses was 0.05. For the graphical representation, each histogram bar represents the index calculated as follows:

$I = VR_{\text{stimulation}} - VR_{\text{acclimation}}$.

This index shows whether the RV increases or decreases as a result of stimulation (positive values mean that the VR increases after the stimulation; negative values mean that the VR decreases).

5) Ethical note

All animals (fishes and cuttlefish) and the entire protocol were approved by the National Tsing Hua University Institutional Animal Care and Use Committee (IACUC Protocol No. 10510). Throughout the protocol, we followed the published guidelines for the care and welfare of cephalopods to avoid stress in test animals (Fiorito et al., 2015).

Results

1) Innate recognition:

The ventilation rate (VR) of each embryo was measured before stimulation ($VR_{acclimation}$) and after stimulation ($VR_{stimulation}$). By using the index I ($I = VR_{stimulation} - VR_{acclimation}$), we could then see whether the resulting VR decreased or increased as a result of this stimulation.

a) Chemical:

Embryo VR did not change after exposure to blank seawater (“C” group); to non-predator odour (“NP” group) or to odour of the predator fed on shrimps (“P_{shrimp}” group) (Figure 2: C group: Z=0.00; p>0.999; NP: Z=-0.57; p=0.574; P_{shrimp}: Z=-1.134; p=0.257). Embryo VR decreased after exposure to the odour of a predator fed on cuttlefish embryos (“P_{embryo}” group) and to ink odour (“I” group) (Figure 2: P_{embryo}: Z=-2.041; p=0.041; I: Z=-2.650; p=0.008).

b) Visual:

Embryo VR did not change after exposure to a non-predator (“NP” group) (Figure 2: NP: Z=-0.537; p=0.590). Embryo VR decreased after exposure to a predator (“P” group) and to ink (“**I**” group) (Figure 2: P: Z=-2.025; p=0.042; I: Z=-2.83; p=0.0047).

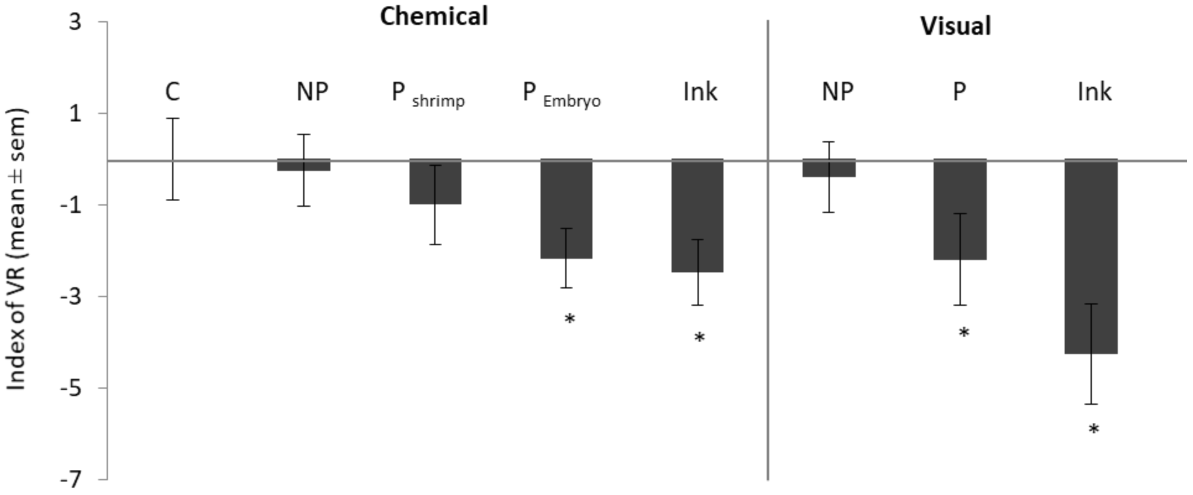

Figure 2: Index of ventilation rate (VR) of embryos exposed to blank seawater (C); non-predator (NP); predator (P - with P_{shrimp} = predator group fed with shrimps (Puffer) and P_{embryo} = predator group fed with cuttlefish embryos; cuttlefish ink (I). Wilcoxon test: *: p<0.05.

2) Learned recognition:

a) Chemical:

After 4 days of repeated exposure to Clownfish odour paired with ink odour, VR significantly decreased when embryos were exposed to Clownfish odour alone on the day 5 (Figure 3: NP+I: Z=-2.157; p=0.031). On the contrary, after 4 days of repeated exposure to Clownfish odour alone, VR did not change if embryos were exposed to Clownfish odour alone on day 5 (Figure 3: NP: Z=-0.303; p=0.762).

b) Visual:

After 4 days of repeated exposure to Clownfish paired with ink, VR significantly decreased when embryos were exposed to Clownfish alone on day 5 (Figure 3: NP+I: Z=-2.395; p=0.017). Conversely, after 4 days of repeated exposure to Clownfish odour alone, VR did not change if embryos were exposed to Clownfish odour alone on day 5 (Figure 3: Z=0.714-; p=0.475).

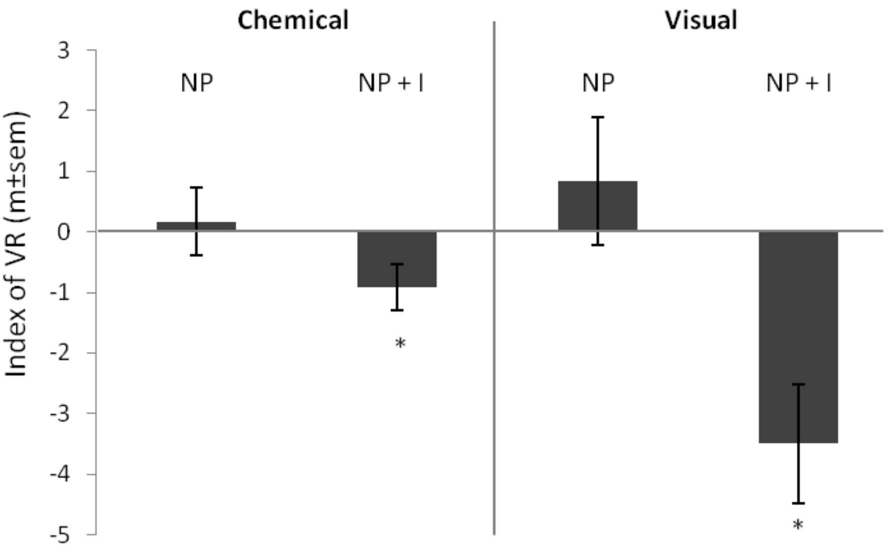

Figure 3: Index of ventilation rate (VR) of embryos after associative learning protocol. Associative learning condition: NP=non-predator over only 4 days (Clownfish, control group); NP+I=non-predator coupled with cuttlefish ink over 4 days. Wilcoxon test: ns: p≥0.05; *: p<0.05.

Discussion

In the first part of this study, we investigated whether embryos are able to innately recognize a predator *via* visual or olfactory cues. We showed that the ventilation rate (VR) of *Sepia pharaonis* embryos significantly decreased when they were exposed concomitantly to potential predators and ink. Changes in physiological parameters such as heart rate or VR often indicate perception (Colombelli-Négrel, Hauber, & Kleindorfer, 2014; Oulton, Haviland, & Brown, 2013) or attention abilities (Porges & Raskin, 1969; Richards & Casey, 1991), notably when the animal is in a dangerous situation. The VR often increases to prepare an individual for flight from a predator (Misslin, 2003). However, predator detection through visual or chemical stimulus may also induce “freezing-like” behaviour (Misslin, 2003) along with a decrease of VR. In mammals, freezing is considered to be a fear response related to a harmful stimulus, characterized by immobility and changes in physiological parameters, such as heart and ventilation rates, and may enhance a prey’s survival when facing predation. In cuttlefish, few studies have focused on changes in VR during such stimulation. In these studies, change of VR indicates visual or chemical perception abilities (Boal & Golden, 1999; Boal & Ni, 1996; King & Adamo, 2006). Indeed, juvenile cuttlefish became motionless (behavioural freezing), hyperinflated their mantle and decreased their VR and heart rate upon presentation of a sudden visual stimulus (rapidly approaching bird cut-out) (King & Adamo, 2006).

Likewise, in adult cuttlefish decreased breathing was associated with a freezing-like response, which would seem adaptive since it could reduce the risk of being detected by movement. Similarly, the reduction of the bioelectric field could well prevent attacks by sharks (Bedore, Kajiura, & Johnsen, 2015). *Sepia pharaonis* eggs are totally transparent; consequently, fewer movements associated with respiratory decrease and hence general activity inside the egg may lower the probability of the embryo being detected by predators, thus increasing its chance of survival. VR is also a sensitive indicator of fish physiological response to stress (Barreto et al., 2003). In their study, Barreto et al. measured the VR of the Nile tilapia (*Oreochromis niloticus*) before and after the presentation of three stimuli: an aquarium with a harmless fish, a predator or water (control). Nile tilapia VR increased significantly in the group visually exposed to a predator when compared with the two other groups, thus indicating its recognition ability (Barreto et al., 2003).

We were able to show that, as in the young of vertebrate species, cuttlefish embryos innately respond to chemical cues from predators but not from non-predators. Indeed, our study shows that embryos respond differently to Puffers fed with frozen shrimps (less harmful) than to Puffers fed with cuttlefish embryos (harmful). The VR significantly increased only when embryos were exposed to the latter. This result suggests that embryos do not respond to the fish odour itself but rather to the degree of danger represented by the predator, and this in connection with its diet. Such specific recognition is in accordance with the results of a study on the Clownfish *Amphiprion percula,* in which the larvae remained indifferent to chemosensory cues from non-piscivorous fishes fed with their usual diet, but significantly avoided chemical cues from piscivorous and non-piscivorous fishes fed with a diet containing a fish product (Dixson, Pratchett, & Munday, 2012).

One of the most noteworthy results of the present study is that predator recognition is not based on chemical cues alone, but also on predatory visual information. Embryo VR decreased when embryos were exposed to the Puffer but not to the Clownfish. This change of VR cannot be attributed to a lack of oxygen, the egg being enclosed in a box. On the first hand we did not observe any change of VR when embryos were exposed to a non-predator and secondly, a lack of oxygen would have been more likely to cause an increase in VR (Randall, & Shelton, 1963). Which visual predatory cues embryos are generally familiar with is a question that remains unanswered. Nevertheless some hypotheses can be advanced.

First, since the size of the fish has been controlled, recognition may be based on the predator’s behaviour. Indeed, behaviour in the experimental tank differed between the two species of fish. The Puffers had been trained to prey on eggs, cuttlefish embryos thus forming the basis of their diet. As a consequence, during exposure the Puffer spent most of the time close to the glass box and directed several attacks on the egg (personal observation). On the contrary, the Clownfish stayed away from the egg closest to the side of the aquarium. It is evident that the threat-level is higher with the Puffer than with the Clownfish. This observation is in accordance with others made on a similar model; juvenile cuttlefish display secondary behaviour (deimatic pattern and inking) when the Puffer fish is close by (Lee, Darmaillacq, Dickel, & Chiao, submitted).

Second, a morphometric analysis of 20 different facial features of reef fishes was carried out in order to assess cues to possible predator recognition, showing that the shape of the fish’s mouth and the distance between the eyes and the mouth could be different between a carnivorous and a herbivorous fish (Karplus & Algom, 1981). This morphological criterion may be sufficient for good visual recognition of a predator.

Our study highlights the fact that embryos innately respond to the sight of an ink cloud as well as to ink odour at a very low concentration, serving as a warning signal. Again, this response is adaptive because it decreases the probability of being detected by predators likely to attack eggs or hatchlings in their vicinity. In fish and amphibian species, young individuals innately respond to chemical alarm cues (pheromones) released by injured conspecifics. In cephalopods, threatened individuals eject clouds of black ink, which would make cuttlefish ink a relevant warning signal (Derby, 2014). Cephalopod ink would thus serve as a defense against predators as a direct predator deterrent and as an alarm cue for conspecifics (Derby, 2014; Hanlon & Messenger, 2018). In *Loligo opalescens* squid ink can cause inking and camouflage (Gilly & Lucero, 1992; Lucero, Farrington, & Gilly, 1994). Furthermore, dopamine at biologically relevant concentrations is sufficient to cause ink ejection (Gilly & Lucero, 1992; Lucero et al., 1994).

In the second part of the study, we were able to show that embryos can learn to distinguish a harmful stimulus when it is paired with ink. Indeed, we showed that the VR of cuttlefish embryo decreased significantly on the simple presentation of a Clownfish (through sight or smell) when it had been coupled with ink over the previous 4 days. It is unlikely that this group should be experiencing any form of sensitisation. Unpublished data show that pairing the Clownfish odour with ink for 2 days leads to the same results in both *S. pharaonis* and *S. officinalis* after only one pairing event. We also showed that embryos exposed to cuttlefish ink once a day for four days do not subsequently respond to a neutral odour (cinnamon; personal observation). In the present study, it is more likely that embryos learn to recognize a new predator by associative learning. Associative learning, defined as the ability to learn either to associate two related events or a form of behaviour and its consequences (Bouton, 2007), has been shown in cuttlefish (adults and juveniles) and other cephalopods including octopuses (O'Brien, Mezrai, Darmaillacq, & Dickel, 2017; Wells, 1968; Young, 1961). Cuttlefish (*S. officinalis*) can learn the visual characteristics of a prey while inside the egg through mere exposure, which would be non-associative learning because spontaneous juvenile food preferences are altered after embryonic exposure to crabs (Darmaillacq et al., 2008, 2017; Guibé et al., 2012)). The present study brings direct evidence that cuttlefish embryos can also learn through classical conditioning. This learning capability is adaptive in that it allows juvenile cuttlefish to gain information relevant to its future environment while still safe inside the egg case, hence improving the survival chances of the hatchlings. These results are in accordance with studies on tadpoles and invertebrate larvae, in which embryos have been shown to learn to recognise new predators when they are paired with alarm cues (mosquitoes: Ferrari et al., 2008; damselfly: Wisenden et al., 1997). Predation is a constant threat faced by prey individuals, so learning about predation before hatching is a great advantage for the survival of young animals, especially when they develop without direct parental care.

To conclude, the ability to detect, identify and learn about potential predators is highly beneficial for the embryo while still in its protective egg case. In a changing environment, these prenatal learning abilities are important when faced with new predators (e.g. invasive species) or predator diet changes. Indeed, in fish the flexibility of feeding behaviour is an important adaptive trait because most natural environments undergo constant spatial and temporal change (Dill, 1983; Vehanen, 2003; Wright, Eberhard, Hobson, Avery, & Russello, 2010). Development in a transparent egg favours the use of visual as well as chemosensory information. Lastly, in this study cuttlefish embryos are shown to learn in 4 days, but unpublished experiments have shown that learning can be considerably faster: 2 days in *Sepia pharaonis* and 1 day in *Sepia officinalis;* this means that embryos are able to learn right up to the time of hatching.

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II. Expériences complémentaires : Apprentissage associatif chez *Sepia officinalis*

Dans la première partie menée chez *Sepia pharaonis*, un non-prédateur a été couplé avec de l'encre de seiche (un signal d'alarme) en utilisant la modalité visuelle ou olfactive. Dans cette deuxième partie, réalisée chez *Sepia officinalis*, une odeur de cannelle a été couplée à de l'encre de seiche. L'odeur de cannelle a remplacé l'odeur de non-prédateur puisqu'elle est plus facilement quantifiable et donc l'expérience est plus reproductible. En effet, l'odeur des poissons est très complexe et potentiellement variable car elle comporte l'odeur de fèces et d'urine mais aussi l'odeur corporelle des individus. Par contre l'odeur d'encre a remplacé l'odeur de prédateur pour des raisons méthodologiques (au moment des expérimentations nous ne disposions pas de ce premier item).

Introduction

Lors d'un conditionnement classique, les individus sont exposés à un stimulus inconditionnel couplé à un stimulus conditionnel. Le stimulus inconditionnel va induire une réponse inconditionnelle chez l'individu alors que, avant tout conditionnement, le stimulus conditionnel n'induit aucune réponse. Après conditionnement, les animaux répondent au stimulus conditionnel qui prédit le stimulus inconditionnel. Les propriétés de cet apprentissage associatif ont été étudiées chez un grand nombre d'espèces. Chez les Invertébrés, ces études ont porté principalement sur l'aplysie, la drosophile et l'abeille qui présentent de nombreux avantages pour une approche réductionniste (Avarguès-Weber & Mota, 2016 pour revue ; Byrne, 2017 ; Byrne & Hawkins, 2015 ; Hawkins & Byrne, 2015). Comme les aplysies et les drosophiles, les abeilles (*Apis mellifera*) sont d'excellents modèles d'Invertébrés pour étudier les mécanismes d'apprentissage visuel et de la mémoire, en raison de leur système visuel sophistiqué et de leurs capacités cognitives impressionnantes associées à un cerveau relativement simple (Avarguès-Weber & Mota, 2016 pour revue). Mais l'apprentissage et la mémoire des mollusques céphalopodes modernes (*coleoidea*) ont fait l'objet d'études approfondies en raison de la complexité du répertoire comportemental des céphalopodes et de leur système nerveux relativement accessible. Ils sont souvent considérés comme les Invertébrés les plus « intelligents », rivalisant avec le niveau d'intelligence de nombreux Vertébrés. A l'instar des céphalopodes adultes et juvéniles, l'embryon de seiche *Sepia pharaonis* est également capable d'apprentissage associatif (**chapitre 4-I**).

Ils peuvent associer un signal d'alarme avec la présence un poisson clown en utilisant soit la modalité visuelle, soit la modalité olfactive.

L'objectif de ces expériences complémentaires est d'analyser les compétences d'apprentissage associatif des embryons de *Sepia officinalis*. Nous allons dans un premier temps démontrer que les embryons de *Sepia officinalis* sont eux aussi capable de conditionnement classique comme les embryons de *Sepia pharaonis*. Ensuite, dans le but de complexifier ce conditionnement, nous allons rajouter une étape à ce conditionnement et ainsi essayer de réaliser un conditionnement de second ordre. Dans la première phase d'entraînement de ce conditionnement, la présentation d'un stimulus conditionnel (SC1) est suivie d'un stimulus inconditionnel. Dans la deuxième phase, un stimulus conditionné de second ordre (CS2) est présenté avec CS1. Enfin, en phase de test, CS2 est présenté seul aux embryons tandis que leurs réponses sont enregistrées. Ce type de conditionnement secondaire a déjà été réalisé chez d'autres espèces, aussi bien chez les poissons (Amiro & Bitterman, 1980) que chez les primates (Cook & Mineka, 1987) mais à notre connaissance il n'a jamais été réalisé ni chez des céphalopodes ni chez des embryons de toutes autres espèces. Comme l'ont montré les études précédentes, nous nous attendons à ce que les embryons de seiches montrent une réponse conditionnelle à CS1 mais aussi à CS2 (Jara, Vila, & Maldonado, 2006).

Matériel et méthodes

Deux protocoles expérimentaux ont été effectués afin de tester les capacités d'apprentissage des embryons au stade 29. Pour ce faire, dès le début du stade 29 les œufs ont été isolés individuellement dans des cristallisoirs de 150 ml dans une pièce à 20°C en luminosité naturelle et les apprentissages ont débuté le lendemain de leur installation (N=8 par groupe). L'eau a été changée tous les matins et la température de la salle surveillée quotidiennement.

1) Odeurs utilisées

Au cours de ces études, trois odeurs ont été utilisées : une odeur de roussette (un prédateur de la seiche ; *Scyliorhinus canicula*), une odeur de cannelle et une odeur de laurier. Avant chaque test, la réponse des embryons naïfs a été analysée en mesurant le rythme ventilatoire 1min avant et 1min pendant la stimulation.

Odeur de roussette : les roussettes ont été élevées par groupe de 8 femelles dans bacs d'eau de mer de 1000 litres (168cm de diamètre ; 61,5cm de hauteur), en circuit d'eau semi ouvert, sous une photopériode LD 12 : 12 et une température de 20 ± 2°C. Elles ont été nourries quotidiennement avec des morceaux de poissons congelés. L'odeur de roussette correspond à l'eau de mer du bac des roussettes prélevée avant chaque test. Pour chaque embryon testé, 3ml d'odeur ont été utilisés.

Odeur de cannelle : la cannelle utilisée correspond à 0,1g de cannelle moulue (©Ducros) diluée dans 150 ml d'eau de mer. Pour chaque embryon testé, 3ml d'odeur ont été utilisés.

Odeur de laurier : le laurier utilisé correspond à 0,1g de laurier moulu (©Ducros) dilue dans 150 ml d'eau de mer. Pour chaque embryon testé, 3ml d'odeur ont été utilisés.

2) Apprentissage associatif

L'odeur de roussette a été utilisée comme stimulus inconditionnel et l'odeur de cannelle a été utilisée comme stimulus conditionnel. Tous les après-midi pendant 4 jours, les embryons ont été exposés à l'odeur de cannelle suivie par l'odeur de roussette avec un intervalle de 3 secondes (l'eau est changée le lendemain matin). Au 5ème jour, le rythme ventilatoire des embryons a été mesuré 1 min avant et 1 min pendant une exposition à de l'odeur de cannelle seule.

3) Apprentissage associatif de second ordre

Cet apprentissage est composé de deux conditionnements l'un à la suite de l'autre. Pour le premier, l'odeur de roussette a été utilisée comme stimulus inconditionnel et l'odeur de cannelle a été utilisée comme stimulus conditionnel pendant 2 jours. Pour le second, l'odeur de cannelle (stimulus conditionnel numéro 1) est couplée avec l'odeur de laurier (stimulus conditionnel numéro 2) pendant 2 jours également. Au 3ème jour, le rythme ventilatoire des embryons a été mesuré 1 min avant et 1 min pendant une exposition à de l'odeur de cannelle seule. Puis au 5ème jour, le rythme ventilatoire des embryons a été mesuré 1 min avant et 1 min pendant une exposition à de l'odeur de laurier seul.

Résultats

1) Avant le conditionnement classique

Avant le conditionnement classique, le rythme ventilatoire des embryons augmentent significativement pendant une exposition à l'odeur de roussette (cf. figure 1 : test de Wilcoxon : $m_{acclimatization}$=65,78 ±3,62 ; $m_{stimulation}$=70,11 ±4,36 ; z=-2,536 ; p=0,0112). Le rythme ventilatoire des embryons reste stable pendant une exposition à l'odeur de cannelle, de laurier et de vanille (cf. figure 1 ; test de Wilcoxon : Cannelle : $m_{acclimatization}$=55,88 ±3,29 ; $m_{stimulation}$=57,25 ±2,51 ; z=-0,943 ; p=0,3454 ; Laurier : $m_{acclimatization}$=67,13 ±3,88 ; $m_{stimulation}$=69,13 ±4,53 ; z=-1,272 ; p=0,2033 ; Vanille : $m_{acclimatization}$=66,13 ±2,32 ; $m_{stimulation}$=63,38 ±2,93 ; z=-0,946 ; p=0,3441).

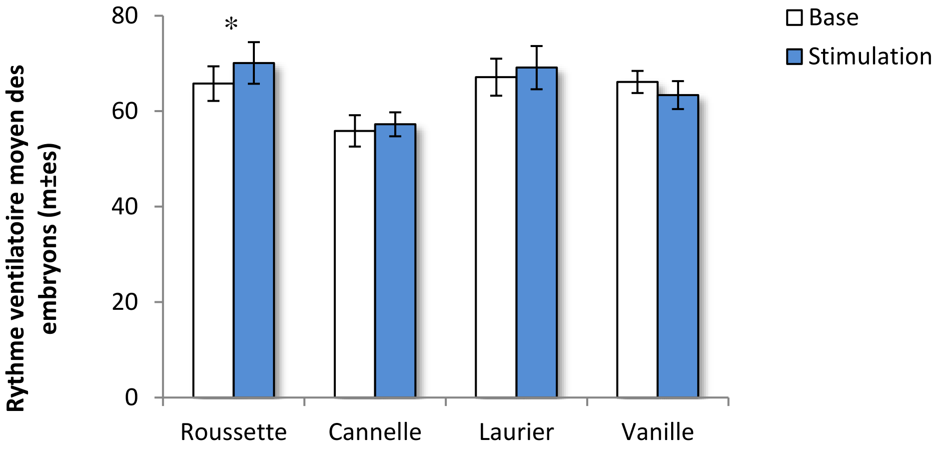

Figure 1 : Rythme ventilatoire des embryons de *Sepia officinalis* avant (rythme de base en blanc) et pendant une exposition (stimulation en bleu) à une odeur de roussette, de cannelle, de laurier et de vanille. Test de Wilcoxon * : p < 0,05.

2) Après le conditionnement classique

Après le conditionnement, le rythme ventilatoire augmente significativement pendant une exposition à de l'odeur de cannelle (cf. figure 2 ; test de Wilcoxon : $m_{acclimatization}$=73,14 ±4,08 ; $m_{stimulation}$=75,71 ±3,97 ; z=-2,375 ; p=0,0176).

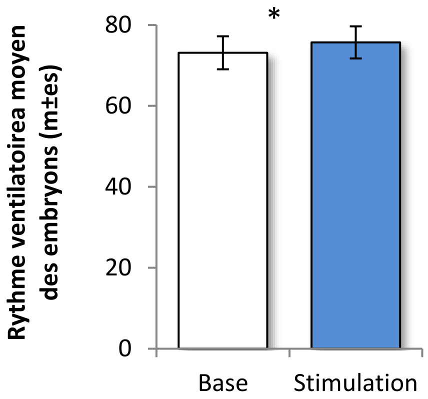

Figure 2 : Rythme ventilatoire des embryons de *Sepia officinalis* avant (rythme de base en blanc) et pendant une exposition (stimulation en bleu) à une odeur de cannelle après le conditionnement classique. Test de Wilcoxon * : p < 0,05.

3) Après le conditionnement classique de second ordre :

Suite au premier conditionnement, le rythme ventilatoire des embryons augmente significativement pendant une exposition à de l'odeur de cannelle (cf. figure 3 ; test de Wilcoxon : $m_{acclimatization}$=52,63 ±2,53 ; $m_{stimulation}$=62,50 ±2,89 ; z=-2,524 ; p=0,0116). Suite au second conditionnement, le rythme ventilatoire des embryons reste stable pendant une exposition à de l'odeur de le laurier (cf. figure 4 ; test de Wilcoxon : $m_{acclimatization}$=69,0 ±2,70 ; $m_{stimulation}$=68,50 ±3,47 ; z=-0,254 ; p=0,7995).

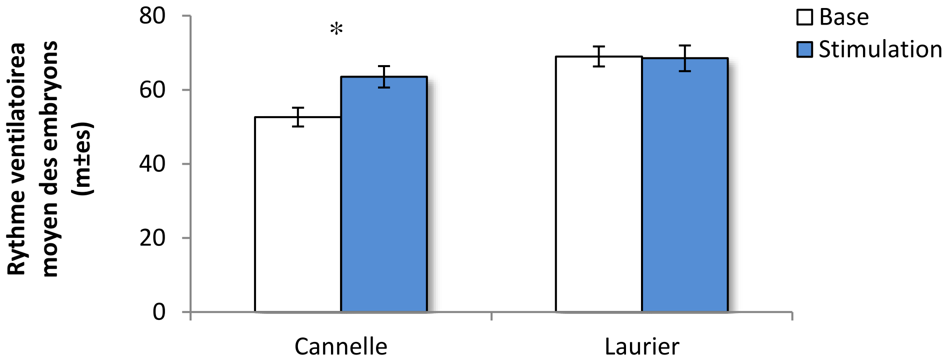

Figure 3 : Rythme ventilatoire des embryons de *Sepia officinalis* avant (rythme de base en blanc) et pendant une exposition (stimulation en bleu) à une odeur de cannelle (après le premier conditionnement) et à une odeur de Laurier (après le second conditionnement). Test de Wilcoxon * : p < 0,05.

Discussion et conclusion

Les résultats de notre étude montrent que les embryons de *Sepia officinalis* sont capables d'apprentissage associatif : alors que le rythme ventilatoire des embryons reste stable face à de l'odeur de cannelle avant le conditionnement, il augmente significativement après le conditionnement (odeur de cannelle couplée à de l'odeur de prédateur pendant le conditionnement). Cependant, nous n'avons pas réussi à mettre en évidence des capacités de conditionnement de second ordre : le rythme ventilatoire des embryons reste stable lorsqu'ils sont exposés à l'odeur de laurier (le second stimulus conditionnel).

A l'instar de *Sepia pharaonis*, les embryons de *Sepia officinalis* sont capables d'apprentissages associatifs. Ils sont capables d'utiliser des indices n'ayant pas de sens écologique (odeur de cannelle par exemple) et de les associer avec une menace (une odeur de prédateur). Même si cette nouvelle odeur n'a jamais été rencontrée précédement, elle va engendrer une réponse conditionnelle une fois associée au danger. Vivant dans un environnement constamment en changement, il est alors primordial pour les seiches d'apprendre très tôt et d'ainsi améliorer leur comportement anti-prédateur et d'augmenter leur chance de survie. Au cours de la seconde expérience complémentaire (conditionnement classique de second ordre), nous n'avons pas observé de réponse au stimulus conditionnel numéro 2 (odeur de laurier). Même s'il est possible que les embryons ne perçoivent pas cette odeur, ce conditionnement de second ordre n'est probablement pas pertinent chez cette espèce. Il est possible qu'à la fin de ce conditionnement de second ordre la cannelle ait perdue son « pourvoir stressant » car elle n'est plus présentée avec la menace. Pour vérifier cette hypothèse il faudrait (1) tester une autre odeur que le laurier et (2) re-tester la cannelle à la fin de cet apprentissage secondaire.

Bilan du chapitre 4 :

La **première partie** de ce **chapitre 4** met en évidence des capacités de reconnaissance innée mais aussi acquise des prédateurs chez les embryons de *Sepia pharaonis*. Ainsi les embryons sont capables d'apprentissage. Ces compétences précoces ont été mises en évidence grâce à un protocole de conditionnement classique que nous avons appliqué pendant 4 jours en utilisant le signal d'alarme comme stimulus inconditionnel et le non-prédateur comme stimulus neutre.

De la même façon, au cours de la **deuxième partie**, ces compétences précoces ont été mises en évidence chez *Sepia officinalis* en utilisant une odeur de prédateur comme stimulus inconditionnel et une odeur de cannelle comme stimulus neutre. Par contre, suite au protocole de conditionnement classique de second ordre, les embryons ne répondent pas au stimulus conditionnel numéro 2 (une odeur de laurier). D'autres essais sont nécessaires avant de pouvoir tirer des conclusions sur cet apprentissage un peu plus complexe.

Chapitre 5

Le stress prénatal module-t-il les capacités d'apprentissage chez la seiche ?

Chapitre 5 – Le stress prénatal module-t-il les capacités d’apprentissage chez la seiche ?

Contexte du chapitre 5 :

Afin d’augmenter leur chance de survie, les animaux peuvent apprendre très tôt à reconnaitre leurs proies, leurs prédateurs, leurs partenaires sociaux etc. Cet apprentissage est possible dès la période prénatale chez de nombreuses espèces (voir **chapitre 1**). Cependant, le stress prénatal peut perturber cet apprentissage. Dans la littérature ces effets sont principalement étudiés chez les mammifères, montrant des effets délétères sur leurs capacités cognitives. Or, chez les ovipares, le stress perçu par les embryons peut aussi influer sur le comportement des jeunes après leur naissance/éclosion comme c’est le cas chez la caille japonaise (C*oturnix c. japonica*). Un stress artificiel (un bruit métallique) va influencer les comportements sociaux et un stress naturel (des vocalisations de prédateur) va moduler l’émotivité des jeunes (Mezrai et al. en préparation). Ces stress perturbent aussi certaines capacités cognitives : des jeunes soumis au stress artificiel et naturel échouent au cours d’un test de détour de locomotion contrairement aux jeunes non stressés avant l’éclosion (Mezrai 2014). L’objectif de ce **chapitre 5** est alors de décrire les effets du stress prénatal sur les capacités d’apprentissage simple et associatif chez la seiche. Une lumière forte a été utilisée comme stress artificiel et une odeur de prédateur comme stress naturel. Leurs capacités d’apprentissage ont été mesurées avec un protocole d’empreinte alimentaire et de conditionnement classique. Pour ce premier test, les embryons ont été exposés à des gammares (*Sepia pharaonis ;* **partie I**) ou à des crabes (*Sepia officinalis ;* **partie II**). Pour le conditionnement classique (**partie I** pour *Sepia pharaonis* et **partie II** pour *Sepia officinalis*), l’encre a été utilisée comme stimulus inconditionnel (l’odeur de prédateur a déjà été utilisé pour stress les embryons soumis au stress naturel) et à une odeur de cannelle comme stimulus conditionnel (stimulus validé au cours du **chapitre 4**).

Ce chapitre est composé d’une étude préliminaire réalisée chez *Sepia pharaonis* et d’un article en préparation sur *Sepia officinalis* :

I - Etude préliminaire sur les effets du stress prénatal chez *Sepia pharaonis*

II – Article 3 : **Mezrai, N.,,** Tchadjiane, C., Dickel L., & Darmaillacq, A.S., in preparation. Prenatal stress effect on simple and associative learning abilities in cuttlefish *Sepia officinalis.*

I. Etude préliminaires sur les effets du stress prénatal chez *Sepia pharaonis*

Un protocole d'empreinte alimentaire a d'abord été réalisé chez les individus non stressés en février 2017 lors de mon séjour à Taiwan. Puis, ce protocole a été appliqué à des individus des groupes stressés et non stressés en février et mars 2018 dans le cadre d'un stage de Master 2 mené par Iris Lemercier. Enfin, les individus des groupes stressés ou non stressés ont été soumis à un conditionnement classique où de l'encre de seiche a été couplée avec une odeur de cannelle.

1) Validation du protocole d'empreinte chez *Sepia pharaonis*

Le protocole d'empreinte alimentaire a été décrit par Darmaillacq et al. (2008) chez les embryons de *Sepia officinalis*. Au cours de leur expérience, les embryons ont été exposés à des petits crabes pendant au moins une semaine avant l'éclosion. Puis, 7 jours après leur éclosion, les juvéniles ont passé un test de préférence alimentaire où ils étaient exposés à des crabes ou des crevettes. Les jeunes seiches exposées aux crabes avant l'éclosion ont montré des préférences significatives pour ces proies alors que les seiches naïves (non exposées à des proies pendant la période prénatale) ont montré des préférences pour les crevettes (Darmaillacq et al., 2008).

Méthode utilisée

Le dispositif et le protocole sont alors les mêmes que l'étude de Darmaillacq et al. (2008) à la différence que les embryons n'ont pas été exposés à des crabes mais à des gammares (*Gammarus sp*). Les crabes ne sont pas disponibles à cette période de l'année et les gammares sont de petits crustacés également consommés par les seiches. Afin d'exposer les embryons à ces proies, les œufs sont installés dans des aquariums de 50x30x25cm directement avec les gammares. Cette exposition prénatale débute dès le stade 27 et ce en continu jusqu'à l'éclosion. Dès l'éclosion, les jeunes ont été isolés des gammares et un test de choix alimentaire a été effectué 7 jours après l'éclosion (gammares *versus* crevettes ; cf. figure 1 et 2). Le dispositif expérimental et le protocole utilisés sont les mêmes que ceux décrits dans le **chapitre 2**.

Résultats :

Les seiches non exposées aux gammares avant l'éclosion préfèrent significativement les crevettes à ces derniers (Figure 35 ; test binomial : $N_{crevette} = 11$ *versus* $N_{gammares} = 1$; $p = 0{,}0063$). Par contre, si les seiches ont été exposées à des gammares avant l'éclosion, elles ne montrent plus aucune préférence pour les crevettes 7 jours après l'éclosion (Figure 35 ; test binomial : $N_{crevette} = 16$ *versus* $N_{gammares} = 14$; $p = 0{,}8555$).

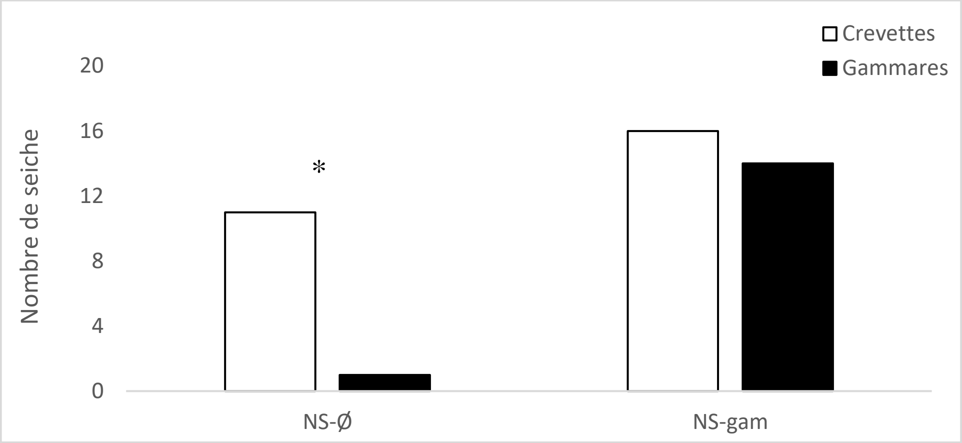

Figure 35 : Nombre d'individu qui s'oriente préférentiellement vers les crevettes (blanc) ou les gammares (noir) au cours du test d'empreinte alimentaire. Le test a été réalisé chez des seiches non stressés de 7 jours ayant été exposés à des gammares avant l'éclosion (NS-gam) et chez des seiches naïves (NS-Ø). Test binomial * : p < 0,05.

Conclusion :

Les jeunes seiches *Sepia pharaonis* non stressées et non exposées aux gammares avant l'éclosion ont une préférence innée pour les crevettes. Ces résultats sont en accord avec ceux obtenus par Darmaillacq et al. (2004) montrant que les seiches *Sepia officinalis* préfèrent aussi les crevettes aux crabes. Nous avons également montré que cette préférence innée disparait si les jeunes seiche *Sepia pharaonis* ont été exposées à des gammares avant l'éclosion. Contrairement à *Sepia officinalis* où les jeunes préfèrent les crabes après le protocole d'empreinte, les préférences alimentaires de *Sepia pharaonis* sont supprimés suite à ce protocole (les jeunes ne préfèrent ni les crevettes ni les gammares).

2) Effet du stress prénatal sur l'empreinte alimentaire chez *Sepia pharaonis*

Méthode utilisée

Suite à ce premier essai, le protocole d'empreinte alimentaire a été réalisé chez des seiches exposées ou non à un stimulus stressant avant l'éclosion afin de voir les effets du stress prénatal sur cet apprentissage simple. Pour ce faire, trois groupes ont été étudiés : un groupe d'embryons exposés à un stress naturel (groupe « SN »), un groupe d'embryons exposés à un stress artificiel (groupe « SA ») et un groupe témoin non stressés avant l'éclosion (groupe « NS »). Les embryons du groupe « SA » et « SN » subissent un stress chronique du stade 21 au stade 27. Le groupe SN est exposé à une odeur de prédateur, le poisson ballon (*Arothron manilensis*). Ils ont été incubés dans le bac du prédateur (50x30x25cm), de façon à ce que les embryons puissent percevoir les stimuli chimiosensoriels mais pas visuels (cf. Figure 36). Quant au groupe SA, les œufs ont été incubés dans un même bac mais sous une lumière forte qui s'allume de façon aléatoire dans la journée (15 min répartie 4 fois par jour ; cf. Figure 36). Les embryons du groupe « NS » sont placés dans un même bac sans stimulation particulière.

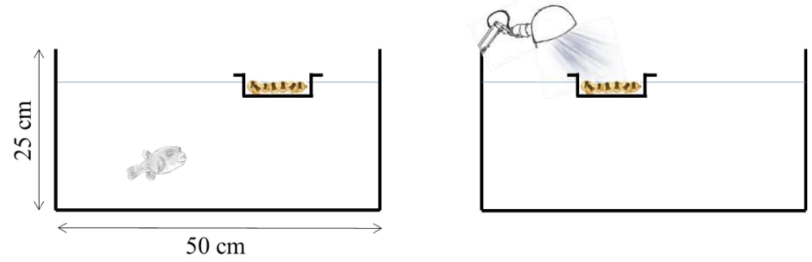

Figure 36 : Schémas des bacs d'incubation des embryons du groupe SN (gauche) et SA (droite). Schémas réalisés par Iris Lemercier.

Résultats :

Les seiches du groupe NS non exposées aux gammares avant l'éclosion tendent à préférer les crevettes aux gammares (Figure 37 ; test binomial : $N_{crevette}$ =24 *versus* $N_{gammares}$ =12 ; p=0,065) alors que les seiches du groupe NS exposées aux gammares avant l'éclosion n'ont aucune préférence entre ces deux items (Figure 37 ; test binomial : $N_{crevette}$ =11 *versus* $N_{gammares}$ =18 ; p=0,2649). De plus, les individus du groupe SA et SN non exposés aux gammares avant l'éclosion préfèrent significativement les crevettes aux gammares (Figure 37 ; test binomial : SA : $N_{crevette}$ =18 *versus* $N_{gammares}$ =4 ; p=0,004 ; SN : $N_{crevette}$ =21 *versus* $N_{gammares}$ =3 ; p=0,0003). Par contre, les individus du groupe SA exposés aux gammares avant l'éclosion n'ont aucune préférence entre ces deux proies (Figure 37 ; test binomial : SA : $N_{crevette}$ =13 *versus* $N_{gammares}$ =9 ; p=0.523) mais les individus SN exposés aux gammares avant l'éclosion préfèrent les crevettes (Figure 37 ; test binomial : SN : $N_{crevette}$ =15 *versus* $N_{gammares}$ =5 ; p=0,041).

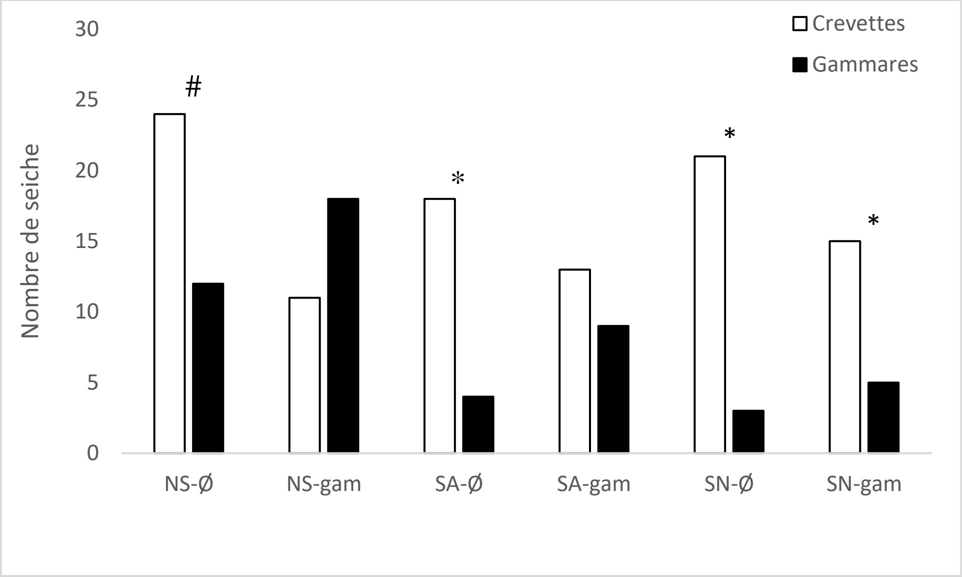

Figure 37 : Nombre d'individu qui s'oriente préférentiellement vers les crevettes (blanc) ou les gammares (noir) au cours du test d'empreinte alimentaire. Le test a été réalisé chez des seiches de 7 jours non stressées (NS) ou stressées avec un stress artificiel (SA) ou un stress naturel (SN) avant l'éclosion. Avant l'éclosion, les seiches ont été exposées à des gammes (gam) ou non (Ø). Test binomial * : $p < 0,05$; # : $0,05 \leq p \leq 0,07$.

Conclusion :

Chez *Sepia pharaonis*, le stress prénatal artificiel (lumière forte) ne semble pas avoir d'impact sur l'empreinte alimentaire. A l'instar des jeunes seiches du groupe non stressé, elles ne montrent pas de préférence entre les crevettes et les gammares. Par contre il est possible que le stress naturel (odeur de prédateur) ait un impact sur l'empreinte alimentaire puisque même après un protocole d'empreinte, les jeunes seiches exposées à des gammares avant l'éclosion préfèrent tout de même les crevettes aux gammares.

3) Effet du stress sur l'apprentissage associatif chez *Sepia pharaonis*

Méthode utilisée

La procédure de stress utilisée pour mesurer les effets de stress prénatal sur les capacités d'apprentissage associatif est la même que dans la partie précédente (stress artificiel ou naturel du stade 21 au stade 27). Dans premier temps, il a fallu s'assurer que les embryons répondaient ou non aux stimuli conditionnel et inconditionnel que nous voulions utiliser. Pour ce faire, des embryons naïfs de chaque groupe ont été exposés à de l'encre et de l'odeur de cannelle et leur rythme ventilatoire (RV) a été mesuré avant et pendant la stimulation. Puis, en fonction des résultats obtenus, nous avons réalisé l'apprentissage associatif à partir du stade S27 : une odeur de cannelle couplée avec de l'encre de seiche a été présentée une fois par jour pendant 4 jours aux embryons. La phase de test s'est déroulée le 5ème jour au cours duquel, le rythme ventilatoire a été mesuré avant et pendant l'exposition à l'odeur de cannelle seule. Enfin, ce même apprentissage a été réalisé sur 2 jours (le test a eu lieu le 3ème jour).

<u>Résultats :</u>

Le RV des embryons du groupe NS (non stressé) diminue significativement lorsqu'ils sont exposés à de l'encre (Figure 38 ; test de Wilcoxon : N=16 ; p=0,0080 ; z=-2,650) et reste stable lorsqu'ils sont exposés à une odeur de cannelle (Figure 38 ; test de Wilcoxon : N=11 ; p=0,0881 ; z=-1,706). En ce qui concerne les embryons du groupe SA (stress artificiel) et SN (stress naturel), leur RV reste stable lorsqu'ils sont exposé à de l'encre ou à de l'odeur de cannelle (Figure 38 ; Tests de Wilcoxon : SA : encre : N=8 ; p=0,2282 ; z=-1,205 ; SN : encre : N=16 ; p=0,530 ; z=-0,628 ; cannelle : N=11 ; p=0,6733 ; z=-0,422). En raison de problème méthodologique (mortalité accrue des embryons) l'odeur de cannelle n'a pas pu être testé sur les individus du groupe SA.

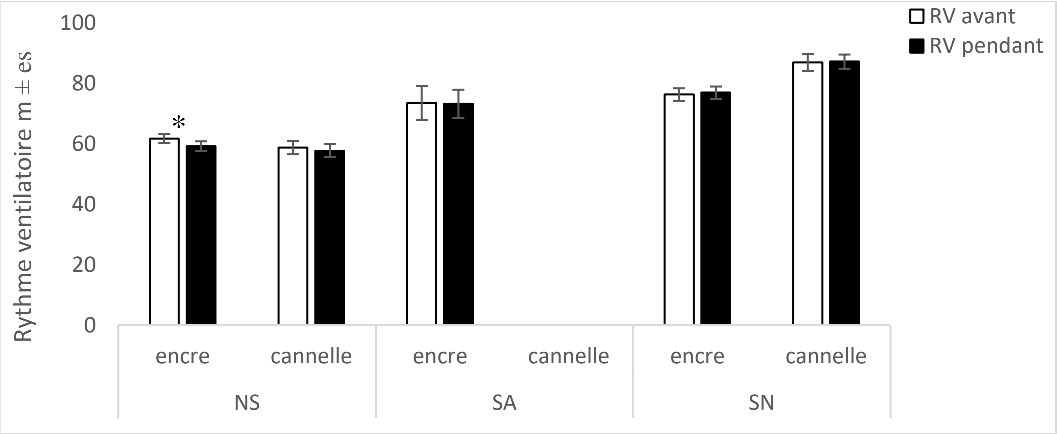

Figure 38 : Rythme ventilatoire avant (blanc) et après (noir) une exposition à de l'encre de seiche ou de la cannelle chez les embryons de seiche non stressés (NS) ou stressés avec un stress artificiel (SA) ou un stress naturel (SN). Test de Wilcoxon : * p<0,05.

Suite aux expériences préliminaires, le conditionnement classique n'a été réalisé que sur le groupe d'embryons NS (non stressés). Après appariement de l'encre et de la cannelle pendant 4 jours, le RV diminue significativement avant et pendant l'introduction de l'odeur de cannelle au 5eme jour (Figure 39 ; test de Wilcoxon, N=12 ; p=0,0448 ; z=-2,007). Après appariement de l'encre et de la cannelle pendant 2 jours, le RV diminue également significativement avant et pendant l'introduction de l'odeur de cannelle au 3eme jour (Figure 39 ; test de Wilcoxon : N=6 ; p=0,0422 ; z=-2,032).

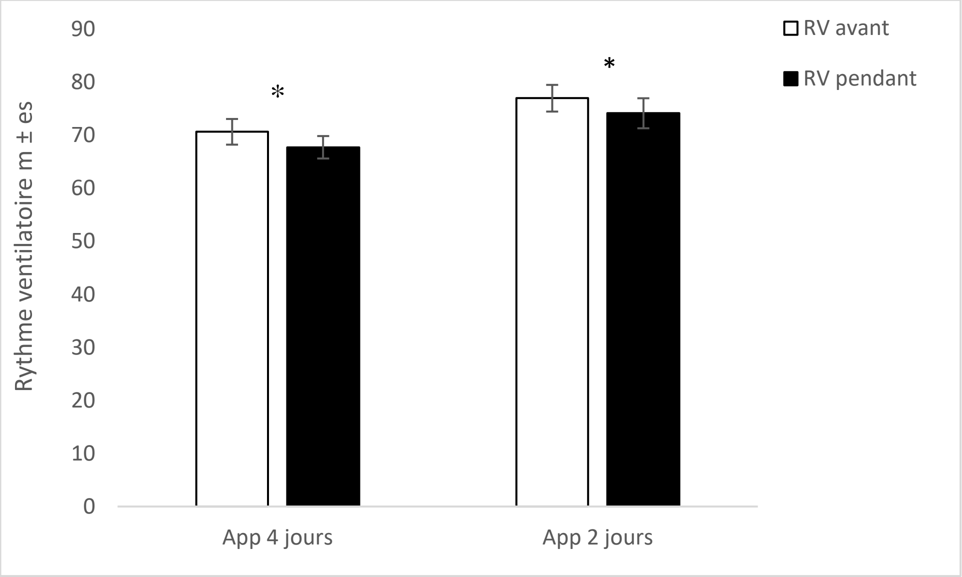

Figure 39 : Rythme ventilatoire avant (blanc) et après (noir) une exposition à de la cannelle après le conditionnement classique de 4 jours ou de 2 jours chez les embryons de seiche non stressés. Test de Wilcoxon : * p<0,05.

Conclusion :

Les embryons de *Sepia pharaonis* sont capables d'associer une odeur de cannelle avec un signal d'alarme (de l'encre de seiche). Cet apprentissage associatif peut se faire en 4 jours mais aussi en 2 jours. Cependant, nous ne pouvons pas tirer de conclusion quant aux effets du stress sur ces capacités d'apprentissage. Effectivement, nous n'avons pas pu utiliser l'encre de seiche comme stimulus inconditionnel puisque cet item n'induisait aucune réponse de la part des embryons. Même s'il est possible que ce manque de réponse à l'encre de la part des embryons SA et SN soit une conséquence du stress prénatal, d'autres expériences (réplica et/ou essais avec d'autres odeurs pouvant être utilisées comme stimulus inconditionnel) sont nécessaires pour compléter ces données chez *Sepia pharaonis*.

II. Prenatal stress effect on simple and associative learning abilities in cuttlefish *Sepia officinalis*.

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ABSTRACT:

For many years, embryos have been believed to born without a functional memory but prenatal learning may bring benefits for survival. There are many forms of learning, including imprinting (non-associative learning) and associative learning but they are very few study on embryo because of their inaccessibility. Imprinting is characterized by the establishment of a persistent preference for something during a limited sensitive period, usually early in development. Imprinting for prey has been demonstrated in *S. officinalis*. In hatchlings, the "innate" preference for shrimp can be changed by visual exposure to crabs shortly before and/or after hatching. In associative learning, animals learn about the relation-ship between two stimuli or events. The properties of learning have been studied in a wide range of species and modern cephalopod have received intensive study because of their complex behavioral repertoire and relatively accessible nervous systems. However, many studies have demonstrated that prenatal stress may modulate the learning of juveniles both in humans or animals. The aim of our study is to know if prenatal stress can influence simple and associative learning abilities on embryo and juvenile's cuttlefish. To do that, an artificial and natural stress was used by stressing the embryos during the prenatal period. Then, an imprinting paradigm and a classical conditioning protocol was used to measure their learning abilities. As expected, this study shows simple learning skills in cuttlefish embryo: cuttlefish exposed to crab during prenatal period prefer this item rather than shrimps. But, surprisingly, we have shown that prenatal stress did not affect this prenatal learning. Furthermore, the results show for the first time that newly-hatched cuttlefish are capable of associative learning and that prenatal stress can modulate learning depending of the nature and the intensity.

Keywords:

Imprinting – associative learning – *Sepia officinalis* – natural and artificial stress

INTRODUCTION

For many years, embryos have been believed to born without a functional memory. However, research has confirmed that prenatal learning may bring benefits for survival. There are many forms of learning, including imprinting (non-associative learning) and associative learning (classical conditioning for example) but they are very few study on embryo because of their inaccessibility. Indeed, despite increasing empirical evidence that prenatal experience influences postnatal abilities (predator recognition for example: Ferrari & Chivers, 2009a, 2009b, 2010; Ferrari, Crane, & Chivers, 2016; Ferrari, Manek, & Chivers, 2010; Golub, 2013; Mathis, Ferrari, Windel, Messier, & Chivers, 2008; Saglio & Mandrillon, 2006), few studies have tested explicitly learning on embryos. Most studies showing evidence for embryonic learning exposed individuals to a stimulus prenatally, but only tested their discriminatory capacity towards the stimuli after birth or hatching. Imprint can illustrate this point. It is characterized by the establishment of an irreversible preference for something during a limited sensitive period, usually early in development (Lorenz, 1937; Sluckin, 2017). Contrary to perceptual learning, imprinting can be defined by 5 particular criteria: 1) there is no reinforcement; 2) imprinting takes place during a sensitive period and 3) is indelible; 4) this preference will be generalized to other objects sharing similar characteristics and 5) imprinting has consequences on later behaviors in the life (Sluckin, 2017). So, learning takes place before birth/hatching and juveniles are tested after. This simple learning has been demonstrated in a large range of species (Lorenz, 1937; Sluckin, 2017) like on the cephalopod *Sepia officinalis* for the first time by Darmaillacq (2004; 2005). Darmaillacq show that on hatchlings, the "innate" preference for shrimp could be change by chemical and/or visual exposure to crabs shortly before and/or after hatching (Darmaillacq, Chichery, & Dickel, 2006; Darmaillacq, Chichery, Poirier, & Dickel, 2004; Darmaillacq, Chichery, Shashar, & Dickel, 2006). This induced preference fit the criteria for imprinting: it lasted for at least three days, persisted after the cuttlefish had consumed a shrimp and was only induced during a short sensitive period early in the life of the cuttlefish (Darmaillacq, Chichery, & Dickel, 2006; Darmaillacq et al., 2004; Darmaillacq, Chichery, Shashar, et al., 2006; Darmaillacq, Lesimple, & Dickel, 2008; Darmaillacq, 2005). This sensitive period for prey preference induction begin before hatching and closes about 6 hours after sunrise on the day of hatching (Darmaillacq, Chichery, Shashar, et al., 2006; Darmaillacq et al., 2008).

One the other hand, associative learning was very rarely study on embryo (rat: Smotherman, 2002; Smotherman & Robinson, 1985, 1988, 1992; Smotherman et al., 1991) and never done on cephalopod embryo or hatchling. However, learning and memory in modern cephalopod mollusks (coleoids) have received intensive study because of cephalopods' complex behavioral repertoire and relatively accessible nervous systems. They are often considered as the most sophisticated invertebrates, rivaling the level of intelligence of many vertebrates. Many scientific work led by J. Z. Young, M. J. Wells, A. Packard, B. Boycott, H. Maldonado, and J. B. Messenger, invested in the subject in the middle of the previous century. They were interested in these animals because they believed that the simplicity of the invertebrate nervous system, together with the complex behavior of coleoids (Turchetti-Maia, Shomrat, & Hochner, 2017).

It is now recognized that individuals' learning and memory abilities can be disrupted by stress, especially by prenatal stress. Although definition of stress is sometimes controversial, it refers to a series of physiological, morphological and behavioral changes that arise in the face of external challenges in an attempt to restore homeostasis or to mitigate the impact of the stressor. When it is chronic, repeated, changes can persist over time (Jones, 1996). Experiments in mammals and birds have shown changes in locomotors behavior, exploration, play, fear of novelty but also on learning and memory abilities (Benoit, Rakic, & Frick, 2015; Braastad, 1998; Weinstock, 2017). More recently on cuttlefish, O'Brien et al. (2017) highlight that juveniles exposed to a natural stressor, predator odor, showed no differences from controls, while embryos exposed to an artificial stressor, bright light, differed in their predation behavior (O'Brien et al., 2017b). In view of these recent bibliographic data we can ask ourselves if prenatal stress also modulates learning abilities. The aim of our study is two-fold: (1) study if prenatal stress can influence simple learning abilities (imprinting) on juvenile's cuttlefish. And (2) investigate associative learning abilities in 1-day old cuttlefish and know if prenatal stress can modulate these early learning abilities. These two questions were tested on unstressed and stressed cuttlefish during prenatal period (natural stress: predator odor or artificial stress: bright light).

MATERIALS AND METHODS

1) Biological materials

Experimental model

Model species is the common cuttlefish (*Sepia officinalis*). All the eggs studied were laid in natural environment in Ouistreham (Calvados, France) and were transferred before organogenesis to the CREC on July 2, 2018 (Centre de Recherches en Environnement Cotier, Calvados, France). Eggs were maintained in floating, circular baskets (3.8L; maximum 100 eggs per baskets) at 20 ± 2°C temperature in 65L (80x60x40 cm) tanks constantly renewed by natural seawater from a flow-through system with vigorous aeration from an air stone and exposure to the natural light cycle.

Embryonic development

It took 1 month to complete embryonic development at 21°C water temperature (lemaire, 1970). Based on morphological characteristics, the embryonic development of *S. officinalis* is divided into 3 periods and 30 stages: *segmentation* (stages 1-9), *gastrulation* (stages 10-17) and *organogenesis* (stages 18-30) (Lemaire, 1970). During the embryogenesis the different sensory systems begin to develop and become mature (Romagny et al., 2012). Response to touch and odors is possible by the 23rd and response to visual information is made possible by the 25th stage via early maturity of the visual system and increased transparency of the egg membrane due to osmotic swelling (Darmaillacq et al., 2008; Paulij, Bogaards, & Denucé, 1990; Romagny et al., 2012). A recent study, highlight than response to odors is possible at stage 22 and respond to light at stage 24 (Mezrai et al. submitted).

2) Prenatal stress

3 groups of cuttlefish (around 150 eggs per group) were studied: two groups of stressed embryos (Artificial Stress « **AS** » and Natural Stress « **NS** ») and one group of un-stressed embryo (**US**). AS eggs were exposed to white LED light (18 Watts, 20.7klux, approximately 10 cm from surface of water) in 15 min bursts eight times every 24h on a schedule that was re-randomized daily. NS eggs were exposed to 2 liters of water from 8 small-spotted catshark (*Scyliorhinus canicula)* tanks (1000L). Every day, at different moment during the day, we exposed embryos to 1.5l of water. Prenatal stress was applied from stage 23 to stage 30.

3) Imprinting: experimental protocol

This experiment was replicate once. One week before hatching (stage 30), eggs were placed in circular apparatus (15 eggs maximum per apparatus) with crabs (2 to 4 mm; 3 crabs per eggs) during 1 week at least (cf. Figure 1 A). No crabs were placed for control group (cf. Figure 1 B).

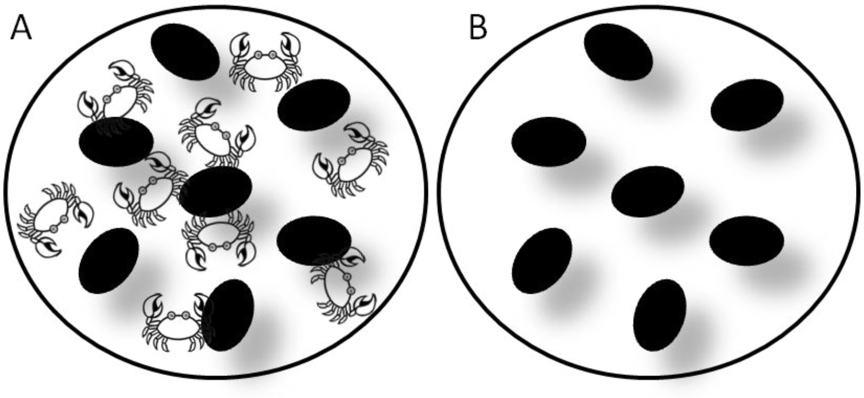

Figure 1: Schematic representation of the apparatus with eggs (black circle) and crabs during imprinting (A: experimental group with crab's exposure; B: control group without crab's exposure).

Every morning, hatchlings were collected and individually isolated in opaque black plastic tank (6x6x5cm) and were not fed until testing seven days later to ensure a sufficient food motivation. Cuttlefish can survive such fasting since they hatch with their internal yolk reserves (Boletzky 1975; Dickel, Chichery & Chichery 1997; Boletzky 2003). On day seven after hatching, juveniles from each group were submitted to a two-way choice discrimination test between two preys (2 crabs and 2 shrimps) as described by Darmaillacq et al (2004). The apparatus (cf. figure 2) consisted of a black rectangular PVC arena ending in 3 separate adjacent compartments. The transparent glass used in the procedure between preys and cuttlefish did not affect light polarization. Prey positioning was switched randomly to eliminate the possibility of a choice based on prey position and laterality. Initially, preys were hidden behind black PVC and the cuttlefish was places in the corner, equidistant from the two prey types. After 30 min acclimatization period, the black PVC was removed and the cuttlefish could see preys. During 15min test, cuttlefish could choose between shrimp (3-4mm) or crabs (2-4mm). For each group we noted the first choice and the choice latency.

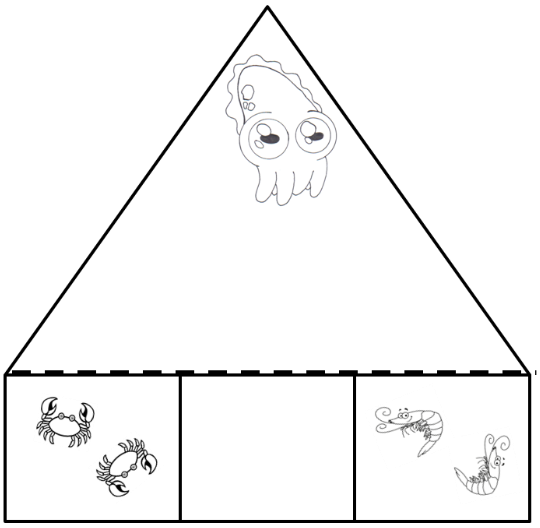

Figure 2: Schematic representation of the two-way choice discrimination. Cuttlefish could choose between crabs and shrimps. Preys were placed randomly to the right and to the left to avoid laterality bias. Preys were isolated from cuttlefish by transparent glass (dotted line).

4) Associative learning: experimental protocol

Conditioning phase

A standard conditioning protocol has been used on newly-hatched cuttlefish. The cinnamon odour (**C**) was used as a conditional stimulus and cuttlefish ink (**I**) was used as an unconditional stimulus (experimental group). In order to do this, juveniles follow this associative learning protocol once a day for 4 days. At 4:00pm we add 3ml of cinnamon and 3ml of ink in the crystallizer (3 sec between the two presentations) and every morning at 9:30am the water was changed with clear seawater.

We studied 8 groups:

"C + I" group: we followed the same protocol explained previously. We made a cinnamon exposure followed by cuttlefish ink once a day for 4 days. Cinnamon odor was obtained by mix 0.3g of cinnamon powder (cannelle moulue by ducros®) and 150ml of seawater. Ink was obtained by mix 3ml of ink and 150ml of seawater (ink was taken from an ink sac of an individual previously euthanized for another experiment - principle of reuse; these cuttlefish hatched in July 2018; 5cm mantle length).

"C + I $_{\text{bis}}$" group: the previous experiment was replicate to validate the protocol.

"C $_{\text{pre-test}}$" and "I $_{\text{pre-test}}$" control group: Two pre-test was done with cinnamon alone and Ink alone. We want to confirm that cinnamon does not induce response (conditional stimulus) and that ink induces response (unconditional stimulus) on naive juvenile cuttlefish (that have never perceived theses stimulations.

"C + SW" control group: We want to demonstrate that several exposures to cinnamon does not induce sensitisation. This simple learning is the increased likehood of an animal responding to a stimulus (Hanlon & Messenger, 2018).

"C + I $_{\text{differed}}$" control group: Here we did a differed exposition to cinnamon and ink. Cinnamon is presented at 14:00 and ink at 16:00. We want to make sure that the response observed after the conditioning are due to the two stimuli together.

"C + I $_{\text{one day}}$" group: For this group, we replicated the experimental group "C + I" but in just one day of conditioning protocol. We wondered if babies' cuttlefish can learn in one day.

"C+I $_{\text{one day - strong stress}}$" group: "C + I $_{\text{one day}}$" group was replicated with a strong prenatal stress. For AS, instead of 15 min bursts eight times every 24h we stress embryo between 9:00am and 6:00pm. For NS, instead of 1.5l of water with predatory odor, all the eggs were placed in the predatory tank between 9:00am and 6:00pm (eggs were protected from predator but can smell and see their shadow).

Testing phase

The day following the end of the conditioning phase all cuttlefish were tested with the cinnamon odour alone. Under binocular loupe (Loupe binoculaire numérique Jeulin®; Ref: 571265), data collection was carried out by counting manually the ventilation rate one minute before (baseline rate) and one minute after the stimulation time.

Measured variable

For the first time on cephalopod, we develop classical conditioning on newly-hatched cuttlefish by using the ventilation rate analysis (VR). Unlike heart rate, VR is easily and directly observable in cuttlefish without specialized equipment. VR can also be used to monitor more subtle responses to low intensity stimuli (Boal & Ni, 1996). In embryos, VR can be easily observed under a microscope as rhythmic motion of the collar flaps, which circulate oxygenated water to the gills, or as the movement of the funnel in response to the pressure changes of inhalation and exhalation (Mezrai, Arduini, et al., submitted; Mezrai, Chiao, et al., submitted).

5) Statistical analyses

Given the sample size, nonparametric statistical methods were used to analyse data. Concerning the imprinting test, the first choice was compared with a binomial test and the latency was compared using a Mann-Whitney test. For the associative learning test, mean ventilation before and after stimulation time were compared using a Wilcoxon test. The α level for all analyses was 0.05. Finally, the data were expressed as mean and standard error of the mean (m ± sem). Statistical analyses were run using Statview© version 5.0 and R version 3.2.3 (2015-12-10).

6) Ethical note

This research was conducted in accordance with Directive 2010/63/EU, under the approval of the Comité d’Éthique NOrmandie en Matière d’EXperimentation Animale (CENOMEXA) #54 (agreement number A14384001).

RESULTATS

1) Imprinting test

First choice:

When juveniles were not exposed to crabs during prenatal period, they significantly prefer shrimps to crabs (cf. figure 3; binomial test: US-Ø: number of successes = 10, number of trials = 11, p-value = 0.01172; AS-Ø number of successes = 11, number of trials = 12, p-value = 0.006348; NS-Ø: number of successes = 15, number of trials = 19, p-value = 0.01921). Conversely, when juveniles were exposed to crabs during prenatal period, they significantly prefer crabs to shrimps (cf. figure 3; binomial test: US-crab: number of successes = 11, number of trials = 13, p-value = 0.02246; AS-crab: number of successes = 11, number of trials = 13, p-value = 0.02246; NS-crab number of successes = 14, number of trials = 16, p-value = 0.004181).

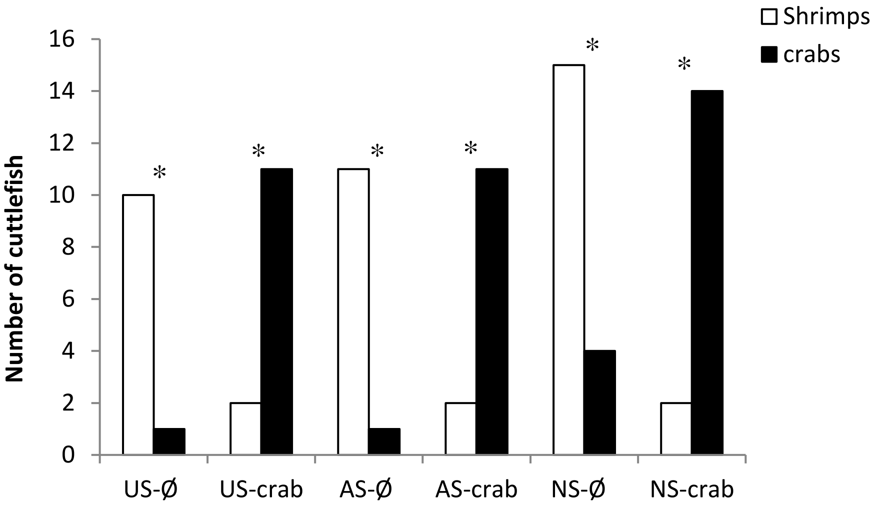

Figure 3: Preference for crabs (white) and shrimps (black) by 7-day-old cuttlefish. In unstressed cuttlefish (US; control group); stressed cuttlefish AS (artificial stress) and stressed cuttlefish NS (natural stress). "Ø" means cuttlefish not exposed to crabs and "crab" means cuttlefish exposed to crabs during prenatal period. Binomial test: * $p < 0.05$.

Latency:

There is no difference in latency between juveniles US and SA exposed or not to crabs (Mann-Whitney test: US: $N_{US-Ø}=11$; $N_{US-crab}=13$; p=0.3103; z=-1.015; AS: $N_{AS-Ø}=12$; $N_{AS-crab}=13$; p=0.4627; z=-0.734). On NS, juveniles exposed to crab are significantly faster than those not exposed to crabs (Mann-Whitney test: NS: $N_{NS-Ø}=19$; $N_{NS-crab}=16$; p=0.0322; z=-2.142). Furthermore, there is no difference between the unstressed and the stressed (Mann-Whitney test: US-crab *vs.* NS-crab: p=0.7419; z=-0.329; US-crab *vs.* AS-crab: p=0.2811; z=-1.078; NS-crab *vs.* AS-crab: p= 0,1089; z=-1.603; US-Ø *vs.* NS-Ø: p=0.9283; z=-0.090; US-Ø *vs.* AS-Ø: p=0.6664; z=-0.431; NS-Ø *vs.* AS-Ø: p=0.5533; z=-0.593).

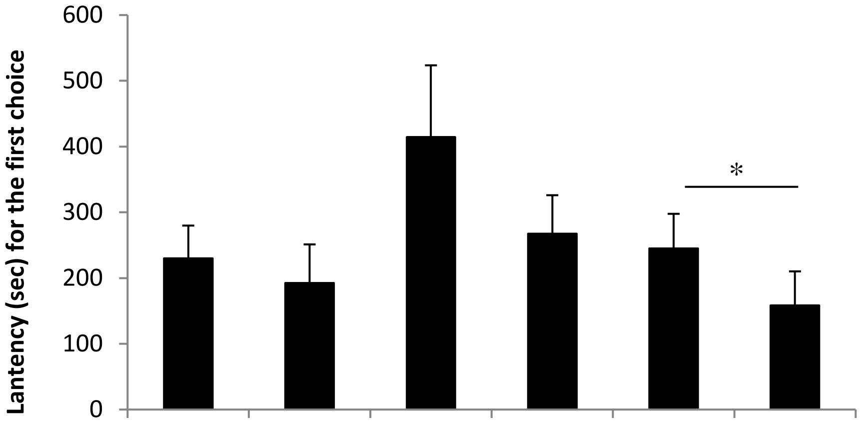

Figure 4: Latency (sec) to make the first choice by 7-day-old cuttlefish. In unstressed cuttlefish (US: control group); stressed cuttlefish AS (artificial stress) and stressed cuttlefish NS (natural stress). "Ø" means cuttlefish not exposed to crabs and "crab" means cuttlefish exposed to crabs during prenatal period. Mann-Whitney test: * $p < 0.05$.

2) Associative learning test

Before conditioning learning, ventilation rate of cuttlefish exposed to cinnamon did not change (Figure 1: Wilcoxon test: C group: N=10 z=-1.067; p=0.2859; AS group: N=10 z=-0.980; p=0.3270; NS group: N=10 z=-1.169; p=0.8658). But the ventilation rate decrease when cuttlefish were exposed to ink (Figure 1: Wilcoxon test C group: N=10 z=-2.501; p=0.0124; AS group: N=10 z=-2.245; p=0.0247; NS group: N=10 z=-2.673; p=0.0075).

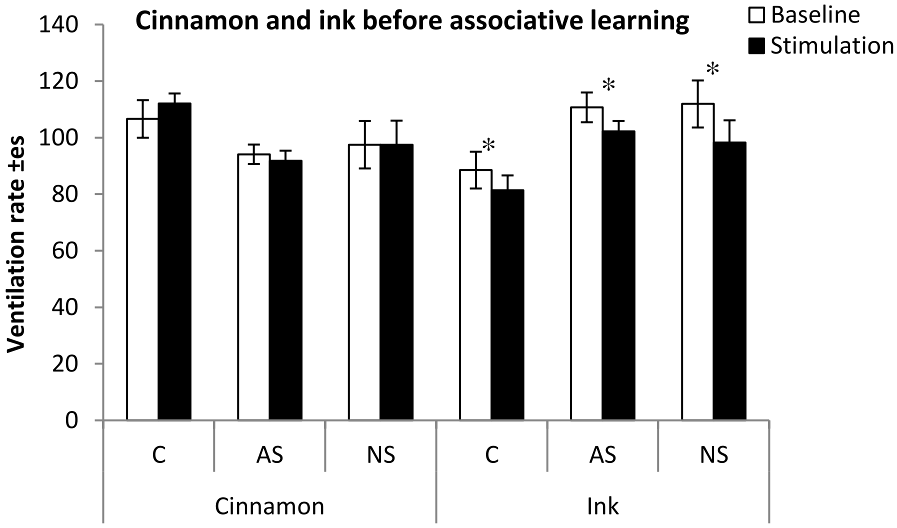

Figure 1: Ventilation rate before (white) and during (black) exposition to cinnamon and ink on unstressed cuttlefish (control: C) and stressed cuttlefish (Artificial Stress: AS and Natural Stress: NS). Wilcoxon test: * means a significant increase ($p<0.05$).

After conditioning learning, stressed and unstressed cuttlefish's ventilation rate decrease when they are exposed to cinnamon odour alone (Figure 2: Wilcoxon test: C group: N=10 z=-2.293; p=0.0218; AS group: N=10 z=-2.703; p=0.0069; NS group: N=10 z=-2.703; p=0.0069). We observed the same result when we replicated the experiment (Figure 2: Wilcoxon test: C group: N=10 z=-2.652; p=0.0080; AS group: N=10 z=-2.814; p=0.0049; NS group: N=10 z=-2.077; p=0.0378).

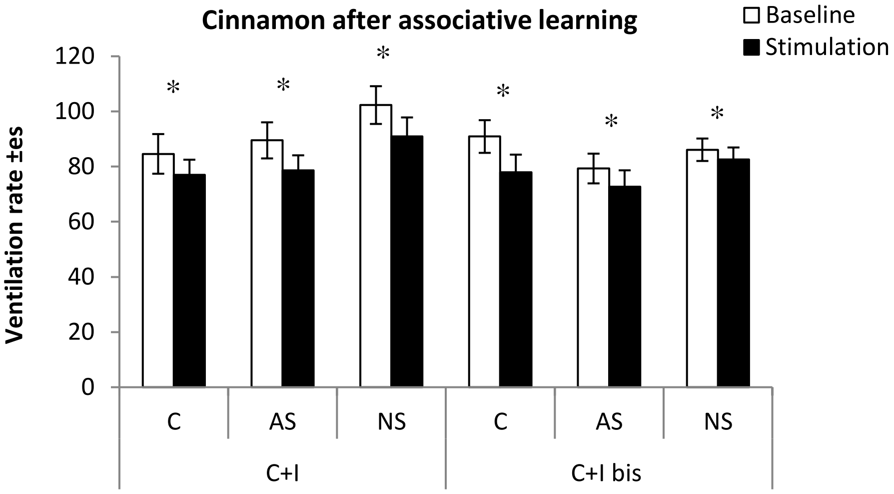

Figure 2: Ventilation rate before (white) and during exposition to cinnamon (black) after associative learning on unstressed cuttlefish (control: C) and stressed cuttlefish (Artificial Stress: AS and Natural Stress: NS). C+I: cuttlefish exposed to cinnamon coupled with ink and C+I bis: replicated experiment. Wilcoxon test: * means a significant increase ($p<0.05$).

Concerning control group "C + SW", stressed and unstressed cuttlefish's ventilation rate did not change when they are exposed to cinnamon odour alone (Figure 3: Wilcoxon test: C group: N=10 z=-0.943; p=0.3454; AS group: N=10 z=-0.631; p=0.5282; NS group: N=10 z=-0.339; p=0.7344). We observed the same results with control group "C + I_{differed}" (Figure 3: Wilcoxon test: C group: N=10 z=-1.332; p=0.1829; AS group: N=10 z=-0.841; p=0.4004; NS group: N=10 z=-1.122; p=0.2620).

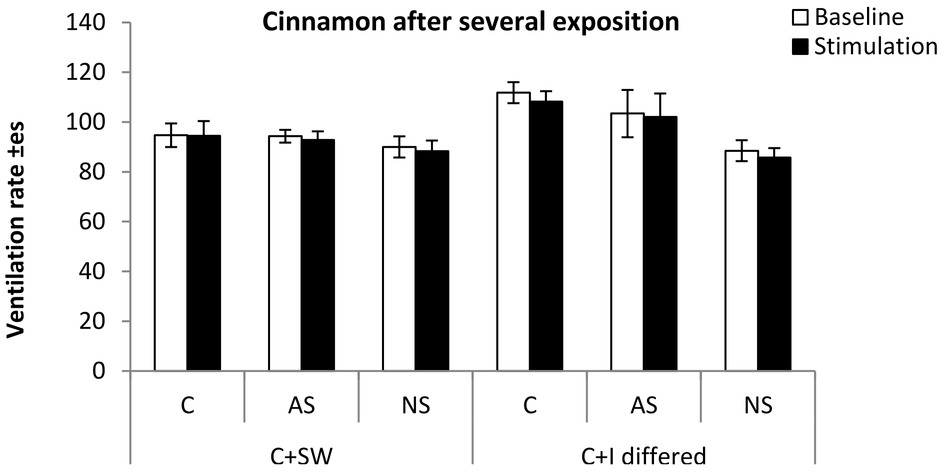

Figure 3: Ventilation rate before (white) and during exposition to cinnamon (black) after several exposition to cinnamon on unstressed cuttlefish (control: C) and stressed cuttlefish (Artificial Stress: AS and Natural Stress: NS). C+SW: cuttlefish exposed to cinnamon coupled with sea water and C+I differed: cuttlefish exposed to cinnamon coupled with ink 2 hours later. Wilcoxon test: * means a significant increase ($p<0.05$).

If associative learning spent just one day (instead of 4 days), stressed and unstressed cuttlefish's ventilation rate decrease when they are exposed to cinnamon odour alone (Figure 4: Wilcoxon test: C group: N=10 z=-2.703; p=0.0069; AS group: N=10 z=-1.994; p=0.0461; NS group: N=10 z=-2.550; p=0.0108). But if associative learning spent just one day and prenatal stress are strong (during all the day), only C and NS cuttelfish's ventilation rate decrease when they are exposed to cinnamon odour alone (Figure 4: Wilcoxon test: C group: N=10 z=-2.668; p=0.0076; AS group: N=10 z=-1.246; p=0.2127; NS group: N=10 z=-2.524; p=0.0.0116).

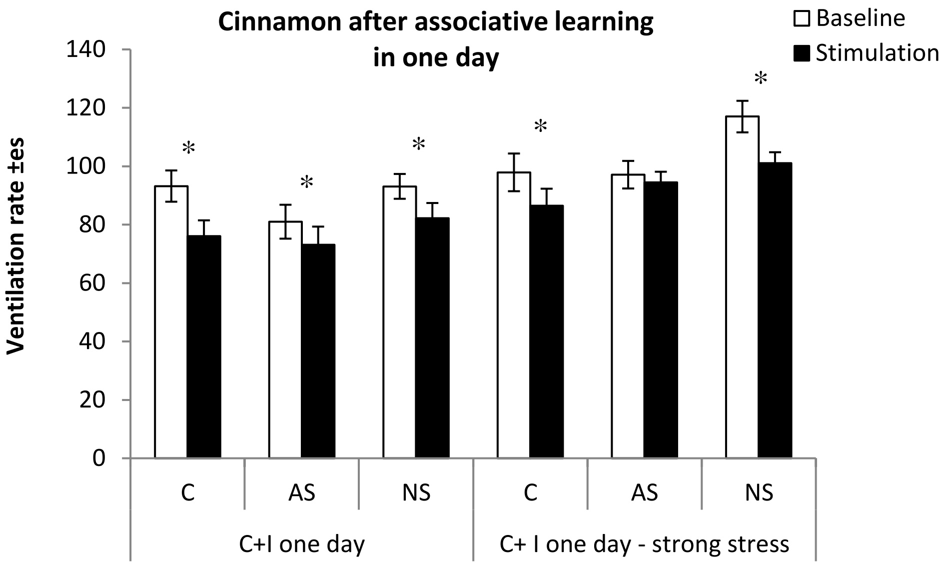

Figure 4: Ventilation rate before (white) and during exposition to cinnamon (black) after associative learning in one day on unstressed cuttlefish (control: C) and stressed cuttlefish (Artificial Stress: AS and Natural Stress: NS). C+I one day: cuttlefish exposed to cinnamon coupled with ink during one day and C+I one day – strong stress: cuttlefish exposed to cinnamon coupled with ink and in which stress were stronger than previously (all day prenatal stress exposure). Wilcoxon test: * means a significant increase (p<0.05).

DISCUSSION

1) Does prenatal stress influence simple learning abilities?

This study shows simple learning abilities in cuttlefish embryo. If they are exposed to crabs before hatching, 7-day-old cuttlefish prefer this item rather than shrimps, which are preferred by naïve individuals. This result is in accordance to the last experimentation (Darmaillacq, Chichery, & Dickel, 2006; Darmaillacq et al., 2004, 2008; Darmaillacq, Chichery, Shashar, et al., 2006; Darmaillacq, 2005; Dickel, Darmaillacq, Jozet-Alves, & Bellanger, 2013; Guibé et al., 2012). Then, we have shown that prenatal stress did not affect this prenatal learning. This result is very surprising in view of the current literature on the subject. In many taxa, prenatal stress has strong effects on the development of individuals and their ability to learn (Braastad, 1998; Weinstock, 2008, 2017). In human, stress early in life dramatically affects motor and cognitive development (Moss et al., 2017) and in particular the prefrontal cortex and executive function (Neuenschwander et al., 2018). Fetal exposure to prenatal maternal stress can have lifelong consequences (Barrett, Sefair, & O'Connor, 2017; Benoit et al., 2015; Bergh, Dahnke, & Mennes, 2018). In our study we expected to see learning differences between our groups so why do they learn similarly? Unlike previous studies, we are working on cuttlefish, an oviparous species. Unlike mammals, where embryo develops inside the maternal organism, oviparous embryos develop in an egg and therefore they do not perceive the same types of stress. In mammals, in case of chronic stress, the hypothalamic-pituitary-adrenal axis is hyperactivity and the secretion of glucocorticoids (cortisol, corticosterone) increases and becomes harmful for the body: decrease in energy resources and weakening of the immune system and modulate their behavioral development (Braastad, 1998; Miller et al., 2007). In oviparous, this maternal stress can affect embryos only during the laying phase. Indeed, the composition of the eggs can be affected by stressful living conditions of the laying female and can influence the development of young. Thus, stressed females in the laying phase can produce eggs richer in corticosterone and sex steroids (testosterone, androstenedione) and have more emotional young (Groothuis et al., 2005; Guibert et al., 2010; Welberg & Seckl, 2008). In our study, the eggs did not come from stressed mothers, but the stress was applied directly to the embryos. Although this stress may also have effects on the development of young people (Carlsen & Lickliter, 1999; Lickliter, 1994, 2000, 2005, 2011; Sleigh & Casey, 2014; Sleigh & Lickliter, 1995, 1996) it is possible that the effects of this embryonic stress are less strong than maternal stress and so do not have effect on simple learning. A second hypothesis is that the stress applied is not strong enough to have an impact on this simple learning. The experiments conducted by O'Brien et al. notes the effects of artificial stress on camouflage and predation and few effect of natural stress (O’Brien et al., 2018; O’Brien, Jozet-alves, et al., 2017).

2) Can prenatal stress influence learning abilities?

Our results clearly demonstrate that newly-hatched cuttlefish are able to do associative learning. This is the first time, to our knowledge, that this phenomenon has been demonstrated in new-hatch cuttlefish. As we can see in our data, at first, cuttlefish did not react to cinnamon odor while they did to ink (decrease of ventilator rate). And then, after association with ink, an alarm signal in cephalopod (Derby, 2014), they react to cinnamon alone. The stressful power of ink is transmitted to cinnamon. This phenomenon is possible in 4 days association but also in 1 day association. If we focus on control group we can see that sensitization to cinnamon did not occurs. Even after several expositions to cinnamon alone, cuttlefish did not react to this stimulus. Furthermore, the combination of the two stimuli must be relatively close in time as shown our results. The association is not possible if ink is presented two hours after cinnamon. In our data, we observed a decrease of VR. It can be compared to an attention phenomenon like in human infant (Richards & Casey, 1991) but also to a freezing-like behaviour, an adaptive response enabling adult cuttlefish to not be detected by predators (Bedore et al., 2015). Two recent studies, in the process of publication, have shown that this response is adaptive, thus allowing embryos to not be detected by predators (Mezrai, Arduini, et al., submitted; Mezrai, Chiao, et al., submitted). RV decreases significantly if the egg is transparent and embryos perceive a predatory odor.

The present data indicates also that prenatal stress can have an impact on learning depending of intensity and nature. On the first hand, when moderate stress is applied, all cuttlefish from group stressed and unstressed are capable of associative learning. On the other hand, when the stress becomes stronger, stressed embryos with light (AS) are not able to do this complex learning in one day. Did light stress disrupt memory skills? Stress during prenatal period can have profound effects in humans. For example, it has been linked to disorders in behavior, cognition and emotion, such as attention deficit hyperactivity disorder (ADHD), post-traumatic stress disorder (PTSD), depression, anxiety and schizophrenia (Charil, Laplante, Vaillancourt, & King, 2010; Weinstock, 2017). Conversely, some stressors are essential and can prepare embryos for their future postnatal life. Perceiving predator odors before birth/hatch can give advantages for their survival. A previous study on cuttlefish has shown that moderate artificial stress can improve prey detection (predation) but impair the camouflage (primary defense) in contrast to moderate natural stress (O'Brien, Jozet-alves, et al., 2017).

Many studies also highlight a change in sensory development when there are prenatal stimulation before birth/hatching.As the establishment of the various sensory systems follow a chronological order, and invariant with some degree of overlap, certain environmental stimuli can reorganize the development of other sensory systems. In the literature, Gottlieb and then Lickliter and collaborators have frequently demonstrated this phenomenon by using sub- or over-environmental stimulation (Carlsen & Lickliter, 1999; Gottlieb, Tomlinson, & Radell, 1989; Honeycutt & Lickliter, 2001; Jaime & Lickliter, 2006; Lickliter, 1994, 2000; Lickliter & Lewkowicz, 1995; Sleigh & Lickliter, 1996, 1998). For example, visual stimulation can influence the postnatal hearing preferences in bobwhite quail. Chicks incubated in the dark preferentially directed towards to specific maternal call at 24h, 48h, 72h and 96h old even if they are associated with non-specific visual cues (scaled quail - *Callipepla squamata*). Conversely, individuals incubated in the light are oriented towards specific vocalizations (associated with non-specific visual cues) only to 24h and 48h of life. These individuals show no preference to 72 hours of life and are oriented towards specific female (associated with non-specific vocalizations) to 96 hours of life (Lickliter, 1994). This experiment highlights the effect of prenatal visual stimuli on the hierarchy of indices used in postnatal social context. Individuals receiving early visual stimulation use visual cues in social recognition. *Sepia officinalis* Cuttlefish embryonic development follow the same chronological order: they can smell before they can see (Mezrai, Chiao, et al., submitted; Romagny et al., 2012). It is possible that the light (used by stressing the embryo) have stimulated visual development to the detriment of olfactory modality. SA cuttlefish would then have more trouble on associative learning related to the olfactory modality. In order to verify this hypothesis, it would be interesting to replicate this experiment and using only the visual modality (non-predatory video associated with cuttlefish ink, for example).

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Bilan du chapitre 5 :

Au cours de la **première partie** nous avons montré que *Sepia pharaonis* est capable d'empreinte alimentaire. De plus, il a été mis en évidence que le stress prénatal artificiel (lumière forte) ne semble pas avoir d'impact sur cet apprentissage simple contrairement au stress naturel (odeur de prédateur) qui semble le perturber (même après une exposition à des gammares pendant la période prénatale les jeunes de 7 jours préfèrent les crevettes à ces derniers). Les effets du stress prénatal sur l'apprentissage associatif n'ont cependant pas pu être testés au cours de ces expériences. En effet, les embryons ayant été exposés à des stress naturel ou artificiel ne répondent pas au stimulus inconditionnel. Il faut poursuivre ces expériences avant de savoir si cette « non-réponse » à l'encre est due à la procédure de stress ou bien à un problème méthodologique.

La **deuxième partie** de ce chapitre, réalisée chez *Sepia officinalis*, montre que le stress prénatal n'affecte pas les capacités d'empreinte alimentaire chez la seiche *Sepia officinalis*. Les individus stressés et non stressés exposés aux crabes avant l'éclosion préfèrent significativement les crabes aux crevettes après l'éclosion. De plus, nous avons vu que la seiche est capable d'apprentissage associatif dès son premier jour de vie postnatale. Le RV des jeunes seiches diminue significativement face à la cannelle après le conditionnement classique. Une seule exposition suffit même pour induire une réponse à la cannelle. Le stress prénatal, lorsqu'il est modéré, n'influence pas ces capacités d'apprentissage. Cependant, lorsqu'il est artificiel et plus fort il perturbe cet apprentissage.

Chapitre 6

Discussion générale
&
Perspectives

Chapitre 6 – Discussion générale et perspectives

Ce travail de thèse avait pour objectif de mettre en évidence les capacités de perception, et d'apprentissage chez les embryons d'un céphalopode : la seiche. Les embryons de cet ovipare sont relativement accessibles puisqu'ils se développent dans des œufs plus ou moins transparents. Nous avons également voulu savoir si les stimulations environnementales, lorsqu'elles sont fortes peuvent induire un stress prénatal et ainsi moduler les apprentissages périnatals des seiches. Cette étude a été réalisée chez *Sepia officinalis* (en France) et *Sepia pharaonis* (à Taiwan) dans le but de comparer le développement de ces deux espèces très semblables (morphologie et comportement) mais qui se développent dans des œufs dont la transparence de la capsule diffère (*Sepia officinalis* se développe dans des œufs noirs et *Sepia pharaonis* dans des œufs blancs transparents). Les résultats ont montré que les systèmes chimiosensoriel et visuel des deux espèces sont fonctionnels avant l'éclosion (**chapitre III**) et que les seiches peuvent reconnaitre de façon innée des dangers (**chapitre III et VI**). De plus, les embryons de seiche sont capables d'apprendre à reconnaitre de nouveaux dangers grâce à des apprentissages associatifs (**chapitre VI**). Enfin, nous avons montré que le stress embryonnaire mesuré au cours de ce travail de thèse semble avoir des effets très modérés sur les capacités d'apprentissage périnatal (**chapitre V**).

I. Capacité de perception et d'apprentissage embryonnaire

1) Calendrier de développement des systèmes sensoriels

Le développement des systèmes sensoriels des Vertébrés se développent pendant la période prénatale en suivant une séquence chronologique et invariante (système tactile et vestibulaire, système olfactif et gustatif, système auditif et enfin système visuel : Bremner et al., 2012; Carlsen & Lickliter, 1999; Gottlieb, 1968, 1976a; Hepper, 2015; Lickliter, 2000; Romagny, Darmaillacq, Guibé, Bellanger, & Dickel, 2012; Spreen, Risser, & Edgell, 1995). Chez la seiche *Sepia officinalis*, Romagny et ses collaborateurs ont montré en 2012 que les embryons répondent à des odeur de prédateur au stade 23 et à de la lumière au stade 25 : une stimulation induisait une augmentation de la fréquence de contraction du manteau (Romagny et al., 2012). Cependant au cours de leur étude, seuls 3 stades ont été analysés : 23, 25 et 30. Leurs résultats n'indiquent donc pas le moment exact de la première réponse. De plus, la fréquence de contraction du manteau est un paramètre comportemental surtout observable en cas de très forte stimulation. Nous avons alors mis au point un protocole afin de mesurer le rythme ventilatoire (RV) sur des embryons le plus tôt possible et ce jusqu'à l'éclosion. Contrairement aux contractions du manteau, la mesure du RV est plus fine et est détectable beaucoup plus tôt dans le développement des embryons (du stade 21 au stade 30 pour *Sepia pharaonis* et du stade 22 au stade 30 pour *Sepia officinalis*). Par ailleurs, ce paramètre physiologique peut aussi être utilisé chez les juvéniles (Boal & Golden, 1999; Boal & Ni, 1996).

Au cours de ce travail de thèse, nous avons alors mis en évidence que les systèmes sensoriels se développent pendant la période prénatale mais que cette séquence chronologique peut changer en fonction de l'espèce (**chapitre III** – article 1). Chez *Sepia officinalis*, le système chimiosensoriel est fonctionnel avant le système visuel alors que chez *Sepia pharaonis* le système visuel va être fonctionnel avant le système chimiosensoriel. Notre étude a effectivement montré que les embryons de cette espèce, se développant dans des œufs transparents, répondent à des stimuli lumineux au stade 22 alors qu'ils ne répondent qu'au stade 23 à des odeurs de prédateur. Inversement les embryons de *Sepia officinalis* répondent à des odeurs de prédateur au stade 22 et à la lumière au stade 24 (Mezrai, Chiao, et al., soumis). Ces résultats peuvent être expliqués par des caractéristiques écologiques différentes entre les deux espèces. Les œufs de *Sepia pharaonis* sont transparents et pondus près des fonds sous des roches ou des coraux (Gabr, Hanlon, Hanafy et El-Etreby, 1998). Pouvoir voir plus tôt semble être un avantage important dans la reconnaissance des proies et des prédateurs. Inversement, les œufs de *Sepia officinalis* sont noirs et ils sont pondus sur des algues ou d'autres supports verticaux (Boletzky, 1983). Il pourrait donc être important pour eux d'apprendre les signaux olfactifs plus tôt. Nous pouvons aussi imaginer une hypothèse alternative. Comme les œufs de *S. pharaonis* sont transparents et que la lumière traverse la capsule, le système visuel est stimulé davantage. Comme chez les oiseaux, ces premières stimulations visuelles pourraient favoriser le développement du système visuel et/ou moduler le développement du système olfactif (Carlsen et Lickliter, 1999; Gottlieb et al., 1989; Honeycutt et Lickliter, 2001; Jaime et Lickliter, 2006; Lickliter, 1994, 2000. ; Lickliter et Lewkowicz, 1995; Sleigh et Lickliter, 1998; Sleigh et Lickliter, 1996). Cependant, une expérience complémentaire menée chez *Sepia pharaonis* montre que l'incubation dans l'obscurité totale ne modifie pas la chronologie du développement des systèmes sensoriels de réponses à la lumière au stade 22 et à l'odeur de prédateur au stade 23 (Mezrai unpublished data). Ce changement dans la chronologie serait par conséquent plus un changement au sein de l'histoire de l'espèce (évolutif) plutôt que liée au développement ontogénétique de l'individu.

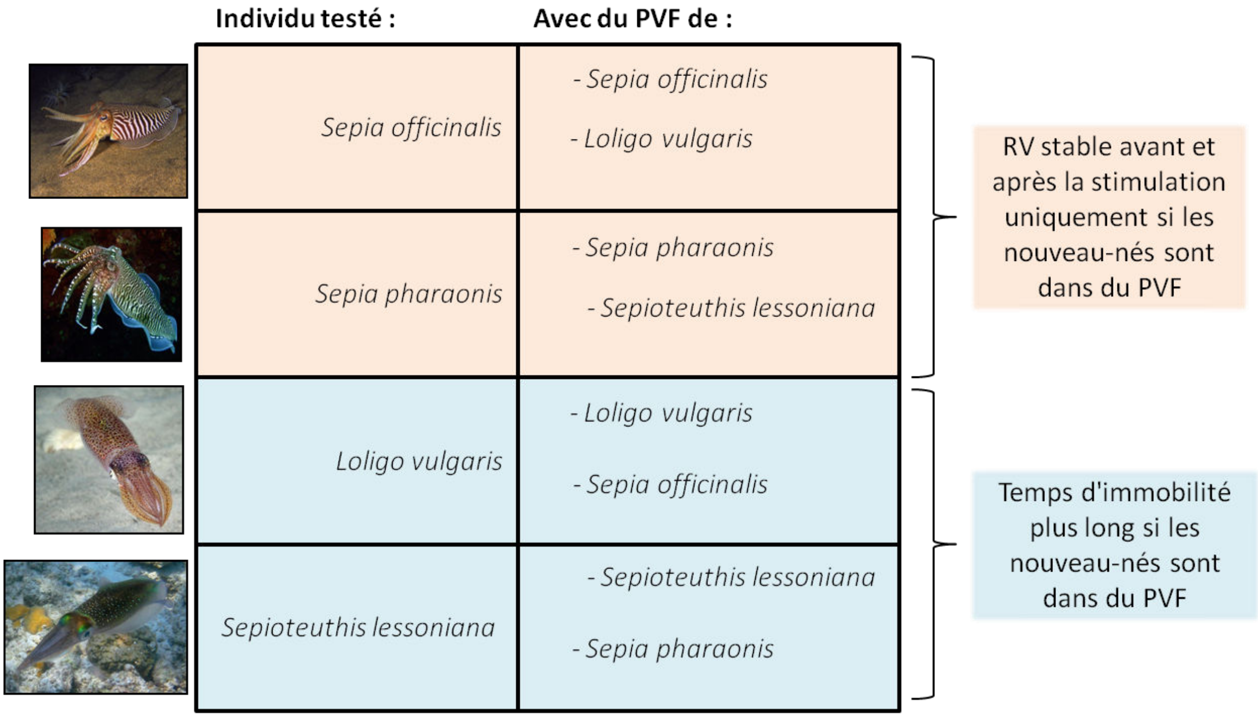
2) *Un tranquillisant dans le liquide périvitellin des seiches ?*

Un autre point d'intérêt dans nos résultats (**chapitre III** – article 1) est la disparition de la réponse au stade embryonnaire tardif en cas de stimulation induisant une augmentation du rythme ventilatoire. Effectivement, au stade 30, qui est le dernier stade avant l'éclosion, le rythme ventilatoire des embryons reste stable suite à une stimulation lumineuse chez les deux espèces et suite à une exposition à une odeur de prédateur chez *Sepia officinalis* (il n'y a plus d'augmentation du rythme ventilatoire). Par contre, chez *Sepia pharaonis*, le rythme ventilatoire diminue tout de même au stade 30 suite à l'exposition à l'odeur de prédateur. Ce résultat, tout à fait surprenant, peut être expliqué par la présence potentielle d'un tranquillisant naturel dans l'œuf à la fin du développement. En effet, chez le calmar (*Loligo vulgaris*), il a été démontré que le liquide périvitellin (PVF) contient ce tranquillisant qui entrerait en action à la fin du développement embryonnaire (Marthy et al., 1976; Weischer & Marthy, 1983). Il préviendrait les éclosions prématurées et réduirait la détection des embryons par les prédateurs, offrant ainsi des conditions optimales pour la survie des juvéniles (Boletzky, 2003; Marthy, Hauser et Scholl, 1976; Weischer et Marthy, 1983). Il est possible que ce tranquillisant soit également présent dans les œufs de seiche. Sa présence pourrait expliquer pourquoi les embryons ne répondent plus à l'odeur de prédateur ni à la lumière au cours des derniers stades de développement. Afin de répondre à cette interrogation, des premières expériences ont été menées au cours de cette thèse montrant un effet potentiel de ce tranquillisant dans les œufs des céphalopodes. Pour ce faire, nous avons prélevé et testé le PVF tardif de différents céphalopodes sur des nouveau-nés de différentes espèces :

- Le PVF de *Sepia officinalis* a été testé sur les seiches *Sepia officinalis* et sur les calmars *Loligo vulgaris* (deux espèces présentes en France).
- Le PVF de *Sepia pharaonis* a été testé sur les seiches *Sepia pharaonis* et sur les calmars *Sepioteuthis lessoniana* (deux espèces présentes à Taiwan).
- Le PVF de *Loligo vulgaris* a été testé sur les calmars *Loligo vulgaris* et sur les seiches *Sepia officinalis* (deux espèces présentes en France).
- Le PVF de *Sepioteuthis lessoniana* a été testé sur les calmars *Sepioteuthis lessoniana* et sur les seiches *Sepia pharaonis* (deux espèces présentes en France).

Les nouveau-nés expérimentaux étaient placés dans de l'eau de mer mélangée à du PVF (50% de PVF et 50% d'eau de mer) alors que les nouveau-nés contrôles étaient dans de l'eau de mer.

Tous les individus ont été testés individuellement sous une loupe binoculaire. Pour les seiches, nous avons appliqué une stimulation lumineuse suite à une période de familiarisation de 5 minutes et nous avons mesuré le rythme ventilatoire avant et après cette stimulation. Pour les calmars, nous avons simplement mesuré l'activité locomotrice des individus du groupe expérimentale et contrôle. Les résultats, présentés sur la Figure 40, montrent que chez les seiches, seuls les nouveau-nés témoins ont un rythme ventilatoire qui augmente significativement après une stimulation lumineuse. Chez les calmars, le temps d'immobilité des individus au cours de ce test était significativement plus long si les individus sont placés dans du PVF. Ces résultats appuient l'hypothèse qu'il y aurait un tranquillisant dans les œufs des céphalopodes comme l'ont décrit Marthy et ses collaborateurs (Marthy et al., 1976; Weischer & Marthy, 1983).



Individu testé :	Avec du PVF de :
Sepia officinalis	- *Sepia officinalis* - *Loligo vulgaris*
Sepia pharaonis	- *Sepia pharaonis* - *Sepioteuthis lessoniana*
Loligo vulgaris	- *Loligo vulgaris* - *Sepia officinalis*
Sepioteuthis lessoniana	- *Sepioteuthis lessoniana* - *Sepia pharaonis*

RV stable avant et après la stimulation uniquement si les nouveau-nés sont dans du PVF

Temps d'immobilité plus long si les nouveau-nés sont dans du PVF

Figure 40 : Schéma récapitulatif des résultats obtenus au cours des études sur l'effet tranquillisant du PVF des céphalopodes. PVF : liquide périvitellin ; RV : rythme ventilatoire.

Ce liquide transparent est visqueux, hypertonique et est présent en quantité plus importante lors des derniers stades embryonnaires. Au cours de sa thèse, Cornet et ses collaborateurs ont identifié 17 protéines dans ce liquide prélevé à la fin du développement embryonnaire des seiches *Sepia officinalis* (Cornet, 2015). Il comporterait des protéines de structures (apparentées à la tectorine ou au collagène-α1) ; une protéine caractéristique de nombreux mucus impliquée dans la protection des épithéliums (une mucine) ; une protéine retrouvée dans la capsule d'œufs de calmar où elle joue un rôle de phéromone (une β-microséminoprotéine) ; des protéines connues pour leurs potentiels antibactériens (une Waprin-like par exemple) ; etc… (Cornet, 2015). Finalement, même si nous commençons à connaitre un peu le rôle de ce liquide (tranquillisant : Marthy et al., 1976; Weischer & Marthy, 1983 ; protection de l'embryon vis-à-vis des pathogènes : Cornet, 2015) nous ignorons encore comment et à quel moment ce tranquillisant se retrouverait dans l'œuf (synthèse dans l'œuf au cours du développement embryonnaire ? transmission maternelle ?).

3) Reconnaissance innée des dangers chez l'embryon

Nous avons également montré qu'au cours de cette période précoce, les embryons de seiches sont capables de reconnaitre des prédateurs sans expérience au préalable **(chapitre III)**. Chez *Sepia officinalis*, le rythme ventilatoire augmente du stade 22 au stade 29 en cas d'exposition à une odeur de prédateur, mais ne change pas si elle est exposée à une odeur de non-prédateur. Par ailleurs, le rythme ventilatoire de *Sepia officinalis* augmente lorsque les embryons sont exposés à des proies (petits crabes et crevettes). Ces résultats sont en accord avec l'étude de Boal and Ni (1996) où des juvéniles ont un rythme ventilatoire qui augmente face à des proies, des conspécifiques familiers et des non-familiers (Boal & Ni, 1996), Ici, l'augmentation du rythme ventilatoire peut être interprétée comme un phénomène d'attention visuelle ou olfactive face à des proies ou des prédateurs.

Chez *Sepia pharaonis*, suite à une exposition à une odeur de prédateur, le rythme ventilatoire des embryons augmente aux stades 23 et 24 et diminue à partir du stade 25 et ce jusqu'au stade 30. En parallèle, le rythme ventilatoire des embryons de *Sepia pharaonis* augmente au stade 23 face à des odeurs de non-prédateurs mais ne change pas à partir du stade 25. Il est alors probable que *Sepia pharaonis* perçoit d'abord les odeurs sans les reconnaitre (stades 23 et 24) et qu'elle les distingue plus tard (au stade 25). L'augmentation refléterait une détection de l'odeur alors que la diminution indiquerait une reconnaissance. En effet, la diminution du rythme ventilatoire peut être comparée à un comportement de « freezing », qui est une réponse adaptative diminuant la probabilité de se faire détecter par les prédateurs chez les seiches adultes (Bedore et al., 2015). Ainsi, les adultes de *Sepia officinalis* réduisent leurs signaux bioélectriques *via* un comportement de freezing en réponse à une exposition visuelle de prédateur (requins marteaux, Bedore et al., 2015). Le fait que *Sepia pharaonis* ait un rythme ventilatoire qui diminue et que *Sepia officinalis* ait un rythme ventilatoire qui augmente suite à une exposition à une odeur de prédateur peut s'expliquer par la différence de capsule. La capsule d'œuf de *Sepia officinalis* est noire et donc la diminution du rythme ventilatoire chez cette espèce peut ne pas être nécessairement adaptative contrairement à *Sepia pharaonis* où les embryons sont visibles de l'extérieur. Afin de vérifier cette hypothèse, il faudrait étudier une troisième espèce de seiche qui cache ses œufs non pas en pondant des œufs noirs mais en les recouvrant de sable comme *Sepia lycidas* ou *Sepia esceulenta.* Chez ces deux espèces, les œufs sont blancs mais la femelle va déposer du sable sur la couche la plus externe de la capsule de l'œuf (Natsukari & Tashiro, 1991). Au fur et à mesure du développement embryonnaire l'œuf va grossir en volume et le sable va alors se détacher (observation personnelle chez *Sepia lycidas*) et les embryons seront alors visibles de l'extérieur.

Les résultats obtenus au sein du **chapitre III** soulignent des capacités de reconnaissance innée d'un autre type de danger : de l'encre de seiche. Effectivement, comme Derby l'a décrit dans sa revue (2014), l'encre est un signal d'alarme fort chez les céphalopodes et ses effets seraient interspécifiques (pour dissuader les prédateurs) mais aussi intraspécifiques (pour prévenir les congénères du danger ; Derby, 2014). Au cours de nos expériences les embryons de *Sepia officinalis* et *Sepia pharaonis* ne réagissent pas à l'odeur de congénères blessés contrairement aux amphibiens qui utilisent cet indice comme signal d'alarme (Ferrari & Chivers, 2009a, 2009b; Ferrari et al., 2010; Garcia et al., 2017). Par contre, lorsqu'ils sont exposés à de l'encre de seiche, leur rythme ventilatoire diminue significativement suite à cette stimulation (*Sepia officinalis* : stade 29 et *Sepia pharaonis* : stade 25). Cette réponse, également semblable au « freezing », est observable si l'encre est présentée de façon visuelle (nuage noir) ou chimique (odeur d'encre). Contrairement aux signaux d'alarme utilisés par les autres animaux, l'encre de seiche est la seule à pouvoir être détectée grâce à deux systèmes sensoriels différents (utilisation d'un signal d'alarme sonore ou visuel chez les primates non-humains : Seyfarth, Cheney, & Marler, 1980 ; ou d'une phéromone d'alarme chez les poissons (Commens & Mathis, 1999; Sorensen & Wisenden, 2015). Les embryons de seiche ont donc la capacité de percevoir et de reconnaitre ce signal d'alarme en utilisant plusieurs modalités sensorielles leur permettant d'anticiper l'arrivée des prédateurs à des distances variables de jour comme de nuit. En effet, *Sepia officinalis* est une espèce nocturne en été et en automne, devenant diurne en décembre et arythmique en janvier (Oliveira, Grano-Maldonado, Gonçalves, Frias, & Sykes, 2017). Ce changement de rythme chronobiologique est en relation étroite avec les principaux synchroniseurs environnementaux comme la photopériode et la température (Oliveira et al., 2017). La capacité d'utiliser plusieurs indices visuels ou olfactifs va alors permettre aux seiches de reconnaitre et ainsi éviter les prédateurs à tout moment de la journée. Malgré de nombreuses études sur ce signal d'alarme, nous ignorons encore ce qui induit une réponse chez les céphalopodes. Serait-ce vraiment la dopamine comme chez le calmar *Loligo opalescens* (Gilly & Lucero, 1992; Lucero, Farrington, & Gilly, 1994) ? Ou bien serait-ce le mucus présent en quantité significative dans les jets d'encre ? Comme le décrit Derby dans sa revue, le mucus est très peu étudié dans la littérature et nous ignorons quels sont les produits chimiques présents dans ces sécrétions (Derby, 2014). Il est probable qu'il s'agisse de beaucoup plus que du mucus, qui, avec la poche à encre, produit un riche éventail de molécules bioactives (Derby, 2014).

4) Reconnaissance acquise des dangers chez l'embryon

La reconnaissance « innée » des prédateurs peut conférer des avantages importants dans la survie des jeunes puisque les comportements défensifs qui en résulteront seront très rapides car indépendants de l'apprentissage. Cependant, dans un environnement pouvant changer constamment, il est important pour les embryons de pouvoir s'adapter en apprenant à reconnaitre de nouveaux dangers. Cet apprentissage va être important pour l'embryon après sa naissance/éclosion car il va lui permettre d'adopter des comportements anti-prédateurs efficaces ; ce qui est d'autant plus vrai pour les espèces qui se développent sans soins parentaux postnatals. Cet apprentissage va également être primordial avant sa naissance/ éclosion puisque, même si l'embryon est « protégé » par la capsule de son œuf, il peut tout de même être victime de prédation. Utiliser un comportement anti-prédateur approprié (e.g. diminution de son activité et de son rythme ventilatoire) peut lui permettre d'éviter la prédation.

L'apprentissage prénatal peut se faire par association entre différents événements comme cela a été précédemment montré chez l'embryon de rat (Smotherman, 2002; Smotherman & Robinson, 1985, 1988, 1992; Smotherman et al., 1991). Pourtant, l'apprentissage associatif est très rarement étudié directement chez l'embryon en raison de son inaccessibilité. La plupart des études mettent indirectement en évidence ces capacités d'apprentissage associatif chez l'embryon : les apprentissages sont faits avant l'éclosion mais le test n'est réalisé qu'après (e.g. apprentissage associatif avec une odeur de prédateur couplée à un signal d'alarme : Ferrari & Chivers, 2009a, 2009b, 2010; Ferrari et al., 2010; Garcia, Urbina, Bredeweg, & Ferrari, 2017). Chez les céphalopodes, aucune étude ne s'est intéressée à ces capacités cognitives précoces, notre étude (**chapitre IV**), est alors la première à entreprendre cet apprentissage associatif chez l'embryon. Pour la première fois un protocole de conditionnement classique a été réalisé et validé chez deux espèces de seiche (*Sepia officinalis* et *Sepia pharaonis*). Sur la base des résultats du **chapitre III**, notre protocole consistait à associer un stimulus neutre avec un danger (de l'encre de seiche ou une odeur de prédateur par exemple) en utilisant la modalité visuelle et/ou chimique. Nos résultats soulignent l'importance des stimulations environnementales prénatales des embryons de seiche leur permettant ainsi d'augmenter leur chance de survie pendant leur vie postnatale.

Apprendre dans un environnement constamment en changement est primordial pour la survie des individus. Ces capacités précoces seront ainsi bénéfiques pour les jeunes en cas d'arrivée de nouveaux prédateurs (des espèces envahissantes par exemple) ou en cas de changement de régime alimentaire des prédateurs. En effet, chez les poissons, la flexibilité du comportement alimentaire est un trait adaptatif important car la plupart des environnements naturels changent dans le temps et dans l'espace (Dill, 1983; Vehanen, 2003; Wright, Eberhard, Hobson, Avery, & Russello, 2010). Dans notre étude, les embryons de seiche apprennent en 4 jours, mais nous avons montré dans le **chapitre 5** qu'ils peuvent apprendre en 2 jours (*Sepia pharaonis*) ou en 1 jour (*Sepia officinalis*) lorsqu'une odeur de cannelle est associée à de l'encre (une seule présentation).

Les capacités cognitives précoces que nous avons soulignées au cours de nos tests d'apprentissage nous permettent de (1) prouver que la seiche est aussi capable d'apprendre dans l'œuf et que cet apprentissage est adaptatif et primordial pour la survie des jeunes et (2) réutiliser ce test cognitif très simple à mettre en place pour tester les effets de l'environnement sur le développement des individus (e.g. étudier les effets du stress sur les capacités d'apprentissage).

II. Effet du stress sur les apprentissages prénatals

Comme nous l'avons détaillé dans l'introduction (**chapitre I**), un des objectifs de ce travail de thèse étaient d'étudier les effets du stress embryonnaire sur les capacités d'apprentissage chez la seiche (projet ANR -13- BSV7- 0002 PReSTO'Cog 2014-2017). Ces effets ne sont que très peu étudiés dans la littérature. La très grande majorité des expériences porte sur les effets du stress maternel et non du stress perçu directement par l'embryon. Pour mettre en évidence ces effets sur la seiche, trois groupes ont été étudiés : un groupe non stressé (contrôle) ; un groupe stressé avant l'éclosion avec de la lumière forte (SA) et un groupe stressé avant l'éclosion avec une odeur de prédateur (SN). Puis, deux protocoles expérimentaux ont été utilisés : (1) le protocole d'empreinte alimentaire décrit par Darmaillacq et collaborateurs en 2008 et (2) le protocole de conditionnement classique que nous avons mis au point chez les deux espèces (**chapitre IV** et **V**). Néanmoins, les essais réalisés chez *Sepia pharaonis* ont été non concluants puisqu'il s'est avéré compliqué de tester leurs capacités de réponses, d'apprentissage et les effets du stress dans les temps impartis (expériences menées au cours du stage de Master 2 par Iris Lemercier). Des réplicas sont alors nécessaires pour tirer des conclusions sur cette espèce.

1) Stress et empreinte alimentaire

Comme nous l'avons vu précédemment, l'empreinte est une forme d'apprentissage simple caractérisée par l'établissement d'une préférence durable pour un objet au cours d'une période sensible limitée, généralement au début du développement (Bolhuis, 1991; Lorenz, 1937; Sluckin, 2017). Cet apprentissage simple a déjà été validé chez les nouveau-nés de seiche *Sepia officinalis* dans des études menées par Darmaillacq et ses collaborateurs (Darmaillacq, Chichery, & Dickel, 2006). Cette étude met en évidence une empreinte alimentaire persistante au cours de la première semaine de vie qui se met en place pendant une période sensible et sans renforcement (Darmaillacq, Chichery, & Dickel, 2006). Ce protocole a également été utilisé chez les embryons montrant que les préférences alimentaires ultérieures des jeunes sont aussi influencées par l'expérience prénatale (Darmaillacq et al., 2008).

Durant ce travail de thèse, nous avons montré que le stress prénatal n'affecte pas cet apprentissage simple chez *Sepia officinalis*. Les embryons stressés et non stressés préfèrent significativement les crevettes comme premières proies mais ces préférences changent pour les crabes s'ils sont exposés à ces derniers pendant au moins une semaine avant l'éclosion. Chez

Sepia pharaonis, nous avons d'abord mis en évidence des capacités d'apprentissage simple en utilisant le protocole d'empreinte alimentaire (**chapitre V**). Ainsi, les jeunes seiches préfèrent les crevettes aux gammares comme premières proies mais cette préférence innée disparait si les embryons sont exposés aux gammares pendant au moins une semaine avant l'éclosion. Puis, Iris Lemercier a montré que le stress naturel (odeur de prédateur) pouvait potentiellement perturber cette empreinte alimentaire. Cependant, ces résultats sont à prendre avec prudence puisqu'elle n'a pas montré de préférence significative pour les crevettes chez les individus non stressés. Les effets du stress embryonnaire semblent être très modérés chez la seiche. Nous pouvons alors émettre deux hypothèses. La première est que le stress appliqué aux embryons n'est pas suffisamment fort pour perturber leurs capacités d'apprentissages. La deuxième hypothèse est que cet apprentissage simple est beaucoup trop robuste pour que ce type de stress puisse avoir un effet sur lui. Afin de vérifier ces hypothèses il est nécessaire de répliquer cette expérience en appliquant un stress plus fort sur les embryons (appliquer le stress pendant toute une demi-journée par exemple).

2) Stress et apprentissage associatif

Au cours du **chapitre V**, les capacités d'apprentissage associatif des seiches des groupes stressés et non stressés ont été évaluées en utilisant un protocole de conditionnement classique. Au cours de celui-ci, les nouveau-nés ont été exposés à une odeur de cannelle couplée à de l'encre de seiche pendant 4 jours. Puis, au 5$^{\text{ème}}$ jour, ils ont tous été testés avec une odeur de cannelle seule et nous avons observé que tous les juvéniles ont un rythme ventilatoire qui diminue significativement suite à cette exposition de cannelle seule. Ce résultat ne met pas en évidence de phénomène de sensibilisation puisque les seiches ne répondent pas à l'odeur de cannelle après les expositions à la cannelle et à l'encre en différée dans le temps. Ce protocole met bien en évidence des capacités d'apprentissages associatifs chez le nouveau-né. Cependant, ici encore nous n'avons pas réussi à démontrer de différence d'apprentissage entre les individus des groupes stressés et non stressé. Là encore il est possible que le stress ne soit pas assez fort pour moduler ces apprentissages. Nous avons donc répliqué l'expérience en réalisant le conditionnement classique en 1 essai et nous avons appliqué les stress prénatals sur les demi-journées (stress plus forts). Suite à ce changement, seuls les jeunes ayant été stressés avec la lumière, ne répondent pas à l'odeur de cannelle. Le stress artificiel, semble donc avoir affecté les capacités d'apprentissage associatif.

Les résultats obtenus chez *Sepia officinalis* n'ont pas pu être comparés à ceux de *Sepia pharaonis* puisqu'au cours des premiers essais, les seiches des groupes stressés avaient un rythme ventilatoire stable lorsque qu'elles étaient exposées à l'encre. Ce stimulus ne pouvait alors pas être utilisé comme stimulus inconditionnel. Il est cependant possible que le stress ait une influence sur la réponse des seiches à l'encre en modulant leur réponse émotionnelle comme c'est le cas chez la caille japonaise (Mezrai et al, soumis). Afin de vraiment savoir si les individus ont été stressés ou non il faudrait entreprendre des dosages hormonaux (dosage de corticostérones sur les embryons des différents groupes stressés et non stressés).

3) Effet du stress chez les espèces ovipares

Comme nous l'avons décrit dans le **chapitre I**, le stress maternel a de forts effets chez toutes les espèces étudiées au sein de ce projet ANR : des éclosions prématurées (O'Brien et al., 2018, 2017) ; des modification de la réactivité émotionnelle et de l'hyperactivité (De Haas et al., 2017) et des déficits cognitifs et cérébraux (Charrier et al., en préparation; Colson et al., 2017) (cf. Figure 41). De plus ces effets ont des conséquences à long terme puisqu'ils peuvent perdurer sur la seconde génération (Charrier et al., en préparation). Ces effets maternels seraient dus aux hormones qui se transmettent de la mère aux jeunes. Effectivement, chez les oiseaux il a déjà été démontré que la corticostérone et les stéroïdes sexuels (testostérone, androstènedione) se retrouvent dans les œufs et que leur quantité sera modulée par le stress vécu par la femelle pondeuse (Groothuis et al., 2005; Guibert et al., 2010; Henriksen et al., 2011; Houdelier et al., 2011). Chez les céphalopodes, une étude a également montré des niveaux élevés de corticostérone mesurés dans les fèces du poulpe géant du Pacifique (*Enteroctopus dofleini*) après une injection d'hormone surrénale (ACTH) (Larson & Anderson, 2010). Cependant, O'Brien, au cours de sa thèse a fait des essais de mesure de cette hormone chez des seiches stressées et non stressées pendant la période de ponte mais les résultats n'ont pas été concluants puisqu'aucune différence n'a été observée entre les seiches stressées et non stressées.

Quant aux stress embryonnaires, leurs effets sont plus modérés et la nature du stress (artificiel ou naturel) est cruciale. Les stress embryonnaires naturels altèrent les capacités cognitives et modulent la réactivité émotionnelle chez la caille, le poisson zèbre et la truite arc-en-ciel (Mezrai et al., en préparation ; Poisson et al., 2017). De plus, ils vont moduler l'activité locomotrice des poissons zèbres et altéré le développement cérébral des seiches (croissance des différents lobes du cerveau retardé). Les stress embryonnaires artificiels vont quant à eux moduler le comportement prédateur des seiches (O'Brien et al., 2017), influencer la réactivité émotionnelle des poules, des cailles et des poissons zèbres (Bertin et al., 2018; Mezrai et al., en préparation), augmenter la motivation sociale des cailles (Mezrai et al., en préparation) ; provoquer une hyperactivité chez le poisson zèbre et induire des déficits cognitifs chez les oiseaux et les poissons et perturber le développement cérébrale des seiches (cf. Figure 41). Dans l'ensemble le stress embryonnaire semble être délétère pour le jeune (sauf quelques exceptions : amélioration de la prédation chez la seiche).

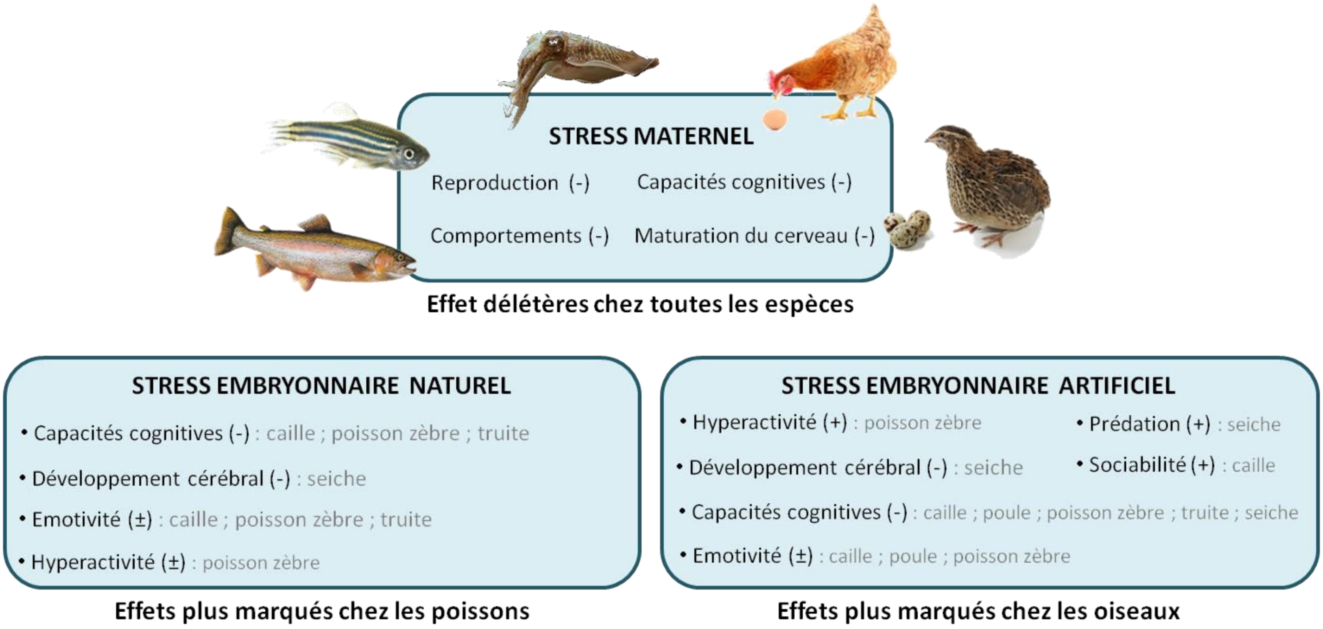


Figure 41 : Schéma récapitulatif des principaux résultats du projet Presto'Cog (d'après le compte rendu de fin de projet ANR-13-BSV7-0002). Le symbole (-) signifie une diminution ; le symbole (+) une augmentation et (±) indique une augmentation ou une diminution selon les espèces.

III. Conclusion

L’embryon de seiche, bien qu’il soit enfermé dans son œuf, n’est pas isolé de son environnement. La capsule n’est pas une barrière puisqu’elle laisse passer de nombreuses informations sensorielles. L’embryon va alors percevoir et ainsi apprendre de son environnement et ces apprentissages vont lui permettre d’augmenter ses chances de survie avant et après l’éclosion. Ils peuvent par exemple induire des comportements anti-prédateurs *in ovo* (e.g. « freezing »). Après la naissance, le jeune se servira de ces apprentissages prénatals pour reconnaitre et éviter les prédateurs. Il y a donc une continuité transnatale sensorielle et mnésique avant et après l’éclosion des seiches. L’embryon de ce céphalopode est alors doué de sensibilité et ce dès des stades relativement précoces. Pourtant, ils ne sont pas inclus dans la dernière version de la directive européenne 2010/63/EU. Au sein de cette directive, le statut des embryons de céphalopode n’est pas clairement établi et pourtant la définition de celle-ci est fondamentale. Au sein de cette directive il est stipulé que : ***« la présente directive s’applique aux animaux suivants : (a) animaux vertébrés non humains vivants, y compris : (i) les formes larvaires autonomes ; et (ii) les formes fœtales de mammifères à partir du dernier tiers de leur développement normal et (b) les céphalopodes vivants ».*** Nous ne savons donc pas avec exactitude à partir de quand les céphalopodes sont considérés comme étant « vivants » ? Pour l’heure, en raison du manque de preuves scientifiques, les embryons d’Invertébrés ne sont inclus dans la réglementation qu’à partir de l’éclosion des individus. Notre étude a alors apporté des informations objectives sur les capacités cognitives d’embryons d’Invertébrés mollusques. Pour des raisons éthiques, il serait maintenant bienveillant d’utiliser le principe de précaution et d’ainsi protéger les embryons de toutes espèces de façon équitable.

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Annexes

Annexe 1

Darmaillacq, A.-S., **Mezrai, N.,** O'Brien, C. E., & Dickel, L. (2017). Visual ecology and the development of visually guided behavior in the cuttlefish. *Frontiers in Physiology*, *8.*

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REVIEW
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Visual Ecology and the Development of Visually Guided Behavior in the Cuttlefish

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Cuttlefish are highly visual animals, a fact reflected in the large size of their eyes and visual-processing centers of their brain. Adults detect their prey visually, navigate using visual cues such as landmarks or the *e*-vector of polarized light and display intense visual patterns during mating and agonistic encounters. Although much is known about the visual system in adult cuttlefish, few studies have investigated its development and that of visually-guided behavior in juveniles. This review summarizes the results of studies of visual development in embryos and young juveniles. The visual system is the last to develop, as in vertebrates, and is functional before hatching. Indeed, embryonic exposure to prey, shelters or complex background alters postembryonic behavior. Visual acuity and lateralization, and polarization sensitivity improve throughout the first months after hatching. The production of body patterning in juveniles is not the simple stimulus-response process commonly presented in the literature. Rather, it likely requires the complex integration of visual information, and is subject to inter-individual differences. Though the focus of this review is vision in cuttlefish, it is important to note that other senses, particularly sensitivity to vibration and to waterborne chemical signals, also play a role in behavior. Considering the multimodal sensory dimensions of natural stimuli and their integration and processing by individuals offer new exciting avenues of future inquiry.

Keywords: cephalopod, vision, embryo, brain, polarization, camouflage, behavioral plasticity

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INTRODUCTION

One of the most remarkable experiences one can have as a SCUBA diver is an encounter with a cuttlefish. Not only is it unexpected (during daytime, cuttlefish are mostly camouflaged, and only an experienced eye is likely to spot one), but you have a strange feeling of being observed! Indeed, the eyes of the cuttlefish are large and captivating (**Figure 1**). They are single-chambered camera-type eyes whose structure strikingly resembles that of vertebrates. This convergence is unique among invertebrates and was probably driven by shared ecology and competition with fish (Packard, 1972). Another indication of the importance of vision to cuttlefish, though other senses are important, is the size of the optic lobes. These two bean-shaped lateral nervous structures process visual information and occupy 140% of the whole central nervous system (Nixon and Young, 2003; **Figure 2**). The primary purpose of the visual system is to recognize objects so that individuals may interact with them appropriately and execute the behaviors necessary for survival. Vision plays a crucial role in the early life stages, as functional vision is essential for perception of prey, predator

FIGURE 1 | Eyes of the cuttlefish *Sepia elongata* caught off the coast of Eilat (Gulf of Aqaba, Israel; photo AS Darmaillacq).

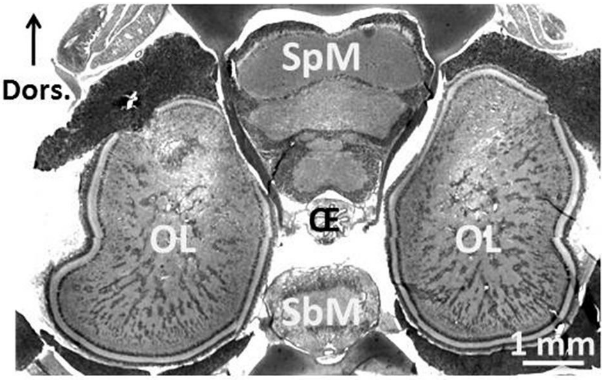

FIGURE 2 | Central nervous system of 3-month-old *Sepia officinalis* cuttlefish. Frontal section. Prenant-Gabe trichrome stain. Abbreviations: OL, optic lobe; SpM, supra-esophageal mass; SbM, sub-esophageal mass; Oe, esophagus. Modified from Jozet-Alves et al. (2012a).

avoidance and visually-guided behavior (e.g., predation, Darmaillacq et al., 2004; camouflage, Zylinski et al., 2012; navigation, Cartron et al., 2012). Consequently, the early development of functional vision is critical because it enhances the chances of survival. Although the visual capacities of cephalopods have been studied extensively in adults, few studies have investigated their development. Indeed, embryos were traditionally considered to possess only limited abilities because of the immaturity of their developing brains. In this review, we will describe how the visual system develops in embryos and how it allows embryonic visual learning. We will also summarize our knowledge of some of the interesting particularities of cephalopods: polarization sensitivity (PS) and contrast perception (Shashar et al., 2002), and that of visual lateralization. Lastly, more recent data regarding the development and plasticity of defensive behavior in juveniles will be presented.

EMBRYONIC DEVELOPMENT OF THE VISUAL SYSTEM AND EMBRYOS' RESPONSES TO VISUAL STIMULI

Development of Sensory Systems

Sepia officinalis eggs are laid in clusters on various kinds of rigid support such as algae, tubeworms, ropes or nets. Unlike other species of *Sepia*, the eggs are usually darkened with maternal ink but become more translucent due to the expansion of the capsule during embryonic development (Boletzky, 2003). *S. pharaonis* eggs are completely translucent.

During the final phase of embryonic development (stages 23–30; Boletzky et al., 2016), rhythmic mantle contractions are visible through the egg capsule after removal of the outer darker envelopes. These can be measured to assess embryonic responses to various external stimuli. Like this, Romagny et al. (2012) showed that in cuttlefish embryos, the order of the onset of function of chemosensitivity, touch and vision follows the same sequence as that of birds and mammals, with the visual system being the last to develop. Neurobiological data illustrating the early development of sensory neurons in embryos support these behavioral observations (Baratte and Bonnaud, 2009). This is another evidence of convergent evolution between cephalopods and vertebrates, perhaps instigated by similar environmental pressures and direct competition (Packard, 1972). Because embryonic development takes place outside of the mother and in the absence of direct parental care, there is strong evolutionary pressure for the rapid development of functional sensory systems, so that predators can be avoided and feeding can begin. Unlike some vertebrate species, in which the visual system is still immature at birth (Bremner et al., 2012), indirect evidence suggests that cuttlefish embryos can discriminate objects outside the egg. However, to date, no systematic study has been conducted on the development of retina morphology and physiology in the embryo (but see Imarazene et al., in press).

Embryonic Visual Responses

There is increasing empirical evidence that prenatal experience influences postnatal perception, cognitive performance and behavior. Embryonic perceptual learning, (tested in neonates) has been demonstrated across many taxa, including insects (Caubet et al., 1992), amphibians (Mathis et al., 2008), rats (Hepper, 1988), dogs (Wells and Hepper, 2006), precocial birds (Sneddon et al., 1998), altricial birds (Colombelli-Négrel et al., 2012, 2014), and humans (Moon et al., 2013).

Studies showed that embryonic visual experience affects both feeding and defensive behaviors. Cuttlefish embryos visually exposed to juvenile crabs for the last week before hatching will prefer crabs to their innately preferred shrimp prey (Darmaillacq et al., 2008). Likewise, cuttlefish innately prefer black crabs to white crabs but will preferentially select white crabs following embryonic exposure to them (Guibé et al., 2012; **Figure 3A**). Thus, it seems that not only do the cuttlefish pay attention to the shape of the prey (crab vs. shrimp) but also to its brightness. The relative importance of shape and brightness can be inferred from the fact that cuttlefish select black

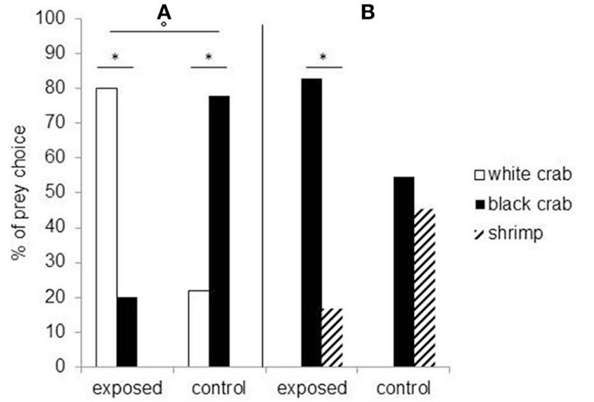

FIGURE 3 | Seven-day-old cuttlefish's prey choice depending on whether they have been exposed to white crabs during embryonic development ("exposed") or not ("control"). **(A)** To the left of the vertical: when they are presented a choice between white and black crabs. **(B)** To the right: when they have a choice between black crabs and shrimp. *Significant prey preference within groups (chi-square exact test: $p < 0.05$) and °significant difference in prey choice between groups (Fisher's exact test: $P < 0.05$). Modified from Guibé et al. (2012).

crabs over shrimp after embryonic exposure to white crabs, suggesting that they are generalizing the characteristics of a learned preference (crab shape) to the closest alternative (black crab) if the preferred item is not present (Guibé et al., 2012; **Figure 3B**).

Juvenile cuttlefish, that spontaneously prefer dark shelters, lose this bias when they have been exposed embryonically to white ones (Guibé and Dickel, 2011). Lee et al. (2012) also showed that cuttlefish raised prenatally in a visually enriched have a preference for high-contrast backgrounds whereas control cuttlefish have no substrate preference. More experiments are needed to study the direct response of the embryo to visual stimuli and the development of related brain structures.

These preferences for certain visual characteristics such as shape and brightness following embryonic exposure are relatively straight-forward. In contrast, chemical exposure to waterborne cues from shrimp or crab alters visual preferences after hatching in a less explicable fashion. Embryonic exposure to crab odor and blank seawater had no effect on the normal preference for shrimp; exposure to shrimp cue however resulted in a reversal of the normal shrimp preference (Guibé et al., 2010). The authors suggested that this is possibly due to cross-modal effects, in which odor cue modulates a primarily-visual preference. Alternatively, it could be that because embryos in this experiment were exposed to the odors of adult shrimp and crabs and they were somehow able to determine the size of the animal by its odor cue, perceiving them as a danger rather than as prey. Repeating these experiments with shrimps and crabs of various sizes could determine whether age causes differences in odor cues that are distinguished by cuttlefish.

DEVELOPMENT OF PS, CONTRAST SENSITIVITY, VISUAL ACUITY AND VISUAL LATERALIZATION

The cephalopod rhabdomeric-type eye has only one type of photoreceptor. The microvilli of neighboring photoreceptors are arranged orthogonally in the retina which confers sensitivity to the linear polarization of light (Shashar et al., 2002), one of the main properties of light in shallow water (Cronin and Shashar, 2001). Cephalopod eyes are positioned laterally on the head allowing both a monocular and a binocular vision.

Spatial Resolution and Polarization Sensitivity

Spatial resolution (or visual acuity), is the ability to discriminate fine detail (Tansley, 1965), and plays an extremely important role in the lives of animals, as it allows them to navigate in space, evade predators, catch prey, and in some species differentiate between males and females. Using an optomotor apparatus and stripes of different width, Groeger et al. (2005) showed that visual acuity improves as cuttlefish grow, ranging from a minimum separable angle of 2.5–0.57° (a decrease in this angle value means a better spatial resolution). A decrease in light intensity affects visual acuity whatever the age of the individual.

Polarization sensitivity (PS) improves the visibility of objects by enhancing the contrast between them and the background. In cephalopods, PS increases the success of predation on transparent prey or silvery fish (Shashar et al., 1998, 2000); in cuttlefish, it may also play a role in communication between adults (Shashar et al., 1996; Boal et al., 2004) and in navigation (Cartron et al., 2012). PS matures gradually after hatching. Cartron et al. (2013a) found that only 20% of cuttlefish hatchlings showed an OMR to a polarized striped pattern when it was rotated slowly. The proportion of cuttlefish responding increased throughout the first month of life (100% by the age of 30 days; **Figure 4**). However, a choice test with fully polarized or depolarized mysids (transparent shrimps) showed that 1 week-old cuttlefish detect polarized shrimp faster than non-polarized, suggesting an earlier maturation of PS (Cartron et al., 2013a). These apparently contradictory results could be explained by the motion of the rotating pattern in the OMR apparatus compared with the more stationary prey. It is possible that polarization contrast is more useful in assessing the shape of prey and that motion can interfere somewhat with this ability. This deficiency could be mitigated by the fact that polarization is not the only quality of light to which cuttlefish are sensitive. Though colorblind (Mäthger et al., 2006; but see Stubbs and Stubbs, 2016), cuttlefish are sensitive to contrast. Indeed, most hatchling cuttlefish (75%) showed an OMR to the black, white and gray striped pattern rotating at the lowest velocity, with the proportion reaching 100% by the age of 1 month. Thus, it can be hypothesized that polarization and luminance signals are processed separately and may play different roles in vision as observed in insects (Pfeiffer et al., 2005). In the desert locust *Schistocerca gregaria* for instance, a group of neurons in the central complex (a neuropil in the center of the brain), has been found to be

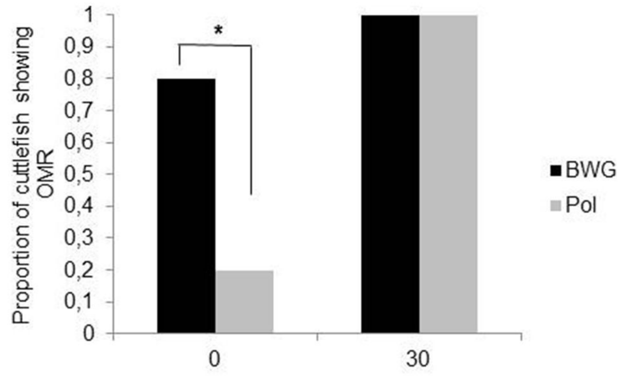

FIGURE 4 | Proportion of the cuttlefish ($N = 10$ per group) that showed an optomotor response (OMR) to BWG (luminance only; black) or Pol (polarization; gray) patterns rotating at a velocity of 30 deg s^{-1}, at hatching (0) and at the age of 30 days. Asterisks indicate a significant difference in the percentage of cuttlefish showing an OMR between the BWG and Pol patterns McNemar's test, ($P < 0.05$). Modified from Cartron et al. (2013a).

sensitive to polarized light while neighboring neurons are not (although all neurons responded to unpolarized light). More experiments, notably electrophysiological and immunochemistry investigations, are needed in order to determine the neural pathways for polarization and luminance information processing in cuttlefish.

Ontogenesis of Visual Lateralization

Cerebral lateralization, a trait that is widespread in animal kingdom (Vallortigara and Rogers, 2005; Frasnelli et al., 2012), is often revealed behaviorally by motor and perceptual asymmetries. In cuttlefish, adults have a preference for turning right or left (side-turning preference) in a T-maze (Alves et al., 2007), which can be the result of an eye use preference as in octopus (Byrne et al., 2002, 2004). In juveniles, Jozet-Alves et al. (2012b) showed that although cuttlefish do not show any side-turning preference in a basic T-maze, they do develop a left-turning bias when shelters are available at the end of the maze's arms from the age of 3 to 60 days. Interestingly, when cuttlefish have been exposed to a predator odor before hatching, they preferentially turn to the left in the simple T-maze (Jozet-Alves and Hebert, 2013); this suggests an influence of environmental factors on the ontogenesis of visual lateralization in cuttlefish. This may be adaptive for young cuttlefish to decide rapidly which shelter to choose specially in a risky situation where predators are potentially present around.

Influence of Environmental Constraints on PS and Visual Lateralization

S. officinalis, the European cuttlefish, is widespread in the English Channel, the Atlantic Ocean and the Mediterranean Sea where the turbidity can be high. On the other hand, *S. pharaonis* and *S. prashadi* are found in the Red Sea, on coral reefs, where the water is clearer. All these species are able to detect a polarized stimulus at higher turbidity levels than an unpolarized one (Cartron et al., 2013b,c), indicating that PS can improve the capacity for object detection through turbid waters when intensity information alone is insufficient. *S. officinalis* can detect objects, whether polarized or unpolarized, at higher turbidity levels than the other two (Cartron et al., 2013b). It is thus likely that PS, which is present in most cuttlefish species (but see Darmaillacq and Shashar, 2008), is a product of natural selection driven by visual features of the species' environment. This hypothesis is supported by the fact that the *S. officinalis* used in this experiment were lab-reared individuals that had never encountered turbidity, yet were still better-equipped to discriminate objects under these conditions.

DEFENSIVE BEHAVIOR

Cephalopods are known for their skills in quickly changing skin patterns in response to environmental change, a property referred to as "dynamic camouflage" (Hanlon and Messenger, 1996; Hanlon, 2007). This dramatic behavior is made possible by their unique skin structure that comprises three layers of cells: the chromatophores (containing dark-brown, reddish-orange or yellow pigments), within the most superficial dermis of the dorsal part of the mantle and arms, under the direct control of the brain; the iridophores, underneath, that reflect environmental light to create iridescence (particularly prominent on the ventral part); and the leucophores, the deepest, that reflect mainly white. Together with textural, postural and locomotor components, these chromatic elements constitute the "body pattern" of cuttlefish (Hanlon and Messenger, 1988). Body patterns displayed in a chronic fashion are mainly used for crypsis in juveniles as a primary defense strategy to avoid detection. Cuttlefish adopt a brightness similar to the substrate (general color resemblance), or a display disruptive colorations that breaks up the outline of the body so that the overall form of the animal is lost (Hanlon et al., 2009). The disruptive pattern has been the most studied. In the lab, it has been shown that artificial backgrounds such as 2d checkerboards can elicit this pattern (Chiao and Hanlon, 2001; Chiao et al., 2007). More, several authors (Chiao and Hanlon, 2001; Barbosa et al., 2007, 2008) showed that both check size and achromatic contrast affected the body patterns. Other characteristics of the objects present in the vicinity of cuttlefish are taken into account by juveniles such as the presence of egdes, the spatial phase and the three dimensionality (Chiao et al., 2005; Zylinski et al., 2009; Ulmer et al., 2013).

Other body patterns (such as the deimatic and flamboyant displays) are shown in a more acute manner (only for a few seconds) and are used mainly as "secondary" defense strategies after a cuttlefish has been detected. Cuttlefish can also adopt a deceptive resemblance to natural objects in the environment (e.g., floating algae) to deceive potential predators or prey. In juvenile cuttlefish, uniform and mottle patterning are generally displayed on uniform/fine sandy backgrounds (**Figure 5A**) while disruptive coloration occurs on more patchy/contrasted substrates (**Figures 5B,D**). Uniform, mottle and disruptive patterns are usually mixed to varying degrees (Hanlon et al., 2009;

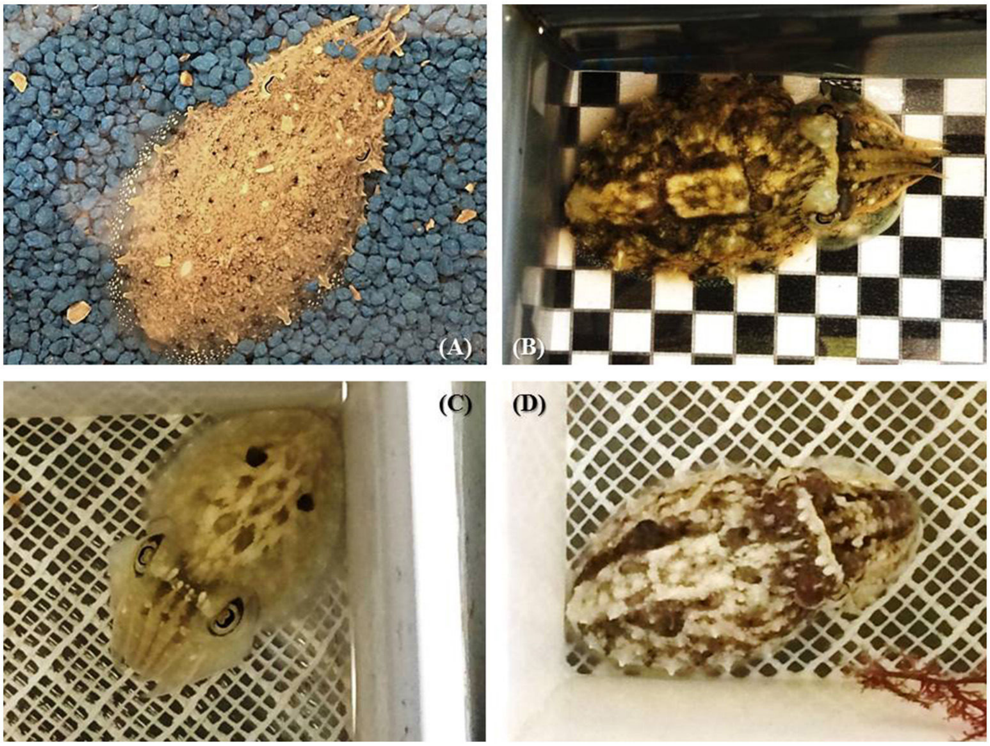

FIGURE 5 | The diversity of body patterns displayed by 2-month-old cuttlefish (ca. 3–4 cm dorsal mantle length). **(A)** stipple-uniform pattern elicited on uniform blue gravel; **(B)** disruptive pattern elicited on a black and white checkerboard combined with mottle pattern; **(C)** deimatic pattern following exposure to a "threat" **(D)** mottle coloration with some components of the disruptive pattern (i.e., white square, white head bar, and paired black dots). Note that patterns are not always fully expressed but exist in combination with others and may or may not directly reflect the visual background.

Figures 5B,C,D), making camouflage "efficiency" very difficult to define or measure (see discussion in Hanlon et al., 2009). Last, in adults, body patterning plays a large role in intra-specific signaling, especially in agonistic and courtship behavior (Hanlon and Messenger, 1988). While social interaction between hatchlings appears to be non-existent (see Holmes, 1940; Hanlon and Messenger, 1996), it is still possible that body patterning also plays a role in signaling between young cuttlefish. This remains unclear as inter-individual communication has never carefully investigated in juvenile cuttlefish, and scarcely even in adults (see Boal et al., 2004).

Functional chromatophores first appear *in ovo* during stage 25 of embryonic development, when the dorsal mantle length of the animal is about 2 mm (Bonnaud-Ponticelli and Boletzky, 2016). While the total number of chromatophores increases with age, their density progressively decreases from 400 to 500/mm^2 at hatching to 35 to 50/mm^2 in adults (Hanlon and Messenger, 1988). Nevertheless, both juveniles and adults possess a high density of cells that allow them to express an infinite range of gradations of various components of their body patterns, depending on background and lighting (Hanlon and Messenger, 1988). Thirteen "typical" body patterns have been identified in adults, but since the body patterning related to sexual behavior is absent in juveniles, the number of color, postural-kinetic, and structural components is lower—only nine distinct patterns (Hanlon and Messenger, 1988). Qualitative changes in body patterning also occur in juveniles. For example, when a late juvenile (about > 6 weeks) or adult is threatened by a small predator, it often displays a "deimatic pattern" in an attempt at intimidation: it flattens its body and flashes two big spots against a white dorsal mantle in a manner resembling eyes (**Figure 5C**). In younger animals, this pattern appears very rarely (Thorpe, 1963; Hanlon and Messenger, 1988), and though the postural components are the same as in adults they flash not two but six dark spots (Hanlon and Messenger, 1988; Mangold, 1989) until about 2 weeks of age. While this version of the deimatic display is used sometimes, newly-hatched cuttlefish are more likely to respond to potential danger with a general darkening or blanching of its body or a cryptic flamboyant display (Hanlon and Messenger, 1988).

One wonders whether body patterning development in juvenile cuttlefish is rigidly fixed or is more influenced by prior individual experience. Simple observations of body patterning in early juveniles speak to this question: when placed on the same background different individuals display different body patterns, suggesting that the response is partially determined by previous experience. Other anecdotal and experimental evidence has the opposite implication however. Hanlon and Messenger (1988) released young cuttlefish (from <1 to 17 weeks of age) previously reared in captivity into the field and observed that they concealed themselves effectively against every substrate encountered and were extremely difficult to see by human observers. Unfortunately, the personal histories of individuals were not described (i.e., whether they were reared in groups or in isolation, the amount of time spent in the wild before the behavioral observations, etc.), so we cannot make any definitive conclusions. Still, this observation suggests that body patterning development could be hard-wired since the impoverished artificial conditions of rearing do not seem to have any deleterious effects on the concealment skills in juveniles.

More controlled experiments also support an innate origin. Cuttlefish were reared in either "impoverished" conditions (housed individual tanks on a dark uniform background) or in "enriched" conditions (housed in groups in a variegated environment with sand, stones, shells, and artificial seaweeds) for 2 months (Poirier et al., 2005). Later, individuals from each group were tested on either a uniform gray substrate or checkered black and white background. In juveniles, a uniform background should elicit a uniform or slightly mottled body pattern (but see discussion in Hanlon et al., 2009), while a disruptive color pattern seems most adaptive against a contrasted background. The authors then assessed camouflage efficiency of by measuring the hue and intensity of various components of body patterning, on both uniform and contrasted substrates. At hatching, many cuttlefish display disruptive patterning regardless of background type. But starting at 15 days of age, cuttlefish previously reared in enriched conditions were better able to match both background types. Cuttlefish raised in enriched conditions also had greater cell proliferation in the optic lobes than those of cuttlefish from impoverished conditions. This makes sense, as the optic lobes are key structures controlling body patterning in cephalopods (Nixon and Young, 2003). Further evidence for greater innate or "hard-wired" control of body patterning comes from experiments with potential predators, in which *S. officinalis* was found to show the deimatic pattern toward small, low-threat teleost fish but not toward larger more dangerous predators such as sea bass or small sharks (Langridge et al., 2007; Langridge, 2009). Moreover, these reactions occur the first time such threats are encountered, suggesting innate recognition of threat type.

While the preponderance of evidence suggests that body patterning is preprogrammed the fact that different individuals may use a different concealment strategies when placed in the same environment (Poirier et al., 2004), suggest some amount of experience-dependence, potentially through learning and phenotypic plasticity, although we cannot rule out the possibility that these inter-individual differences are the result of genetic history or parental experience. These data lead us to conclude that body patterning in cuttlefish is definitely not a simple stimulus-response process, as it is commonly presented in the literature. It probably involves a complex integration of visual information, genetic history and individual experience (West-Eberhardt, 1989), possibly even before hatching (**Figure 6**). Thus, further investigation of body pattern development could lead to insight not only about camouflage and defense, but also to a better understanding of learning, plasticity, decision making and higher-order cognitive processes in cephalopods (Vitti, 2012; Skelhorn and Rowe, 2016).

FIGURE 6 | Stage 30 embryo (less than 1 cm) showing a mottle-disruptive coloration inside the egg. It has also squirted ink; note the cloud of ink in the perivitellin fluid. Note that the embryo is seen from under through a peeled *S. officinalis* egg (photo C.E. O'Brien).

CONCLUSION: EMBRYONIC ECOLOGY

In this review, we discussed the fact that the visual system is functional well before hatching, as indicated by indirect evidence from embryonic visual learning. By stage 25, the embryo's eyes are mature enough to perceive light and also to discriminate stimulus shape, movement and brightness. Unfortunately, little is known about the direct response of embryos to such stimulations and about the development of the brain structures that process visual information in cuttlefish, namely the optic lobes. The fact that cuttlefish are able to attend to and learn from their biotic and abiotic environment during the final stages of their embryonic development from the relative safety of their egg suggests that prenatal learning plays a large facilitative role in finding food and shelter after hatching. This ability may also enable prenatal social learning. Eggs are laid in clusters, and as a consequence, embryos are likely to be able see each other during development. Social rearing conditions after birth are known to have strong effects on growth and memory (Dickel et al., 2000), so the possibility of prenatal effects exists. No studies have yet addressed this, and experiments to test the effect of embryonic development in isolation on postembryonic behavior are needed.

Many questions about the development of vision in cuttlefish remain to be explored. For instance, do females actively choose their egg-laying site in order to increase offspring learning and survival (i.e., non genetic maternal effects)? Cuttlefish reproduce only once in their lifetime and hence, have only a single opportunity to produce offspring. This, combined with the potential for juvenile behavior to be shaped by embryonic learning, implies that strong selection pressure (based on the presence of predators, shelters or prey for juveniles) is exerted on females' decision. Since it has long been assumed that invertebrate behaviors are mostly genetically programmed, attention should be paid to such previously-neglected effects.

This synthesis highlights the importance of vision in embryo and juvenile cuttlefish behaviors. However, like other animals, cuttlefish live in a multisensory world, and even if vision appears predominant, their behaviors may be influenced by other senses. In most animals, the senses are not equal in their ability to provide accurate information about the environment (Bremner et al., 2012). For example, in a turbid environment, relying only on vision may be risky, and other senses may play a greater role. Komak et al. (2005) have demonstrated that young cuttlefish are sensitive to local water movements thanks to specialized cells on the arms and the head that are analogous to the lateral lines of fish. Water movement detected by these cells could alert cuttlefish to the presence of prey or predators before it is possible to see them. The importance of particular senses may also vary throughout the life of an individual. In cuttlefish, given the opacity of the egg capsule, the sensory world of embryos is probably dominated by chemosensory information. This likely changes as soon as the cuttlefish leaves the egg. Assessing the relative importance of vision and its interactions with the other senses through multimodal perception in different situations and at different ages offers exciting new tracks of research such as prey and predator recognition through visual and/or chemical information.

AUTHOR CONTRIBUTIONS

All authors read and approved this version of the ms; ASD, wrote the main part of the article; NM, wrote the section about embryonic responses; LD, wrote the section about body patterns; CEO, co-wrote the section about embryonic behavioral response and copy-edited the ms.

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Annexe 2

O'Brien, C. E., **Mezrai, N.,** Darmaillacq, A.-S., & Dickel, L. (2017). Behavioral development in embryonic and early juvenile cuttlefish (*Sepia officinalis*). *Developmental Psychobiology*, *59*(2), 145–160.

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RESEARCH ARTICLE

WILEY Developmental Psychobiology

Behavioral development in embryonic and early juvenile cuttlefish (*Sepia officinalis*)

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Though a mollusc, the cuttlefish *Sepia officinalis* possesses a sophisticated brain, advanced sensory systems, and a large behavioral repertoire. Cuttlefish provide a unique perspective on animal behavior due to their phylogenic distance from more traditional (vertebrate) models. *S. officinalis* is well-suited to addressing questions of behavioral ontogeny. As embryos, they can perceive and learn from their environment and experience no direct parental care. A marked progression in learning and behavior is observed during late embryonic and early juvenile development. This improvement is concomitant with expansion and maturation of the vertical lobe, the cephalopod analog of the mammalian hippocampus. This review synthesizes existing knowledge regarding embryonic and juvenile development in this species in an effort to better understand cuttlefish behavior and animal behavior in general. It will serve as a guide to future researchers and encourage greater awareness of the utility of this species to behavioral science.

KEYWORDS
defense, learning, memory, plasticity, predation, welfare

1 | INTRODUCTION

The common cuttlefish, *Sepia officinalis* (Linnaeus, 1758), along with other cephalopods, possesses a centralized nervous system capable of learning and memory, advanced sensory systems, and a highly sophisticated behavioral repertoire that is comparable to that of vertebrates (Hanlon & Messenger, 1996; Packard, 1972). The life history and habits of cuttlefish enable such behavioral research to be pursued through both field and laboratory study. With this invertebrate model, we can address questions about complex behavior and learning in a marine mollusc, a group genetically very distant from more traditional models, such as birds and rodents. This phylogenic distance provides an alternative perspective that is critical to understanding the ways that natural selection, ancestral history, and non-hereditary processes interact to shape animal behavior.

S. officinalis is particularly well-suited for the study of behavioral ontogeny. Like many fish, this species has gelatinous, semi-permeable eggs and rapidly-developing sensory abilities that allow exceptional sensory access to the surrounding environment during the final stages of embryonic development (Romagny, Darmaillacq, Guibé, Bellanger, & Dickel, 2012). Coupled with their oviparity and the lack of direct maternal care, these characteristics allow stimuli to be applied directly to embryos rather than being transmitted and mediated by the mother, as in rodents and birds. At hatching, this species possesses one of the richest behavioral repertoires in the animal kingdom. This review provides an overview of existing knowledge regarding the sensory experience, habitat, learning, and behavior of embryonic, hatchling (<1 week after eclosion) and juvenile (up to 17 weeks) cuttlefish to serve as a starting point for further inquiry. In order to properly contextualize the behavior of this less-familiar species, this review also draws insight from and parallels to other species of cuttlefish, as well as some of their closest coleoid cephalopod relatives—octopuses and squids.

2 | EMBRYONIC DEVELOPMENT

2.1 | Embryogenesis and yolk reserves

Spawning of *S. officinalis* eggs occurs in the English Channel in shallow (5–60 m), well-lit coastal waters throughout the spring and summer (Basuyaux & Legrand, 2013; Nixon & Mangold, 1998). Eggs are usually attached in clusters to objects on the seabed, such as algae, seagrass, previously-deposited cuttlefish eggs, cuttlefish traps, other artificial structures, and sessile organisms (Blanc, Du Sel, & Daguzan, 1998; Boletzky, 1983; Nixon & Mangold, 1998; Nixon & Young, 2003). The embryonic development of *S. officinalis* is divided into 3 periods and 30 stages: segmentation (stages 1–9), gastrulation (stages 10–17), and organogenesis (stages 18–30) (Lemaire, 1970). Development proceeds slowly at first, then dramatically increases pace toward the very end, accomplishing the majority of growth and differentiation during the last few stages (Domingues, Bettencourt, & Guerra, 2006; Fioroni, 1990). In the final stages of development, the egg absorbs seawater, increasing the volume of the perivitelline fluid (PVF) filling the capsule. This causes the egg to swell to almost double its original diameter (Richard, 1971), and contrasts sharply with the situation in octopod eggs, which typically remain constant in size during development (Fioroni, 1990). In addition to swelling, excretions of the epidermis digest the inner layers of the egg membrane and, as a consequence, the formerly opaque membrane becomes thinner and partially translucent (Cronin & Seymour, 2000; Richard, 1971). The embryo within becomes visible, giving unprecedented access to late prenatal stages (Figure 1).

Cuttlefish eggs are able to tolerate limited episodes of stress (e.g., prolonged emersion, handling) and still hatch normally (Jones, Ridgway, & Richardson, 2009), but otherwise have a fairly narrow range of physical requirements in terms of temperature and salinity (Boletzky, 1983; Nixon & Mangold, 1998). Within the tolerated temperature range, higher temperatures accelerate growth and result in shorter development times. Because of this, eggs laid in the English Channel during the spring take around 90 days to develop, while those spawned in the summer, when the water temperatures are higher, take 40–45 days, resulting in two cohorts per year (Boletzky, 1983; Bouchaud, 1991).

Throughout prenatal and early post natal development, embryos are sustained by internal and external yolk reserves (Lemaire, 1970). Those that develop more quickly because of higher temperatures hatch with a larger yolk reserve remaining (Boletzky, 1975; Bouchaud, 1991; Bouchaud & Daguzan, 1990; Dickel, Chichery, & Chichery, 1997). However, this more rapid development results in smaller hatchlings (Bouchaud & Daguzan, 1990; Hanlon & Messenger, 1988), and this has implications for survival and the onset of predatory behavior (Boletzky, 1994; Bouchaud, 1991). Water temperature also varies with location, depth, season, currents, tide, and weather, so the timing and position of an egg can strongly affect its later prospects (Bloor, Attrill, & Jackson, 2013).

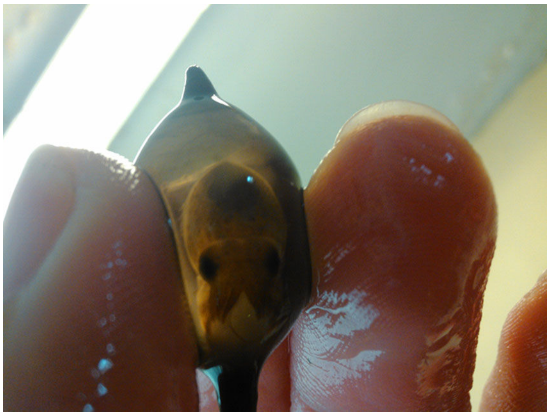

FIGURE 1 Stage 30 *Sepia officinalis* embryo (approx. mantle length 6 mm) seen through the transparent egg membranes. Photo by Nawel Mezrai

2.2 | Sensory systems

Embryos are buffered and protected from the external environment by their egg capsule, but they are not entirely isolated from it. At the beginning of development, the layer of ink in the egg membrane absorbs light and prevents most visual information from penetrating in either direction (Paulij, Herman, Roozen, & Denucé, 1991). In addition, eggs can be laid down to a depth at which only 10% of surface light intensity remains (Bloor et al., 2013). Despite these limitations, the membrane becomes translucent due to egg expansion, the optic lobes and lens mature during stages 20 and 21 (Lemaire, 1970; Lemaire & Richard, 1978), and as a result, reaction to visual stimulation in the external environment is observed by stage 25 (Romagny et al., 2012).

Likewise, *S. officinalis* embryos are able to perceive waterborne chemical cues that diffuse through the egg membrane and tactile stimuli from movement in the external environment by stage 23 (Romagny et al., 2012). Thus, the organogenesis period of embryonic development (stages 18–30) is characterized by a progressive build-up in the amount of sensory information penetrating the egg membrane in conjunction with a gradually-increasingly ability to perceive this information. (It is possible that sensory perception is possible even earlier than stage 23, but the ability to respond is not.)

2.3 | Maternal influence and the prenatal environment

Cuttlefish eggs are abandoned by the mother soon after laying, and thus receive no direct parental care or protection (Darmaillacq, Chichery, & Dickel, 2006). Nevertheless, offspring can be affected by maternal influence. For instance, embryo provisioning in cephalopods affects the size, molecular composition, and ultimately the survival and success of eggs ("maternal effect," Bloor et al., 2013). As one example, nutritional stress in another cephalopod, the dumpling squid (*Euprymna tasmanica*), has been shown to cause females to lay fewer eggs with less yolk that result in lower levels of survival and success (Steer, Moltschaniwskyj, Nichols, & Miller 2004). Among cephalopods, *S. officinalis* have some of the largest eggs, reflecting a high amount of maternal provisioning (Fioroni, 1990).

More importantly, since attachment renders eggs sessile, a female's timing and choice of egg-laying site dictates the environmental conditions experienced throughout egg development (Bloor et al., 2013). Ultimately, these environmental conditions can influence survival, growth rate, hatching time, and post natal behavior. For example, extreme salinities or contamination by pollutants (e.g., heavy metals or pharmaceutical residues), which can slow or interfere with development, may result from being located close to shore (Bidel et al., 2016; Bloor et al., 2013; Di Poi, Bidel, Dickel, & Bellanger, 2014; Paulij, Bogaards, & Denucé, 1990; Paulij, Zurburg, Denuce, & Van Hannen, 1990). As a mostly semelparous species with only a single spawning period at the end of life (Boletzky, 1987), there is presumably strong selective pressure on females to lay eggs in locations, times and in densities that maximize offspring survival (Bloor et al., 2013).

The revelation that perception is possible from within the egg has important implications for the understanding of behavioral development in juveniles. It suggests that *S. officinalis* embryos are developmentally and behaviorally plastic, and demonstrates that they begin adapting to their environment long before hatching. The particular suite of prenatal stimuli experienced by embryos will depend on their location. Field observations concerning the environment around spawning sites are scarce. However, since *S. officinalis* lay their eggs on submerged objects (Boletzky, 1983), and because such objects tend to attract other marine life to the area, it is likely that developing embryos will sense predators, future prey, as well as other species during the course of development (Figure 2). They can then use this information to prepare for the post-hatching environment. Indeed, several experiments have found that predator and prey stimuli strongly influence juvenile behavior (e.g., Darmaillacq, Lesimple, & Dickel, 2008; Guibé, Dickel, & Boal, 2010; Guibé, Poirel, Houdé, & Dickel, 2012; Jozet-Alves et al., 2012; Jozet-Alves & Hébert, 2013). Careful field observations documenting the identity and prevalence of other species at spawning sites would be extremely useful in piecing together a picture of the sensory experience of embryos during development.

One intriguing difference between *S. officinalis* and many other cephalopods is their dark egg capsule. Where most cuttlefish, squid, and octopuses have translucent eggs, the egg membrane in *S. officinalis* is stained with a layer of ink from the mother. It is possible that this ink-staining aids in defense by camouflaging the eggs themselves or by masking the movement of the embryo within (Guerra & González, 2011). The fact that most other cuttlefish species have translucent eggs and employ alternative methods of visual camouflage provides indirect evidence in support of this hypothesis. For instance, the pharaoh cuttlefish (*Sepia pharaonis*) of the Pacific conceals its clear eggs in crevices rather than laying them out in the open like *S. officinalis* does (Darmaillacq, Dickel, & Mather, 2014), while *Sepia esculenta* has a sticky exterior that accumulates a layer of camouflaging detritus (Hanlon & Messenger, 1996). It has also been demonstrated that proteins in the outer egg membrane of *S. officinalis* originating from the mother's nidamental glands have antimicrobial properties (Cornet et al., 2015). Other compounds in the membrane may act as a chemical deterrent to predators (Boletzky, 2003; Derby, Kicklighter, Johnson, & Zhang, 2007; Hanlon & Messenger, 1996).

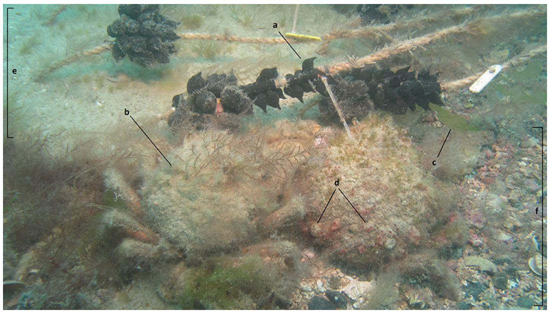

FIGURE 2 Cuttlefish eggs (a) in situ, June 26, 2014 at an artificial laying-site (pre-placed tethers) in the vicinity of Blainville sur Mer, France. Note the crab, *Maja squinado* (b) in the foreground as well as the presence of numerous algaes, including Ulva sp. (c), *Lithophyllum incrustans* (d), various unidentified epibionts and a diversity of surrounding substrate types including sand (e), rocks and shell debris (f). Eggs are approximately 2.5–3.0 cm in length (Boletzky, 1983). Photo by Olivier Basuyaux, of the Synergie Mer et Littoral (SMEL)

Other characteristics of cuttlefish eggs may also play a protective role. As in other species, females may lay their eggs in clusters for protection. Eggs on the inside of the cluster are protected from predators and the external environment by those on the outside. Another hypothesis involves the presence of a tranquilizing compound in the PVF. Such a compound has been identified in squid (Marthy, Hauser, & Scholl, 1976). It is hypothesized to reduce the likelihood of detection and predation by reducing embryo movement and preventing premature hatching (Marthy et al., 1976; Weischer & Marthy, 1983). While this agent has not yet been identified in cuttlefish, its presence is probable and the tranquilizing compound from squid has been shown to reduce activity in cuttlefish and other cephalopods (Weischer & Marthy, 1983).

2.4 | Embryonic movement and sleep

Embryos exhibit a variety of movements and behaviors. Respiration is visible from outside of the egg. In addition, mantle contractions—pumping motions involving the whole mantle and its musculature—are also visible (Corner, 1977). While the function of this behavior is unclear, novel visual and odor stimuli will cause a more or less immediate change in the rate of mantle contractions (Romagny et al., 2012). Finally, embryos also display periods of unprovoked activity including movements of the arms, tentacles, fins and funnel, including twitching, exercise of the muscles controlling the chromatophores, mantle contractions, and apparent stretching of the arms and tentacles (Corner, 2013b). These recurring episodes are analogous to REM sleep in vertebrates and are referred to as "motorically active sleep" (MAS) (Corner, 2013b). This behavior starts sometime before stage 29, and continues into postnatal life (Corner, 2013b). Other cephalopods (squid and octopus) and invertebrates (e.g., nematodes, annelids, cnidarians, and insects) also exhibit similar prenatal or larval behavior (Corner, 2013a).

3 | NON-ASSOCIATIVE LEARNING

3.1 | Habituation

Habituation is a simple form of learning in which an organism ceases responding to a stimulus after repeated or extended exposure to it (Bouton, 2007). The prenatal occurrence of habituation is difficult to ascertain due to the relative inaccessibility of most developing embryos. In species for which this type of prenatal learning has been demonstrated (e.g., superb fairy wrens, rats, humans), inquiry has been largely restricted to chemosensory and vibroacoustic stimuli (e.g., Colombelli-Négrel, Hauber, & Kleindorfer, 2014; Goldkrand & Litvack, 1991; Smotherman & Robinson, 1992; van Heteren, Boekkooi, Schiphorst, Jongsma, & Nijhuis, 2001). In *S. officinalis*, however, the partial translucence of late stage eggs enables observation of the mantle movements of the embryo within and expands the range of stimuli that can be tested to the visual. In response to novel visual, tactile or chemosensory stimulus, embryos will reduce respiration and mantle movements. The subsequent resumption of an increased mantle movement after repeated or chronic exposure to the stimulus indicates habituation. This has been demonstrated in final-stage (30) embryos with repeated exposures to bright light (Romagny et al., 2012). Likewise, stage 30 embryos can be habituated to the sight of *Carcinus maenas* (green crabs) (N. Mezrai, unpub. data). Habituation conserves energy by allowing animals to eliminate unnecessary behavioral responses (Rankin et al., 2009).

3.2 | Prenatal imprinting and exposure learning

Imprinting is another form of learning characterized by the establishment of an irreversible preference for something during a limited sensitive period, usually early in development. This preference is expressed later in life and is considered “indelible” in that it cannot be reversed after the sensitive period (Lorenz, 1937). Often, this preference will be generalized to other objects sharing similar characteristics (Sluckin, 2007). The classic example comes from filial imprinting in precocial birds that imprint on their mother during a particular window after hatching and generalize this preference to sexual partners later in life (Bolhuis, 1991). Other forms include imprinting for prey, habitat, host, or a sexual partner (Bouton, 2007). A similar form of recognition learning is perceptual learning. Like imprinting, perceptual learning may occur early in life and involves a learned preference for something after exposure, but otherwise does not meet the criteria for imprinting, such as indelibility and generalization (Shettleworth, 2009).

Imprinting and perceptual learning for prey preference have been demonstrated in *S. officinalis*. As reported by Wells (1958) and others (e.g., Darmaillacq, Chichery, Poirier, & Dickel, 2004; Darmaillacq, Chichery, & Dickel, 2006; Darmaillacq, Chichery, Shashar, & Dickel, 2006; Guibé et al., 2012) hatchlings have an “innate” preference for shrimp or shrimp-shaped objects (but see below). However, that this preference can be overridden by chemical and/or visual exposure to crabs shortly after hatching. This induced preference fits the criteria for imprinting: it lasted for at least 3 days, persisted after the cuttlefish had consumed a shrimp and was only induced during a short sensitive period early in the life of the cuttlefish (Darmaillacq, Chichery, Poirier et al., 2004; Darmaillacq, Chichery, & Dickel, 2006; Darmaillacq, Chichery, Shashar et al., 2006; Healy, 2006). Interestingly, this sensitive period for prey preference induction seems to begin before hatching: hatchlings from embryos visually exposed to crabs for a week or more prior to hatching (and not after) preferred crabs in a choice test 7 days later (Darmaillacq et al., 2008). Other experiments demonstrated that this sensitive period closes about 6 hr after sunrise on the day of hatching (Darmaillacq, Chichery, & Dickel, 2006).

Imprinting and perceptual learning allow cuttlefish to learn the characteristics of available prey in their environment during the perinatal period (Darmaillacq et al., 2014; Healy, 2006). Shrimp abundance may vary between egg laying sites and perinatal exposure to co-occuring species could transmit information about the relative abundance of predators and prey in the environment. Such information seems especially advantageous to *S. officinalis*, which uses different strategies in response to different species, both predator and prey (described later). Information about species abundance would allow cuttlefish to prioritize the development of one strategy over another. Similar and analogous instances of food imprinting and perceptual learning exist in other phyla (e.g., amphibians, Hepper & Waldman, 1992; birds, Bertin et al., 2010; insects, Quesada & Schausberger, 2012). This suggests that prenatal learning may be a common method of energy conservation and risk reduction during the vulnerable time following hatching or birth throughout the animal kingdom.

The discovery of imprinting in *S. officinalis* may also completely overturn the notion that cuttlefish “innately” prefer shrimp (Wells, 1958). Instead, it may be that the sight of other developing cuttlefish—elongate objects that move along their horizontal axis—actually induces a preference for shrimp through generalization. Indeed, cuttlefish from eggs incubated in isolation tend to not prefer crab or shrimps (L. Dickel pers. obs.). Unfortunately, Wells (1958), the first to record this preference, did not report whether the cuttlefish in his experiments were reared socially or in isolation (Darmaillacq et al., 2014). Regardless of whether this preference is pre-programmed, it does appear that cuttlefish have the innate ability to distinguish between species.

In addition to the capacity to distinguish between different kinds of decapod crustaceans, prey preference could also be induced on the basis of brightness contrast: Where naïve cuttlefish preferred dark to white crabs as their initial meal, embryos and hatchlings exposed to white crabs later preferred these over dark crabs (Guibé et al., 2012). This demonstrates that *S. officinalis* is able to learn about multiple characteristics of prey (shape and/or contrast). Moreover, cuttlefish pre- or postnatally exposed to white crabs preferred black crabs over shrimp, indicating that *S. officinalis* will generalize the characteristics of a learned preference to the closest alternative if the preferred item is not available (Guibé et al., 2012).

One experiment involving *S. officinalis* also investigated the interaction between sensory modalities in the induction of prey choice. Hatchlings from eggs exposed to waterborne chemosensory cues from shrimp, crab and two control cues were tested for visual preference. Cuttlefish that had been incubated with crab or control cues showed either no preference or the “typical” preference for shrimp (Guibé et al., 2010). Cuttlefish that were exposed to chemosensory cues from crabs later showed a visual preference for shrimp (Guibé et al., 2010). These puzzling results suggest that cross-modal effects (VanderSal & Hebets, 2007) are operating between the chemosensory and visual systems and merit further investigation. In cuttlefish, sensory integration occurs in the superior frontal lobes of the brain (Nixon & Young, 2003) and is thus the likely site of these putative interactions (Guibé et al., 2010).

Finally, in addition to the demonstration of prenatal food preference learning, it has been shown that exposure to other ecologically-salient objects in the incubation environment can affect future behavior. Naïve hatchlings were found to spontaneously prefer dark shelters (bivalve shells). Prenatal exposure to white shelters eliminated this preference, resulting in cuttlefish that were equally likely to hide under a black or white shelter (Guibé & Dickel, 2011). Like recognition learning, knowledge about the characteristics of objects in the surrounding environment could be adaptive. In this case, a cuttlefish may be learning that white objects are plentiful in the area, stationary, and may be a source of future refuge.

3.3 | Lateralization

Lateralization is the tendency to process information through one side of the brain in a particular type of situation. Potentially, lateralization yields advantages in speed and efficiency of information processing via the specialization of each side of the brain (Jozet-Alves & Hébert, 2013). Lateralization is seen in numerous vertebrates, including primates, birds, fishes, and amphibians. In these animals, left brain lateralization is often coupled with rapid responses, especially escape reflexes (Jozet-Alves et al., 2012).

In cuttlefish, brain lateralization seems to occur in cuttlefish after prenatal exposure to predator odor (Jozet-Alves et al., 2012; Jozet-Alves & Hébert, 2013). Over the course of the first month of life, juveniles that had been exposed prenatally to predator odor develop a bias for turning toward a shelter on the left, rather than on the right side (Jozet-Alves et al., 2012). This behavior may reflect a preference for using the left eye and left side of the brain (visual input is processed by the same side from which it is perceived in cuttlefish). The tendency to develop a turning bias is another instance of embryos perceiving information from the prenatal environment and adapting accordingly. *Octopus vulgaris*, another well-studied model species in the study of cephalopod behavior, also appears to show brain lateralization in adult individuals' tendency to favor one eye over the other (Byrne, Kuba, & Griebel, 2002). Further study of this phenomenon in both cephalopods and vertebrates could help reveal the selective pressures that promote the evolution of brain lateralization in complex, centralized nervous systems.

4 | HATCHLINGS AND EARLY JUVENILES

4.1 | Eclosion

Left undisturbed, *S. officinalis* eggs will typically only hatch during darkness, especially after a transition from light to dark (Paulij et al., 1991). They initiate eclosion via enzymatic dissolution of the egg envelope (Boletzky & Boletzky, 1973). However, physical disturbance of late-stage eggs (such as handling or an abrupt change in environmental conditions) can induce hatching at any time of day (Domingues et al., 2006; C.E. O'Brien, pers. obs.). Presumably, there are situations that occur in the natural environment, such as strong currents or contact with drifting objects, that could induce premature hatching. Whatever the cause of eclosion, the fact that embryos are capable of prenatal perception ensures that the cuttlefish will experience at least a minimum of transnatal sensory continuity at hatching.

S. officinalis typically measure between 6 and 9 mm in mantle length (ML) at hatching. Unlike octopuses and other cephalopods, cuttlefish do not spend any time as plankton (Nixon & Mangold, 1998) but are potentially vulnerable to strong currents. They are typically found buried in the sand if it is available, especially during the day (Boletzky, 1987). This tendency increases by 85% during the second week of life (Poirier, Chichery, & Dickel, 2004). If sand is unavailable, a juvenile will rest motionlessly on the substrate or an object in the environment (C.E. O'Brien, pers. obs.). Hatchlings are aided in this by a "ventral sucker" formed by the ventral arms and mantle that serves to fight current and maintain position on the substrate (Boletzky, 1974). This transitory sucker is an adaptation specialized for stabilization during the life stage in which the cuttlefish is most vulnerable to dislodgement due to its small size.

Hatching often occurs in areas with sand, mud, stones, algae, or seagrass (Bloor et al., 2013; Jereb & Roper, 2005; Nixon & Mangold, 1998) which give hatchlings numerous opportunities to conceal themselves (Figure 2). The tendency to hatch at night might have an adaptive purpose against visual predators, allowing them to bury in the sand or settle in a dark crevice under the cover of night and is thought to reduce predation (Paulij et al., 1991). As cuttlefish increase in size and hence swimming ability, they can affect more control over their own movements in the water column, and may disperse from the hatching site. For the first week after hatching, young cuttlefish are referred to as hatchlings (Figure 3) and thereafter as juveniles (Figure 4) until they reach 90 days of age (Hanlon & Messenger, 1988).

4.2 | Sensory abilities

Cuttlefish are thought to rely on ocular vision as their primary sense (Hanlon & Shashar, 2003). Basic visual perception is possible well

FIGURE 3 Hatchling cuttlefish (approx. mantle length 10 mm) displaying a disruptive body pattern on a uniform substrate. Photo by Anne-Sophie Darmaillacq

FIGURE 4 Three-month-old juvenile cuttlefish (approx. mantle length 65 mm). Photo by Caitlin E. O'Brien

before birth (Romagny et al., 2012), but at hatching, this ability is still maturing. This has been demonstrated experimentally during the first month post-hatching: the number of hatchlings responding to the rotation of a black, white, and grey cylinder at high speeds increased with age, indicating increasing visual ability (Cartron, Dickel, Shashar, & Darmaillacq, 2013). Likewise, visual acuity as measured by the minimum width of objects that cuttlefish are able to distinguish increases with the size of the animal (Groeger, Cotton, & Williamson, 2005). The level of visual maturity at hatching is sophisticated enough to enable hatchlings to navigate visually in their immediate environment (Jozet-Alves, Modéran, & Dickel, 2008), detect and react to other species (Shashar, Hagan, Boal, & Hanlon, 2000) and to gauge characteristics of the visual environment for body patterning (Chiao & Hanlon, 2001).

Polarization sensitivity (PS) is the ability to distinguish between different linear polarizations of light. If a cuttlefish is placed inside a rotating cylinder with alternating bars of oppositely polarized information, it will usually display an optomotor response, following the motion of the cylinder with eyes and body (Cartron et al., 2013). This apparatus has been used to demonstrate that PS appears in cuttlefish around 7 days post-hatching. This ability matured in a manner similar to that of contrast vision, with the number of individuals reacting to the rotating polarized cylinder increasing with age, although at a slower pace (Cartron et al., 2013). In squid, polarization has been shown to improve the ability to visually detect prey at a distance (Shashar, Hanlon, & deM Petz, 1998). Likewise, *S. officinalis* detect prey faster and preferentially attack them when they reflect polarized light rather than only luminance information (Shashar, Hagan, Boal, & Hanlon, 2000). PS has been hypothesized to aid with capture detection of silvery fish, transparent prey like shrimp and in low-luminance contrast situations (Shashar et al., 1998, 2000; Cartron et al., 2013). Indeed, it has been suggested that the rapid increase in prey detection observed in cuttlefish during the first week after hatching may be concomitant with the maturation of this system (Dickel et al., 1997).

While they are thought to rely primarily on vision, evidence increasingly demonstrates the role of chemoreception for cuttlefish and other cephalopods (Hanlon & Shashar, 2003). We know that this ability is functional before hatching (Romagny et al., 2012) and chemoreceptor cells are present at hatching in the suckers of the arms and tentacles as well as the region surrounding the mouth (Nixon & Mangold, 1998; Sundermann, 1983). However, the relative maturity of this system at hatching is unknown, and more experiments are needed.

Statocysts are the organs responsible for perception of gravity, acceleration, and low frequency vibrations. In addition, cuttlefish possess an analog of the lateral line system in fish, consisting of thousands of sensory hair cells (Budelmann & Bleckmann, 1988). Both of these mechanoreceptive organs are present at hatching. It has been demonstrated that month-old juveniles are able to detect a range of vibrations with one or both of these systems. Low frequency vibrations (20–600 Hz) were shown to induce defensive behaviors such as burrowing, changes in body pattern and movement, although not at every frequency in that range (Komak, Boal, Dickel, & Budelmann, 2005). Interestingly, juvenile cuttlefish (1–3 months old) could not be habituated to vibrational stimuli, even after five consecutive presentations. This is consistent with the hypothesis that mechanoreception plays a role in predator detection, since habituation would be highly inappropriate in this context. Between the ages of 1 and 3 months, juveniles show a decrease in responsiveness to vibrational stimuli. At 3 months of age they cease burrowing in response, despite the fact that the sensory acuity of their statocysts and lateral-line analog is thought to increase with size (Budelmann, 1995). This contrast implies that defensive behavior, especially burrowing, may become less relevant as the cuttlefish increases in size (see later discussion), although mechanoreception continues to play a defensive role (Komak et al., 2005).

4.3 | Body patterning and defense

Juveniles' primary ecological challenge is avoiding predators. Defensive tactics fall into two categories: primary defenses to prevent detection and secondary defenses to affect escape if primary defenses fail (Cott, 1941; Hanlon & Messenger, 1996). Primary defenses are mainly cryptic, including countershading, deceptive resemblance and camouflage. Secondary defenses include inking, jetting, and threat displays (Ferguson & Messenger, 1991; Ferguson, Messenger, & Budelmann, 1994; Hanlon & Messenger, 1988, 1996). Most of the known predators of juveniles are visual hunters (Hanlon & Messenger, 1996), making body patterning a critical aspect of defense.

Body patterns in cuttlefish are created with numerous patches of pigmented cells (chromatophores). When contracted, the pigment of the chromatophore is obscured, creating a light patch. When expanded, the pigment becomes visible and creates a dark patch. Expansion and contraction of these cells are controlled via direct innervation from the brain (Florey, 1969). Different groups of chromatophores are expanded or contracted in unison to create 33 chromatic components. The most prominent of these components is a large white square in the center of the dorsal mantle (see illustrations in Hanlon & Messenger, 1988). These components combine to form a continuum of 13 formally-defined stereotypical body patterns. Among these, there are three main categories of body patterns used to achieve primary crypsis: uniform (entirely dark or light), mottle (a mixture of small dark and light patches), and disruptive (a mixture of large dark and light patches) (Hanlon & Messenger, 1988). Chromatophores begin to appear in stage 25 embryos (Andouche, 2013) and the brain structures controlling body patterning (optic lobes, lateral basal lobes, and chromatophore lobes) are well-developed but not fully mature at hatching (Dickel et al., 1997). As a result, hatchling cuttlefish are capable of almost the entire repertoire of adult body patterns (10 out of 13, Hanlon & Messenger, 1988). This situation differs from that of many Loliginid squid and octopuses, which have few chromatophores at hatching (Fioroni, 1990) and cannot produce full body patterns (Hanlon & Messenger, 1988).

After leaving the egg clutch, a hatchling in the English Channel may settle on a uniform dark background such as mud or a uniform light background such as sand (Blanc et al., 1998). Alternatively, it may come to reside on a heterogeneous background consisting of combinations of algae, rock, shell debris, sand, and mud (Hanlon & Messenger, 1996). In the laboratory, the uniform pattern can usually be prompted by a solid colored artificial background or sand. The mottle pattern is elicited by gravel and artificial checkerboards with squares 4–12% of the size of the juvenile's own white square, while the disruptive pattern is induced by small rocks and squares 40–120% of the size of the cuttlefish's own white square (Barbosa et al., 2007; Mäthger & Hanlon, 2007). *S. officinalis* appears to be employing a rule based on the size of nearby objects and its own increasing size. Notably, this is accomplished without color vision (Mäthger, Barbosa, Miner, & Hanlon, 2006; Messenger, 1977) and without any visual feedback from its own body pattern (Barbosa et al., 2007).

Despite their ability to display almost the entire range of body patterns, newly-hatched cuttlefish often show the disruptive pattern on uniform backgrounds (Figure 3; Dickel et al., 2006; Hanlon & Messenger, 1988; Poirier, Chichery, & Dickel, 2005). Young *S. pharaonis* will also display the disruptive pattern on uniform background (but see discussion below) (Lee, Yan, & Chiao, 2010). This seemingly "inappropriate" behavior may be explained by the fact that cuttlefish have other strategies for crypsis besides simple background matching, including disruptive coloration, deceptive resemblance or a hybrid of the two (Cott, 1941; Hanlon & Messenger, 1988; Hanlon et al., 2009). A hatchling displaying a disruptive body pattern on a uniform background may be unable to produce a uniform pattern or it may be attempting deceptive resemblance of a stone or shell fragment (Hanlon & Messenger, 1988; O'Brien et al., 2016). It is difficult to interpret a cuttlefish's strategy since any particular body pattern may be employed in multiple strategies and several strategies may serve equally well in a given situation. Additionally, the strategy employed by a cuttlefish changes with body size (Hanlon & Messenger, 1988; Lee et al., 2010), the distance of a perceived threat (Shohet, Baddeley, Anderson, & Osorio, 2007) and the type of threat detected (Adamo, Ehgoetz, Sangster, & Whitehorne, 2006; Langridge, 2009; Langridge, Broom, & Osorio, 2007; Staudinger et al., 2013).

Any potential deficiency in crypsis may be partially compensated for by the tendency of *S. officinalis* and *S. pharaonis* to rest on contrasted and black backgrounds when given a choice (Lee, Yan, & Chiao, 2012; Poirier et al., 2004). In particular, *S. officinalis* hatchlings have been observed to settle on the egg clutch from which they recently hatched. On the dark membrane, their tendency to produce disruptive body patterns is sufficient to achieve partial camouflage to the human eye (Dickel et al., 2006). Indeed, human observers releasing hatchlings and juveniles into the field very find them difficult to locate once they have settled on the substrate (Hanlon & Messenger, 1988). In any case, the "inability" to display uniform body patterns and the preference for dark and contrasted substrates disappears after a few months (Allen, Mäthger, Barbosa, & Hanlon, 2009; Hanlon & Messenger, 1988; Poirier et al., 2005). It is possible that this delay in camouflage ability reflects further brain maturation, particularly of the optic lobes (Dickel et al., 1997), and that the preference for dark/contrasted substrates is an adaptation to compensate in the meantime. Further bolstering the idea that this improvement is a reflection of brain maturation is the fact that both *S. officinalis* and *S. pharaonis* from socially- and/or environmentally-enriched backgrounds show different camouflage efficiency than individuals raised in comparatively impoverished conditions (Dickel, Boal, & Budelmann, 2000; Lee et al., 2010; Poirier et al., 2004, 2005).

Juveniles face a diverse set of potential predators with varying sensory acuity and attack strategies, especially teleosts (Blanc & Daguzan, 1999; Hanlon & Messenger, 1988; Langridge et al., 2007; Le Mao, 1985). Naïve cuttlefish are able to distinguish between these predators and other non-predatory fish the first time they encountered them in the field (Hanlon & Messenger, 1988; Staudinger et al., 2013), only displaying body patterns to visual predators and chemosensory ones (Langridge et al., 2007). The tactics and body patterns used for primary and secondary defense change as cuttlefish age and grow. For instance, the deimatic display, consisting of paling, freezing and flattening of the body and the sudden appearance of dark spots on the dorsal mantle, undergoes a metamorphosis during growth: Hatchlings and young juveniles incorporate four pairs of spots into this display, but when the cuttlefish grows to about 3.5 cm ML, the display changes to just one pair of distinct "eyespots" (Hanlon & Messenger, 1996). This pattern is thought to act as a startle or intimidation display, and hatchlings and young juveniles will use it when they encounter a non-predatory fish species (Hanlon & Messenger, 1996; Langridge, 2009). The two-spot manifestation of the pattern is very similar to the deimatic display observed in other cephalopods (e.g., *Octopus bimaculoides*, *Sepioteuthis sepioidea*). Other body patterns expand and take on social meaning in late juvenile and adult cuttlefish (Hanlon & Messenger, 1988, 1996). For instance, the "zebra pattern" (used both as a social signal and a potential form of crypsis), only appears in sexually-mature cuttlefish (Hanlon & Messenger, 1996). The disruptive pattern also changes with age: The number of chromatic components expressed by *S. officinalis* increases over time (Poirier et al., 2005), although the overall strength of expression of the disruptive pattern weakens and the combinations of chromatic components displayed change (Barbosa et al., 2007). Interestingly, the case is the reverse in *S. pharaonis*: like *S. officinalis*, it tends to display the disruptive pattern even on uniform substrates, but unlike *S. officinalis*, shows an increase in the number of disruptive components expressed with age and growth (Lee et al., 2010). Clearly, much remains to be resolved in our interpretation of cuttlefish body patterning.

4.4 | Other defensive behavior

In addition to their camouflage abilities, hatchlings and juveniles possess several defensive behaviors that do not involve body-patterning, although body patterning defense is usually employed concurrently (Hanlon & Messenger, 1996). From the moment of hatching, they are able to use their funnel and fins to dig a shallow depression and cover themselves with sand (Boletzky, 1974; Mather, 1986), while capable of burying, not all hatchlings do so immediately. While capable, and body patterning is often used in conjunction with this tactic. Burrowing entails a three-step sequence that lasts about 5 s, and appears to be prompted by exposure to light and contact with a sandy substrate (Mather, 1986). The behavior can seem highly fixed, with one act highly likely to be followed by the next in the sequence, but in reality a number of external factors are known to modify the pattern (Mather, 1986). For instance, early experience with a sandy bottom improves later burrowing abilities, and the propensity to attempt burrowing increases with age during the first 2 weeks of life (Poirier et al., 2004).

Like many other benthic cephalopods, *S. officinalis* is innately shelter-seeking and photophobic (Nixon & Mangold, 1998). They are not known to establish a den as many species of octopus do, but they will take advantage of objects in the environment for concealment. Unlike fishes and some of their more gregarious decapod relatives (squid), *S. officinalis* has no propensity to group or school (Hanlon & Messenger, 1988), though the limited swimming and dispersal abilities of juveniles sometimes results in the formation of small clusters (C.E. O'Brien. pers. obs.). Instead, they tend to spend the Day buried in the sand or motionless on the surface of the substrate.

Hatchlings also possess a number of secondary defensive tactics that do not involve body patterning. For instance, recent evidence suggests that cuttlefish may be able to counter the electrical detection by sharks and other non-visual predators with a "freeze" response (Bedore, Kajiura, & Johnsen, 2015). Whether this occurs in hatchlings and juveniles has yet to be determined. From hatching, they are also capable of inking and rapid escape via jet propulsion (Bather, 1895). Ink can be used in two ways: as a "smoke screen" to disappear behind or as a pseudomorph, a decoy to misdirect a predator (Hanlon & Messenger, 1996). In conjunction with ink ejection, the forceful expulsion of water from the siphon permits rapid movement away from predators. Often, juveniles will escape via a path that is highly erratic, thus making it difficult for the predator to predict the cuttlefish's location (Hanlon & Messenger, 1988). In confined situations, after ejecting several globules of ink and jetting to another location, a young cuttlefish will sometimes return to a globule of expelled ink and cling to its underside, effectively camouflaging itself as its own ink (C.E. O'Brien, pers. obs.). This behavior is also seen in at least one species of octopus (Moynihan, 1985). Overall, juvenile defensive behaviors are equivalent to those of adults except in scale. As they grow, cuttlefish achieve size refuge from certain predators (Sogard, 1997), while becoming a more attractive meal for others (Bloor et al., 2013).

Overall, the defensive tactics of cuttlefish appear to serve them well. In one of the few existing field experiments with *S. officinalis*, primary crypsis was sufficient to prevent detection by fishes that came into proximity in 40 observed instances (Hanlon & Messenger, 1988). In several dozen instances in which a juvenile was detected by the comber (*Serranus cabrilla*), only 17.1% of attacks were successful (Hanlon & Messenger, 1988). More field experiments and observations of this kind are needed in order to better understand the types and extent of predation pressure experienced by cuttlefish during the first few months of life.

4.5 | Predation

In the late stages of embryonic development, yolk is transported from the outer sac to the inner via the yolk collar (Boletzky, 1983; Boucher-Rodoni, Boucaud-Camou, & Mangold, 1987). Hatchlings are born with internal and sometimes external yolk remaining (Wells, 1958). If present at hatching, the external yolk sac is quickly shed, usually within minutes of eclosion (C.E. O'Brien, pers. obs.), but the internal sac remains for several days (Bouchaud, 1991; Dickel et al., 1997) and intracellular digestion of the yolk continues. The amount of yolk at hatching depends on prenatal temperature and the rate of embryonic development (Dickel et al., 1997). Those that develop at higher temperatures are smaller at hatching (Boletzky, 1994; Bouchaud, 1991; Dickel et al., 1997). For 2–5 days after hatching, hatchlings do not hunt, subsisting on internal reserves and growing relatively slowly (Boucaud-Camou, Yim, & Tresgot, 1985; Messenger, 1973; Nixon, 1985; Richard, 1971; Wells, 1958).

Hatchlings usually begin feeding on prey before their yolk is entirely exhausted (Boletzky, 1975, 1987; Dickel et al., 1997; Wells, 1958). If juveniles have not been able to feed by the fifth day, their cuttlebone becomes positively buoyant, rendering them unable to hunt and they quickly perish (Boucher-Rodoni et al., 1987). At hatching, the digestive gland is not yet fully mature, and it is the initial consumption of food that prompts maturation (Boucher-Rodoni et al., 1987; Yim & Boucaud-Camou, 1980). For several days, prey consumption and yolk absorption continue concurrently (Blanc et al., 1998) and growth proceeds rapidly (Boucaud-Camou et al., 1985). It is unclear why cuttlefish begin consuming food before total yolk absorption. One hypothesis is that initial prey captures are a form of "practice" for hatchlings, in which they refine their predatory abilities during a period in which food consumption is not essential. This possibility is backed by the fact that hatchlings exhibit a very high rate of aborted captures (Dickel et al., 1997).

Most encounters with prey follow a stereotypical sequence initiated by the sight of a prey-shaped stimulus (Wells, 1958): detection, orientation (with or without pursuit) and capture (Messenger, 1968). Detection is marked by the focusing of the eyes, and followed by orientation of the head and whole body toward a potential prey item. The cuttlefish then swims (usually by gently undulating its fins) to within about body length of the prey. At this point, the cuttlefish can employ one of two capture strategies: a "tentacle strike" or a "jumping" attack (Messenger, 1977). In the former, the tentacles are rapidly extended from a pouch below the eyes toward the prey. The suckers on the tentacle clubs adhere to the prey and bring it to the mouth when the tentacles are retracted. In the jumping attack, the cuttlefish positions itself behind the crab (away from the claws) and pounces on it with all eight arms. It then rotates the crab into a position which allows it to bite the junction between the periopods and the main carapace (Chichery & Chichery, 1988). Their saliva contains a toxin which quickly paralyzes the crab, enabling easy consumption.

Cuttlefish employ the tentacle ejection strategy for shrimp and small crabs, and the jumping strategy for large crabs. The "jumping" attack necessitates handling of the crab for proper positioning and to avoid damage from the claws (Chichery & Chichery, 1988). This strategy is thus more time consuming (50 s or more) than a tentacle attack (<300 ms), leaving cuttlefish more vulnerable to their own predators (Chichery & Chichery, 1992). The choice between these two strategies is thus influenced by personal risk, prey size, and prey form, and may also be affected by the speed with which a prey item can potentially escape: regardless of size, fish (which have a rapid escape response) were always captured with the tentacles (Chichery & Chichery, 1992). While both strategies appear pre-programmed, they can also be modified through experience. After the majority of cuttlefish first attacked crabs from the front, leaving them susceptible to pinches from the crabs' claws, the majority later used a dorsal attack (Boal, Wittenberg, & Hanlon, 2000; Dickel, 1997).

Juvenile cuttlefish capture and digest prey items rapidly (Yim & Boucaud-Camou, 1980), consuming about 40% of their body weight daily (Choe, 1966). Rapid consumption, in turn, fuels rapid growth. As in many other marine species, rapid growth can promote survival by minimizing the time to achieve size refuge from certain predators (Sogard, 1997). As in embryos, growth in juveniles can be strongly affected by environmental factors: cooler water temperatures can slow the rate of metabolism (Forsythe et al., 1994) while the use of dark rearing tanks has been shown to increase growth in hatchlings and juveniles (Sykes, Domingues, Márquez, & Andrade, 2011). As early juveniles, cuttlefish typically consume only shrimp, but between the first and second month of life will expand their diet to include crabs and small fish (Blanc et al., 1998; Le Mao, 1985). Notably, this is concurrent with the maturation of the digestive gland (Yim & Boucaud-Camou, 1980), but may also reflect neural maturation and the refinement of attack strategies.

4.6 | Sleep

Juvenile cuttlefish display a nocturnal pattern of activity, with movement peaking during the night (Frank, Waldrop, Dumoulin, Aton, & Boal, 2012), and the Day mostly spent buried or camouflaged (Hanlon & Messenger, 1988). Two potential forms of sleep are present: homeostatically-regulated periods of quiescence as well as a quiescent state associated with rapid eye movement, expansion and contraction of the chromatophores and arm movements that resembles the REM sleep of vertebrates (Frank et al., 2012). The latter is a continuation of the MAS behavior seen in embryos and occasionally escalates to "acting out" of waking behaviors (Corner, 2013b). Evidence for the importance of this behavior comes from experiments showing that when deprived of the ability to rest for 48 hr, the cuttlefish spends more time resting in the subsequent 24 hr, presumably to compensate for the deprivation (Frank et al., 2012). Hatching marks the advent of wake-like behavior, and with age, the incidence of sleep decreases, while wake-like behavior increases (Corner, 2013b).

In contrast to cuttlefish, *O. vulgaris* does not develop sleep-like behavior until well after hatching. Still, the presence of sleep-like behavior in cuttlefish and other invertebrates is interesting from a phylogenic perspective, since it has established that sleep is a feature universal to all animals (Corner, 2013a), and thus probably of early evolutionary origin. The subject of invertebrate sleep is just beginning, and *S. officinalis* is an ideal model with which to study it.

5 | ASSOCIATIVE LEARNING AND MEMORY

Associative learning is defined as a learned link between two events or between a behavior and its consequences (Bouton, 2007). There is a growing body of literature documenting this sophisticated ability in cuttlefish and other invertebrates including octopuses (e.g., Wells, 1968; Young, 1961), gastropods (e.g., Sahley, Rudy, & Gelperin, 1981; Walters, Carew, & Kandel, 1981), bees (e.g., Couvillon & Bitterman, 1980), insects (e.g., Dukas, 1999), and worms (e.g., Avarguès-Weber, Deisig, & Giurfa, 2010; Rankin, Beck, & Chiba, 1990).

In cuttlefish, this phenomenon was first demonstrated in adults and subadults (Darmaillacq, Dickel, Chichery, Agin, & Chichery, 2004) using a taste aversion paradigm: 81% of cuttlefish preferred crabs 1–3 days after attacking a shrimp coated with an unpleasant chemical (quinine). It seems that they had learned to associate their normally-preferred prey (shrimp) with a negative consequence (unpleasant flavor). Associative learning has also been demonstrated in juvenile cuttlefish using a paradigm known as the "prawn in a tube" (PIT) test: a shrimp is placed in a clear tube (glass or plastic) and offered to the cuttlefish. Because of the tube, cuttlefish are able to see the shrimp but unable to capture it despite vigorous attempts to do so. Experiments in which a dishabituatory stimulus failed to reverse PIT learning indicate that this task is learned through association not habituation (Agin, Chichery, Dickel, & Chichery, 2006; Purdy et al., 2006). Other experiments used animals with tentacles surgically removed (Messenger, 1973) and another involved the crab "jumping" strategy instead of tentacle ejection (Cartron, Darmaillacq, & Dickel, 2013). This research confirmed that the associative learning in this task results from an association between the presence of the tube and the lack or food reward, rather from any pain that might be experienced during a failed capture. Cuttlefish are able to detect differences in the polarization of light (polarization vision) and this enables them to detect the presence of the tube (Cartron et al., 2013; Dickel, Darmaillacq, Jozet-Alves, & Bellanger, 2013).

After several unsuccessful attacks on the inaccessible prawn in the tube, adult cuttlefish are able to remember the association for several minutes (Messenger, 1973; Wells, 1958, 1962). If presented a shrimp in a tube between 20 and 60 min after learning, they attack again as if never having learned the task, but if presented a shrimp an hour or more after learning, they again remember not to attack (Messenger, 1971, 1973). This pattern is thought to result from separate short-term and long-term memory (STM and LTM) processes (Dickel, Chichery, & Chichery, 1998).

In contrast to adults, cuttlefish less than 8 days old will continue to attack an inaccessible shrimp in a tube for hours, showing that they have no ability to acquire an association between the presence of the tube and a lack of reward (Agin, Poirier, Chichery, Dickel, & Chichery, 2006; Dickel et al., 1998). After this age, cuttlefish display fully-operational STM (retention for <5 min) with either a 5 or 20 min training session (Agin, Dickel, Chichery, & Chichery, 1998; Dickel et al., 1998). By contrast, a separate LTM (retention for 1 hr or more) emerges about 15 days after hatching and continues to improve over the next several weeks: the ability to retain learning for 1 hr retention reaches maximum levels around 60 days (Dickel et al., 1998), while 24 hr retention matures around 90 days of age (Dickel, Chichery, & Chichery, 2001). Likewise, experiments manipulating environmental enrichment (discussed in the next section) early in life indicated that the period between the first and second month after birth is particularly influential in the development of memory (Dickel, Boal, & Budelmann, 2000). That STM and LTM are two distinct processes is supported by the fact that the regulation of cholinergic enzymes occurs via different mechanisms in STM and LTM (Bellanger, Dauphin, Chichery, & Chichery, 2003).

One question that naturally arises when comparing associative learning and memory with non-associative learning in cuttlefish is the difference in emergence times: imprinting and habituation are possible as early as the prenatal period, while associative learning and memory do not appear for 2 weeks or more. Physiologically, we can point to isometric differences in the development and maturation of the brain lobes associated with certain tasks as potential explanations for these emergence times. Imprinting and habituation are thought to involve brain structures functional before hatching: the optic, basal, and peduncle lobes (Dickel, 1997; Darmaillacq, 2005; N. Mezrai, unpub. data). By contrast, associative learning and memory require the vertical complex (the VL, SFL, inferior frontal lobe, and vertical–subvertical lobe tracts). The vertical lobe complex continues to mature after hatching, increasing 1.7 times in size, much more than the rest of the brain, although the growth is not as great as that of *O. vulgaris*, which shows a 2.5 increase in VL volume (Agin et al., 2006; Dickel et al., 1997, 2001, 2006; Grant, Tseng, Gould, Gainer, & Pant, 1995; Nixon & Mangold, 1998). Additionally, staining with phosphorylated neuro-filament of high molecular weight (NF-H), a marker of neural stability, shows that the VL is still undergoing maturation: none is present in embryos, while adults show a high concentration of NF-H and newly-hatched cuttlefish show only a little (Dickel, 1997; N. Mezrai, unpub. data).

Latencies associated with predation behavior also appear to be explained by isometric differences in brain development. The initial emergence of predatory behavior between 3 and 5 days appears to be correlated with the appearance of the fiber tract between the VL and sub-VL (Dickel et al., 1997). Likewise, hatchlings at first also show long latencies to attack when prey is introduced, a delay that lessens with each subsequent attack (Wells, 1958). This is also probably a reflection of VL maturation. It is worth noting that in this case, "maturation" is only associated with a decrease in attack latency (the time between the detection of the shrimp and capture) and not an increase in accuracy or speed of capture (Wells, 1958). Thus, this delay does not seem to reflect a deficiency in perceptual abilities but rather a lag in behavioral reaction. Finally, although cuttlefish are able to detect and capture prey by Day 3, their ability to pursue prey if it leaves the visual field only develops later (Sanders & Young, 1940). The neural substrates thought to be needed for basic predation behavior (detection, orientation, and capture) are the peduncle, basal, and optic lobes, all of which are mature at hatching (Dickel et al., 1997, 2001). Prey pursuit requires STM, which occurs around 8 days (Dickel et al., 1998) and is correlated with the advent of the VL/sub-VL tract (Dickel et al., 1997).

The immaturity of the VL complex and delay in learning and remembering the negative consequences of a behavior may be ecologically adaptive to the cuttlefish (Darmaillacq et al., 2014). Early predation experiences probably have a strong effect on developing cuttlefish (primacy effect—Burghardt & Hess, 1966). As they are small and inexperienced, these early predation experiences may be unsuccessful and even involve injury to the cuttlefish. Thus, there may be a danger that prey will become associated with a lack of reward or aversive stimuli, which could permanently deter them from pursuing prey. The lack of associative memory before 8 days eliminates this potential. Additionally, the lack of STM which prevents them from pursuing prey that leave the visual field limits them to a "lie in wait" (rather than actively-searching) predatory strategy (Dickel et al., 2006) that renders them less likely to attract the attention of predators.

6 | CONCLUSION

6.1 | Behavioral plasticity

Traditionally, molluscs were thought to have highly rigid, innate, and pre-programmed behavioral regimes. However, for cuttlefish (and other cephalopods), it seems that most behaviors are partially innate and partially plastic. For instance, the actions of burying are highly stereotyped but the latencies to burrow and the durations of particular steps vary with grain size (Mather, 1986). Likewise, assessments of covering abilities reveal that sand burrowing abilities are partially experience-dependent (Poirier et al., 2004). Similarly, after an initial encounter with crabs, most hatchlings learned not to use a frontal attack and instead attack from behind, avoiding the claws (Dickel, 1997). Other behaviors, such as body patterning and predation, also appear to have pre-programmed and plastic facets: different camouflage patterns, postures, and strategies (e.g., inking), while highly stereotyped in their expression, are used in predator-specific contexts (Adamo et al., 2006; Langridge, 2009; Langridge et al., 2007; Staudinger et al., 2013). In the related *S. pharaonis*, exposure to a contrasted substrate as a young juvenile changes later body patterning expression (Lee et al., 2010) and substrate preference (Lee et al., 2012). It seems that initially, juveniles display very stereotyped behavior (e.g., chronic disruptive pattern, no associative learning, no retention). However, with prenatal and early postnatal modifications of prey preference (Darmaillacq, Chichery, Poirier et al. 2004, Darmaillacq, Chichery, & Dickel, 2006, Darmaillacq, Chichery, Shashar et al. 2006), the expansion of the diet around 1 month (Wells, 1962), the increasing range of body patterns (Hanlon & Messenger, 1988), increasing learning and memory abilities (Dickel et al., 1998, 2001) and use of multiple predation strategies (Dickel et al., 1997), behavioral plasticity increases dramatically between the first and second month of life. Experiences prior to this period of plasticity (late prenatal stages and first month of life) are probably critical to the development of these behavioral responses. The goal going forward is to further quantify the role of plasticity and learning in the development of cuttlefish behavior.

6.2 | Implications of artificial rearing

It is judicious to exercise caution when interpreting results from lab-reared animals. First, laboratory conditions may induce behaviors that are not at all adaptive to the natural environment (e.g., reduced reactivity to stressful stimuli). Second, we must consider the fact that cuttlefish hatched in the lab do not undergo natural selection. Cuttlefish in the wild are subject to strong ecological challenges (such as predation and starvation) that quickly eliminate numerous "unfit" hatchlings from the population and leave only a few "fit" individuals. It would be better to conduct behavioral experiments on individuals that survive the gamut of natural selection. Unfortunately, it is very difficult to do this when studying cuttlefish, since the surest means of obtaining a large number of juvenile subjects is to collect eggs. This does not negate the utility of cuttlefish as models, since it applies to many of the animals currently used in research, but must be considered when applying conclusions from laboratory experiments to the natural world.

Thirdly, the natural environment provides numerous sources of stimulation not present in an artificial setting (e.g., epibionts, currents, predators, and prey), and the plasticity of this species manifests in response to the individual experience of each cuttlefish. However, a recent experiment did not find any effects of a standard artificial incubation environment on some basic measurements of growth and behavior in hatchlings (O'Brien et al., 2016). The conclusions of this study were constrained by the fact that embryos had to be removed from the stimulation of the natural environment during their most sensitive period (the last 2 weeks of embryonic development). Thus, if it is the case that the effects of stimulation by a natural incubation environment occur in the last few days of embryonic development, our experiment would have missed them. It is also possible that differences due to prenatal enrichment would have manifested later or in different behaviors than were investigated (O'Brien et al., 2016).

6.3 | Enrichment and welfare

One way to counteract any potential deficits associated with rearing in captivity and improve the quality of experimental data is environmental enrichment. This is defined as providing stimuli (e.g., environmental complexity, novel objects, other organisms, cognitive challenges) that promote the psychological and physiological health of an animal in captivity by allowing it to express behaviors natural to its species (Newberry, 1995). It is generally recognized that enrichment and other such measures aimed at reducing animal distress enhance not only animal welfare, but also the quality of scientific data as well (Andrews et al., 2013). Enrichment may also be a way to promote behavioral plasticity, one of the features that make cuttlefish such interesting subjects of study.

Enrichment seems to improve the efficiency of defensive behaviors in cuttlefish. Juvenile *S. officinalis* reared in tanks enriched with a layer of sand as substrate showed better burrowing abilities (shorter latencies to bury and more complete burying) than juveniles that had only experienced a bare tank (Poirier et al., 2004). Likewise, juveniles raised in social conditions against a variegated background seemed to show improved body patterning abilities compared to those raised in uniform, non-social conditions: displaying an increasing number of disruptive components against a variegated background and more readily adapting to a uniform background with age (Poirier et al., 2005). In experiments with a related species, *S. pharaonis*, juveniles were raised with a varied (checkerboard) or uniform substrate. At 2.5 and 3 months, juveniles raised with the disruptive substrate showed better background matching (stronger disruptive patterns, but see previous discussion in "Body Patterning and Crypsis")—against a checkerboard background than those reared with a uniform substrate (Chiao et al., 2010; Lee et al., 2010). In addition, the expression of N-methyl-D-aspartate (NMDA) receptors—critical to activity-dependent plasticity in the optic lobes—seems to be affected by the contrast of the rearing background in *S. pharaonis* (Lee, Chang, Yan, & Chiao, 2013). Enrichment is also associated with better learning and memory. Juveniles raised with other cuttlefish, sand, and obstacles in the environment were shown to have better memory retention of a learned task (PIT test) than those raised in bare tanks alone or in bare tanks with conspecifics (Dickel et al., 2000). Social enrichment alone was also associated with some memory improvements, but not to the same degree as the combination of enrichment types (Dickel et al., 2000). Finally, growth of *S. officinalis* was higher in socially-enriched conditions, regardless of the presence of objects in the tank, although this was not the case in *S. pharaonis* (Dickel et al., 2000; Lee et al., 2010). The increased growth in *S. officinalis* is thought to be due to an increased level of alimentary motivation induced by the presence of conspecifics (Dickel et al., 2000). These results demonstrate three things as follows: (a) that different types of enrichment affect different aspects of cuttlefish behavior including crypsis, predation, learning, and memory; (b) that there can be an additive effect of environmental enrichment on cognitive abilities; and (c) experiments investigating the progression of learning and memory in juveniles may actually underestimate natural development, since the stimulation and enrichment of the environment are absent.

Research with cephalopods in Europe is now regulated by the European Union. Directive 2010/63/EU mandates that in addition to having basic physical needs met, animals must be provided with "sufficient complexity," "control and choice," and species-appropriate environmental enrichment that is "regularly reviewed and updated." Currently, this directive covers all cephalopods after hatching (Andrews et al., 2013), and thus does not encompass any stage of embryo. However, the existence of prenatal learning and

TABLE 1 A summary of development in *Sepia officinalis*

	Late embryo (stages 23–30)	Hatchling (0–7 days post hatching)	Early juvenile (1–9 weeks)	Late juvenile (10–17 weeks)
Living conditions	(From inside egg) perceives chemosensory, visual and tactile stimuli from immediate environment	Usually on or near the egg, using ventral sucker to adhere to substrate	Some dispersion from egg mass, on the benthos, using ventral sucker to adhere to substrate	Benthos. Substrate may consist of gravel, sand, algae or a mixture thereof
Sensory capabilities	Stage 23: chemosensory and tactile Stage 25: ocular perception of visual stimuli	Contrast and polarization vision functioning but not fully mature, state of chemoreception, statocysts and mechano-receptors unknown	Sensory acuity continues to increase with size	Mature
Feeding	Inner and outer yolk reserves	Inner yolk reserves	Usually shrimp, sometimes crabs	Shrimp, crabs, fishes
Defense	Egg membrane and low activity	PRIMARY: semi-successful camouflage, SECONDARY: inking, escape jetting	PRIMARY: camouflage, burying, shelter-seeking SECONDARY: 8-spot deimatic display, inking, escape jetting	PRIMARY: camouflage, burying, shelter-seeking SECONDARY: 2-spot deimatic display, inking, escape jetting
Learning and memory	Prey imprinting and habituation possible	Prey imprinting and habituation possible	Increasing STM, LTM, and associative learning	Fully mature STM, LTM, and associative learning

Modified from Dickel et al. (2006). Sources are cited throughout the text.

adaptation in cuttlefish may indicate that environmental enrichment during the prenatal period may be necessary for proper development and welfare. Continuing research of the perinatal period would do much to address the knowledge gaps that hinder the development of objective criteria to identify signs of suffering and distress in cephalopods (Fiorito et al., 2014, 2015; Smith et al., 2013). Such inquiry will have practical benefits for aquaculture, restocking, comparative and developmental psychology, and the general study of behavior.

6.4 | Future research

S. officinalis has served as a model organism for the study of invertebrate and animal behavior for decades, yet many basic questions have still to be resolved. Some of the basic characteristics of this species are summarized in Table 1. Throughout this review, we have highlighted some of these questions in order to encourage further study. Such research will yield insight into the evolution of animal behavior from an uncommon perspective. In addition to its potential theoretical contributions, research into the behavioral ontogeny of this species will undoubtedly lead to knowledge that can be used to improve animal welfare, the quality of scientific research, and aquaculture yields.

The relative accessibility of developing embryos is a particular asset to the study of prenatal behavior. Continued characterization of the remarkable sensory and learning abilities of cuttlefish embryos will contribute to our understanding of learning in general. Other questions, such as those regarding the presence of a tranquilizing compound in the PVF and factors controlling the time of hatching, promise to reveal previously unsuspected influences on prenatal development. The high fecundity and hatching success of *S. officinalis* is another major asset of this species, since high numbers of subjects are needed to address questions about individual differences. Focusing on some of the specific topics highlighted here, such as the stereotypy of body patterning and the extent of associative learning, could help reveal the interaction between phenotypic/behavioral plasticity and genetically pre-programmed reactions in shaping individual behavior.

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Annexe 3

O'Brien, C. E., Bellanger, C., Jozet-Alves, C., **Mezrai, N.**, Darmaillacq, A. S., & Dickel, L. (2018). Stressful conditions affect reproducing cuttlefish (*Sepia officinalis*), reducing egg output and quality. *ICES Journal of Marine Science*.

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Stressful conditions affect reproducing cuttlefish (*Sepia officinalis*), reducing egg output and quality

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O'Brien, C. E., Bellanger, C., Jozet-Alves, C., Mezrai, N., Darmaillacq, A.S., and Dickel, L. Stressful conditions affect reproducing cuttlefish (*Sepia officinalis*), reducing egg output and quality. – ICES Journal of Marine Science, doi:10.1093/icesjms/fsy115.

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Stress has profound effects on animals, particularly if it occurs during reproduction or embryonic development. The cuttlefish *Sepia officinalis* is an economically and scientifically important species that is often cultured in captivity. The effects of stressors applied to reproducing females and developing embryos were investigated and compared by measuring the number and quality of eggs produced by females in a typical captive setting with females subjected to stress during egg-laying (confined space and repeated removal from the water) as well as comparing the hatching size and success of embryos subjected to naturally occurring (predator cues) and artificial (random bouts of bright LED light) stressors. Stressed females produced significantly fewer eggs and fewer of those eggs hatched. In addition, nearly a quarter of stressed mothers laid mostly white eggs lacking the dark pigment typical of this species. In contrast to maternal stress, stressors applied directly to embryos had no effect on hatching rate and neither maternal nor embryonic stress was associated with differences in hatching size. Our results suggest that reducing stress during egg-laying can increase aquacultural egg yields in *S. officinalis* and that the presence of pigment-less (white) eggs is a sign of maternal stress during egg-laying.

Keywords: cuttlefish, egg-laying, faecal corticosterone, hatching rate, LED light, maternal stress, predator cues.

Introduction

Organisms have evolved to react to potentially threatening phenomena in their environment (e.g. predators, changes in environmental parameters, unfamiliar sensory stimuli) or to anticipated pain or suffering with physiological or behavioural responses intended to mitigate the resulting negative consequences. Such responses are referred to as "stress" (occurring in response to "stressors"), and involve a variety of physiological changes with which the organism attempts to avoid harm (Schreck *et al.*, 2001). For example, in numerous genera of marine gastropods, the presence of a shell-crushing predator induces defensive features in the snail's shell. In *Nucella* (*Thais*) *lamellosa* for instance, the waterborne effluent of a predatory crab (*Cancer productus*) causes shell-thickening and the development of larger apertural teeth that reduce crabs' ability to consume snails (Palmer, 1985; Appleton and Palmer, 1988). While these responses have evolved to enhance fitness in the face of predators, they also divert resources away from other life functions. In *Littorina obtusata* for example, shell-thickening in response to a crab predator (*Carcinus maenas*) reduces somatic growth, likely resulting in reduced fecundity—a trait directly linked to body size in gastropods (Brookes and Rochette, 2007).

When stress is experienced during reproduction or embryonic development, its effects may be especially profound. In some cases, stress may ultimately increase net reproductive output or offspring success by inducing adaptive responses on the part of the mother or her offspring. For instance, when female sticklebacks (*Gasterosteus aculeatus*) sense predators while spawning, their offspring exhibit stronger anti-predator behaviour (tighter shoaling) than offspring from unstressed females (Giesing *et al.*, 2010). However, the preponderance of literature (primarily regarding fish) suggests that stress reduces reproductive output

(Braastad, 1998; Schreck, 2010). In the red gunard (*Chelidonichthys kumu*) for instance, the stress associated with capture and confinement induced the apoptotic breakdown of oocytes, limiting ultimate reproductive output (Clearwater and Pankhurst, 1997).

In fish, birds and mammals, "stress hormones," including adrenal glucocorticoids, are thought to be the mediator of such stress responses (Moberg, 1991). When exposed to stressors, animals secrete these hormones, which induce changes in behaviour, metabolism and physiology. There are numerous studies associating this "maternal stress" with changes in offspring, and evidence that stress hormones secreted by a mother in response to stress are transferred to her offspring via the placenta or egg yolk to effect some of these changes (Braastad, 1998; Welberg and Seckl, 2001; Henriksen *et al.*, 2011). In addition, the embryos of many of these species are capable of sensing the environment outside the egg or placenta to some degree (Gottlieb, 1976) and could potentially express their own stress response (i.e. secrete their own stress hormones) to external stimuli experienced during development (what we refer to as "embryonic stress").

The cuttlefish *Sepia officinalis* is a cephalopod mollusc inhabiting the eastern North Atlantic, from Norway to Mauritania including the Mediterranean Sea (Jereb and Roper, 2005). A well-established fishery and growing aquaculture interest make it economically important, and it is a model species in biological research (Bloor *et al.*, 2013). *Sepia officinalis* reproduces only once at the end of life and dies very soon after, though egg-laying may last for several days or weeks (Boletzky, 1987). In captivity, *S. officinalis* typically lays a few hundred to a few thousand eggs each (Domingues *et al.*, 2001, 2002; Correia *et al.*, 2005; Sykes *et al.*, 2006, 2009, 2013). Embryonic development lasts between 40 and 90 days, with higher temperatures accelerating embryogenesis (Bouchaud and Galois, 1990; Bouchaud, 1991) but usually yielding smaller hatchlings (Gauvrit *et al.*, 1997). Eggs develop outside the mother and there is no direct parental care during embryonic development or after (Bloor *et al.*, 2013). Stress responses have scarcely been investigated in cephalopods, but there are preliminary indications that there is at least some similarity to the stress responses of vertebrates. In one study, elevated levels of the stress hormone corticosterone were measured in the faeces of the giant Pacific octopus after injection of adrenal hormone (ACTH) or saline solution (Larson and Anderson, 2010). Similarly, an acute instance of air exposure has been shown to elevate noradrenaline in the haemolymph of the octopus *Eledone cirrhosa* (Malham *et al.*, 2002). As in vertebrates, these stress hormones are thought to influence various aspects of cephalopod biology, including the secretion of reproductive hormones (Di Cosmo and Polese, 2016).

Despite interest in culturing cuttlefish, there are only a few studies on the effects of stress on female reproduction or embryonic development in this species to date, and these have focused solely on the effects of conspecific density (Correia *et al.*, 2005; Sykes *et al.*, 2013). We designed an experiment to test the effects of maternal stress on egg output, embryonic survival and growth, and to compare these to the effects of embryonic stress on embryonic survival and growth in *S. officinalis*. Since confined tank space and occasional brief removal from the water are often unavoidable aspects of cuttlefish capture and aquaculture, we used these factors as chronic and repeated acute stressors to investigate maternal stress. We expected that such treatment would reduce egg-laying, since females would likely be forced to expend resources in reacting to the stressors, and that it might also reduce hatching size and success. In a second experiment, we examined the effects of embryonic stress. Since stress responses of embryos are poorly understood, we tested both a naturally occurring and an artificial stressor to represent a range of potential sources of stress present in the natural environment and in captive settings. As a natural stressor, we selected a fish common in the English Channel that preys on small cuttlefish (Blanc and Daguzan, 1999), the seabass, *Dicentrarchus labrax* (Linnaeus, 1758). Such a stimulus should be highly relevant to developing embryos as an indicator of post-natal threat, and seabass cues were predicted to increase size at hatching, since larger hatchlings should theoretically be better equipped to evade attack (i.e. greater speed, size refuge from some predators). As an artificial stressor, we selected bouts of bright artificial (LED) light programmed to switch on for six 15 min bouts (total 90 min) timed randomly throughout the day and night on a daily-changing schedule. This was predicted to have a disruptive effect (e.g. by confusing circadian rhythms) on embryonic development, resulting in lower hatching success. Importantly, previous experiments have shown that late-stage cuttlefish embryos react to both predator odour and bright light with changes in mantle contraction rate (Romagny *et al.*, 2012), indicating a definite ability to perceive these stimuli. Moreover, hatchling cuttlefish are known to respond to environmental enrichment by accelerating growth and neural maturation (Dickel *et al.*, 2000), so it seems logical to predict that embryonic stimuli would affect embryonic growth as well. In comparing the two kinds of stress, we predicted that embryonic stressors would have a stronger negative impact on hatching rate and hatching size than maternal stressors since the very limited resources of embryos would need to be diverted away from growth to mount a stress response.

Finally, we also attempted to find a simple way to measure stress in cuttlefish, a tool that would allow us to assess the suitability of captive conditions for spawning females and eggs. Since measurement of faecal glucocorticoids is used in many species as a non-invasive way to quantify stress (e.g. Tempel and Gutiérrez, 2003; Metrione and Harder, 2011), we tested whether or not faecal corticosterone could be used to assess stress levels in *S. officinalis*. Attempts were also made to test corticosterone levels in embryos directly, but the values obtained fell below the quantification limit of the detection kit. In addition, we examined the amount of unused reproductive material (oocytes) remaining in the females at death, reasoning that stress could reduce the utilization of reproductive capacity. We predicted higher levels of corticosterone and more unused reproductive material in stressed females than in control females.

Material and methods

Females

Broodstock conditions

Throughout May, 2015, and on 10 May 2016, 39 adult female cuttlefish (*S. officinalis*) were captured by cuttlefish trap from the English Channel and transported to the Centre de Recherches en Environnement Côtier (CREC) in Luc-sur-Mer, France. They were maintained in a semi-open flow-through seawater system with a light/dark cycle matched to day length (about 14:10 h) with a mean temperature of 15 ± 1°C. Upon capture, female cuttlefish were mated with males (12 h cohabitation) caught concurrently or already present at the CREC. Females likely also possessed sperm stored after mating with males in the field, since female cuttlefish can store sperm for up to 5 months (Hanlon *et al*., 1999). Each female was fed one large or two medium-sized *C. maenas* (Linnaeus, 1758) per day.

Stress treatments

Females were partitioned into two groups. "Control mothers" (CM, $n = 19$) were maintained in large (1 000 L, 168 cm diameter, 61.5 cm height), round, blue tanks enriched with stones, artificial algae, floating objects, and shaded areas (Figure 1). In 2015, seven CM were housed individually in these conditions, and in 2016, due to a large number of females captured in 1 day, CM were housed in four groups of three (Table 1). A permutation test found no difference in the number of eggs laid per CM female between 2015 and 2016.

In both 2015 and 2016, "stressed mothers" (SM, $n = 20$) were housed individually in small, unenriched square grey bins (65 L, 80 cm × 60 cm × 40 cm) with a water depth of 20 cm. In addition, these animals were subjected to daily "handling" stress; they were gently lifted from the water using a specially made mesh platform for 10 s three times each day (30 s day^{-1} total) during randomly selected 15 min blocks between 09h00 and 18h00. During removal from the water, females would usually eject water from their siphon several times in an attempt to re-enter the water. Often, they would also ink, but usually in small amounts and decreasingly as time progressed. These stress treatments were applied from the day after capture until natural death.

After several days, females began to lay eggs. These were deposited on plastic algae, mesh supports, floating baskets, or simply dropped onto the bottom of the tank. The number of females that produced eggs was recorded both by direct observation of egg-laying and by the presence of eggs within the tank. The number of eggs was counted every morning, and the viability of eggs assessed by visual inspection. The presence of some white eggs (membrane lacking ink) was noted. Some of the eggs produced were too small to be fertilized or showed obvious malformations. Previous experience had shown that such eggs do not develop and these were discounted. We incubated the eggs (see following section) and measured the hatching rate and hatching size of offspring. Only eggs that were part of a cohort of at least 50 eggs laid after at least 1 week of treatment were used in assessments of hatching success and size to ensure sufficient representation of each mother or group of mothers (CM $n = 8$; SM $n = 9$) and sufficient time for the manifestation of any treatment effects (Table 1).

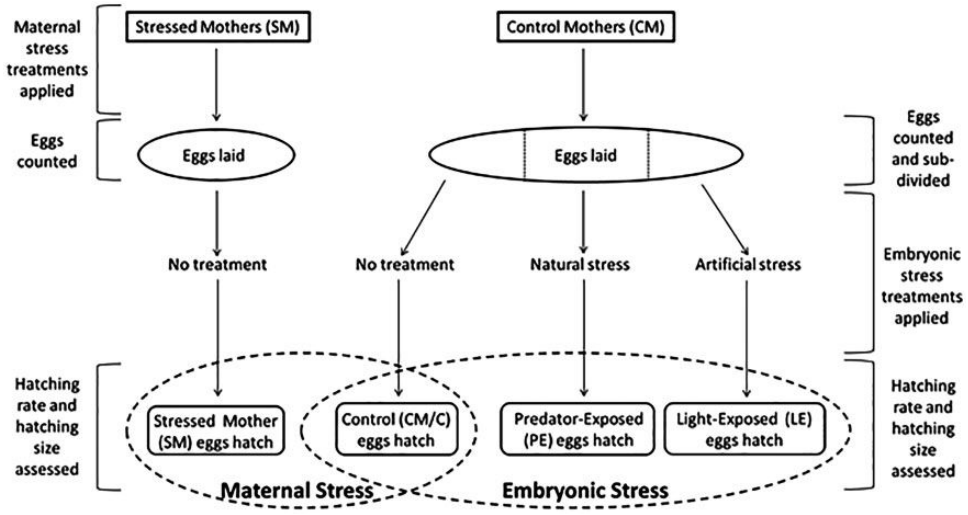

Figure 1. Schematic representation of experimental design. Note that the eggs of the unstressed control females were subdivided to create the three embryonic treatment groups and that both maternal stress and embryonic stress were compared to the same control group of eggs and hatchlings (CM in the maternal stress comparisons, C in embryonic stress comparisons) separately. Adapted from O'Brien *et al.* (2017).

Table 1. Composition of female stress groups by year.

	Control mothers		Stressed mothers	
2015	7 captured		6 captured	
	4 egg-layers	3 non-layers	4 egg-layers	2 non-layers
	3 laid > 50 eggs after 1 week of treatment		*4 laid > 50 eggs after 1 week of treatment*	
2016	12 captured (divided into 4 groups, consisting of three females each)		14 captured	
	11 layers[a]	1 non-layer[a]	8 egg-layers	6 non-layers
	4 group means (total eggs ÷ 3 or 2) > 50 eggs after 1 week of treatment		*6 laid > 50 eggs after 1 week of treatment*	

All data from 2015 to 2016 were pooled for statistical analysis. The sample size used in the different comparisons of females and their offspring vary between tests for several reasons, including whether any individuals did not lay eggs, whether females were housed individually or separately, and whether any egg-layers laid fewer than 50 eggs.

[a]Directly observed.

Faecal corticosterone assays

Faecal collection and extraction methods were adapted from Larson and Anderson (2010). In May and June of 2016, faeces from the spawning females were collected daily from the bottom of every tank using a hand net. Faecal strands were removed separated from other detritus with tweezers and placed in dry 1 ml vials. These were frozen and stored at −80°C for 5–6 months (depending on collection date) while various extraction protocols were tested and the assay was validated. In November 2016, faecal samples were thawed, weighed (0.3–1.2 g wet faeces) and dried (65°C, overnight) to obtain a dry powder. Twelve consecutive days of samples from the 24 individuals tested in 2016 were pooled into four replicates per treatment group (means calculated from faeces of two to four individuals) and into six time points (faeces from two subsequent days) to achieve a usable amount of sample. Extraction was achieved via a specially developed protocol: 90% methanol was added to the dried sample (1 ml 100 mg^{-1} dried faeces), followed by vortexing (20 min) and centrifugation (20 min, 2 800 rpm). Methanol was allowed to evaporate from the resulting supernatant and the sample was subsequently re-suspended in a steroid diluent and stored at 4°C until assay (1 week). After homogenization in an ultrasonic bath (37 kHz, 15 min), an ImmuChem Double Antibody CorticosteroneTM 125I RIA kit (MP Biomedicals, LLC) was used to quantify the corticosterone present in the faeces of each treatment group. A gamma counter measured relative radioactivity of the samples and corticosterone concentrations were calculated via comparison to a standardized curve.

Ovary dissections

"Lifespan after capture" is the number of days between capture and natural death in the facility. At death, we measured female Dorsal Mantle Length (DML, cm) and weight (kg) after water was drained from the body cavity and the outer surface gently dried. The bodies were then frozen in a −20°C freezer. In August 2016, the bodies were thawed and dissected to count the number of oocytes remaining in the pallial cavity.

Eggs

Egg collection

For the first 3 weeks of incubation, eggs were maintained in floating, circular baskets (3.8 L) in the maternal treatment tank in which they were laid (up to 250 eggs per basket) that periodically floated under a stream of flowing water. After a suitable number was collected (about 3 weeks after the first eggs were laid), eggs were moved from these conditions and acclimatized over the course of a day to a mean seawater temperature between 17 and 19°C. Eggs were maintained until hatching in floating mesh trays (55 cm × 70 cm × 7 cm) in 65 L (80 cm × 60 cm × 40 cm) tanks constantly renewed by seawater from a flow-through system with vigorous aeration from an air stone and exposure to the natural light cycle (15:9–16:8 h light:dark). The eggs were arranged in a single layer and gently mixed multiple times a day by hand to promote oxygen absorption and deter epibiont growth.

Stress treatments

Eggs from the nine most fecund SM were allowed to develop without any further treatment (Figure 1), while eggs from the eight most fecund CM were randomly divided into three groups to test the effects of stress applied directly to cuttlefish eggs (Figure 1b). "Predator-exposed" (PE) eggs were placed in a tank with three mature European sea bass (*D. labrax*, approximately 25–35 cm TL), continuously exposing them to the chemosensory, and potentially vibrational and visual, cues emitted by the fish, though they were physically separated by the mesh bottom of the floating trays. Throughout development, "light-exposed" (LE) eggs were exposed to white LED light (18 Watts, 20.7 klux, positioned approximately 10 cm from water's surface) in 15 min bursts six times every 24 h (for a total of 90 min day^{-1}) on a schedule that was re-randomized daily. The third group, CM from the maternal stress part of the experiment, was used as the "control" © group in this phase of the experiment as well. Hatching occurred between 29 June and 5 August 2015 and from 2 to 24 July 2016.

Hatchlings

Hatchlings were counted at 08h00 each morning and used to calculate overall hatching rate. Each hatchling was then gently moved from the hatching tank to a shallow, uniform grey container and photographed with a Panasonic HDC-SD60 camera. Using ImageJ, DML (the tip of the mantle to the edge just behind the eyes) was measured in two photos and averaged. If these two values differed by more than 5% (due to a change in body position or measurement error), a third photo was measured and the mean DML calculated from these three measurements. Very few physical abnormalities were observed, and individuals exhibiting them were excluded from size assessments. Sex discrimination is not possible at this age. A subset of these hatchlings was subsequently used in a series of behavioural tests to gauge the effects of prenatal stress (O'Brien *et al.*, 2017).

Ethical note

This research was conducted in accordance with Directive 2010/63/EU, under the approval of the Comité d'Éthique NOmandie en Matière d'EXperimentation Animale (CENOMEXA) #54 (agreement number A14384001).

Statistics

All statistical analyses were conducted in StatXact®7 (Cytel, Inc.) and R. Because the trends from 2015 to 2016 only differed in a single instance (hatching size following maternal stress), samples were pooled to achieve a usable sample size for statistical analysis. All values are reported as mean ± *SD*.

To compare the number of females that laid eggs, as well as the number of females that laid white eggs with those that laid normal eggs, chi-squared exact tests were used.

The number of eggs per female was calculated from laying females only. In 2016, CM were housed in groups of three, and thus individual counts per female were not possible. Instead, the total number of eggs produced by each tank was divided by three (or two in one case) to yield a mean value for each tank (11 of 12 CM were directly observed by experimenters laying viable eggs in 2016). These were combined with the individual CM egg counts from 2015, and compared to the eggs per female of SM using exact permutation tests for independent samples. We also compared the eggs per female of CM between years with an exact permutation test to test for any effect of housing singly or in groups.

The data for female size (weight and DML), lifespan after capture and the number of remaining oocytes (2016 only) were not normally distributed, so means were compared using exact Pearson permutation tests for independent samples. The sample sizes used to calculate these values varied since some measurements were not possible in certain individuals.

Faecal corticosterone measurements were logit transformed and fitted with logit-log linear regression (log10(corticosterone concentration) ~ treatment + (1 | testing.days)) using the "lme4" package in R.

The hatching rate for SM was calculated as the number of live hatchlings divided by the total number of eggs laid. Due to the large number of eggs laid, not all the eggs from CM were measured for this experiment. Instead, a large subset of the eggs was partitioned into three embryonic stress groups (C, PE, and LE eggs). Females that produced fewer than 50 viable eggs after 1 week of treatment (one CM in 2015 and two SM in 2016) were excluded. 2 × 2 chi-squared tests were used to compare CM and SM, and a Cochran–Mantel–Haenszel chi-squared test was conducted to compare C, PE, and LE eggs.

Hatching DMLs were normally distributed and there was equal variance between treatment groups, enabling parametric analysis. CM and SM were compared using an independent *t*-test, while C, PE, and LE eggs were compared using a two-way ANOVA with stressor type and mother as main factors.

Results

Females

Egg-laying

The proportion of SM (60%) that produced eggs did not differ from CM (78.95%) (two-tailed chi-squared exact test: $\chi^2 = 1.64$, CM $n = 19$, SM $n = 20$, $p = 0.3$; Table 2). 15 CM laid a total of 6 567 eggs while 12 SM laid a total of 1 831 eggs. No significant difference existed in DML, weight at death, lifespan after capture or the amount of remaining reproductive material at death (remaining oocytes) between CM and SM (Table 2). All individuals had ink remaining in their ink sacs at death.

Faecal corticosterone

The mean corticosterone concentration over 6 days in CM was 0.70 ± 0.36 ng mg^{-1} dry faeces and 0.79 ± 0.56 ng cort mg^{-1} dry faeces in SM. No significant difference existed between treatment groups [GLMM: $\chi^2 \geq 0.07$, $n = 4$ (means calculated from the pooled faeces of one to four individuals over 2 days), $p = 0.79$; Figure 2].

Eggs

Egg-laying occurred from 15 May to 9 June 2015 and from 14 to 29 May 2016. CM produced a significantly higher number of eggs per female than SM [CM = 505.23 ± 373.30 per female, $n = 8$ (four individuals from 2015 and four group means from 2016), Table 2]; SM = 152.58 ± 96.93 per female; ($n = 12$; two-tailed exact permutation test: $t \geq 4\,042$, $p < 0.001$). Among CM, there was no difference in eggs per female between years (exact permutation test: $t \geq 2\,675$, $p = 0.69$), supporting our choice to pool these groups despite the differences in housing conditions (individual in 2015, groups of 3 in 2016).

In addition to our planned measurements, we also noted that three of 12 laying SM (25%) produced viable eggs with little or no ink in the egg capsule (Figure 3). A few of the laying CM ($n = 15$) also produced a small number (<25) of grey or white capsules, but these did not develop and were not counted as viable eggs. A comparison of the number of laying females in each group that produced viable white eggs (SM = 3/13, CM = 0/15) did not indicate a statistically significant difference between treatment groups but did constitute a statistical trend (two-tailed chi-squared exact test: $\chi^2 = 4.22$, $p = 0.08$).

Hatchlings

Hatching rate

Significantly more (two-tailed chi-squared test: $\chi^2 \geq 453.50$, $p < 0.0001$; Table 3) CM eggs (57.14%, $n = 1\,876$) produced live offspring than SM eggs (22.27%, $n = 1\,724$).

Among the embryonic treatment groups, there was no significant difference in hatching rates between groups (Cochran–

Table 2. Proportion of egg layers, total number of eggs laid, mean number of eggs per female, size (DML and weight), lifespan after capture (days), and number of eggs laid and remaining oocytes (mean ± *SD*) of female cuttlefish.

	Control mothers	**Stressed mothers**	**Comparison**
Proportion of egg layers	78.95%	60.0%	$p = 0.3$
	$n = 19$	$n = 20$	$\chi^2 = 1.642$
Eggs laid	6 567	1 831	
	(2 675 in 2015, 3 892 in 2016)	(6 54 in 2015, 1 177 in 2016)	
Eggs per female	505.23 ± 373.30	152.58 ± 96.93	$p < 0.001$
	Calculated from 4 individuals + 4 groups of three[a]	Calculated from 12 individuals[a]	$t \geq 4\,042$
DML (cm)	23.29 ± 3.25,	23.03 ± 1.89,	$p = 0.81$
	$n = 17$[b]	$n = 17$[b]	$t \geq 396$
Weight at death (kg)	1.29 ± 0.30,	1.31 ± 0.25,	$p = 0.84$
	$n = 17$[b]	$n = 16$[b]	$t \geq 21.85$
Lifespan after capture (days)	15.63 ± 7.21,	14 ± 5.0,	$p = 0.38$
	$n = 19$	$n = 20$	$t \geq 297$
Remaining oocytes (2016 only)	108.33 ± 33.26,	117.5 ± 48.64,	$p = 0.65$
	$n = 12$[c]	$n = 12$[c]	$t \geq 1\,300$

CM: $n = 19$ females housed individually or in four groups of three; SM: $n = 20$ females housed individually. The proportion of egg layers was tested with a chi-squared exact tests all others with exact permutation tests (these calculations include both egg-layers and non-layers).

[a] See Table 1 for details.

[b] Accurate body measurements were not possible for some specimens due to scavenging activity by crabs.

[c] Due to poor preservation, dissection and measurement was not possible for some specimens.

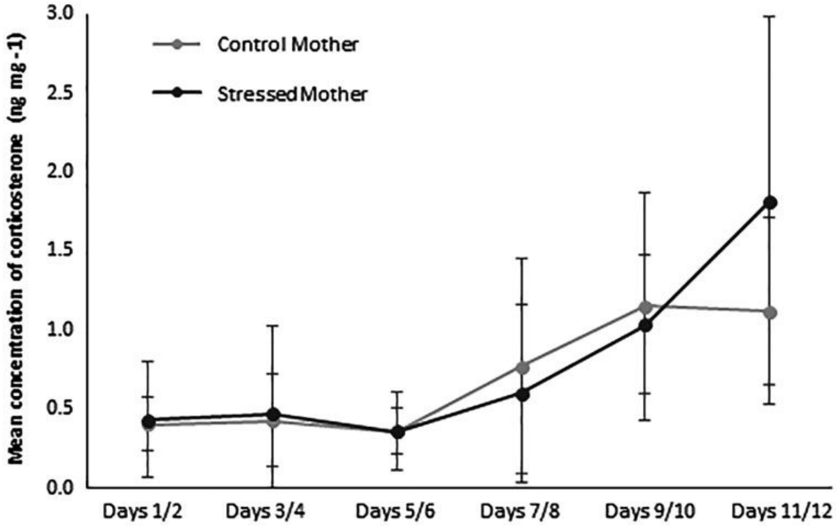

Figure 2. Faecal corticosterone concentration (mean ng corticosterone mg^{-1} dry faeces ±*SD*) over time, measured from faecal samples collected in 2016. No significant difference exists between groups [GLMM: $\chi^2 \geq 0.07$, n per data point = 4 (means calculated from the pooled faeces of one to four individuals over 2 days), $p = 0.79$]. Data are displayed as group means (dots) ± *SD* (whiskers).

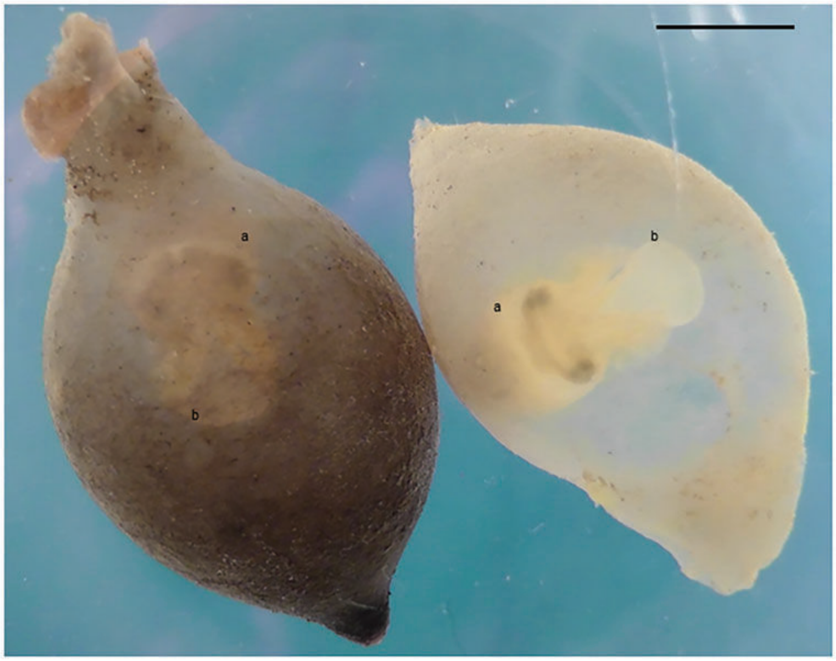

Figure 3. A normal (ink-stained) *S. officinalis* egg (left) and a white egg laid by one of the SM (right). Both eggs are in the final stage of embryonic development (Stage 30) and hatched a few days after the photograph was taken (July 2016). External embryo (a) and yolk sac (b) are visible in both specimens. Bar in upper right corner = approx. 1 cm.

Table 3. Hatching rate and mean hatching size [dorsal mantle length (mm), mean ± *SD*] of eggs and offspring from the maternal stress experiment.

	Control mother	Stressed mother	Comparison
Hatching rate	57.14% $n = 1\,876$	22.27% $n = 1\,724$	$p < 0.0001$, $\chi^2 \geq 453.50$
Hatching size (mm)	8.56 ± 0.75 $n = 72$	8.41 ± 0.92 $n = 97$	$p = 0.26$, $t = 1.13$

Hatching rates were compared with a chi-squared test, while hatching sizes were compared with a two-tailed independent t-test.

Mantel–Haenszel chi-squared test: $\chi^2 \geq 0.84$, $p > 0.99$; Table 4): 57.14% of C eggs ($n = 1\,876$), 59.33% of PE eggs ($n = 1\,876$), and 63.38% of LE eggs ($n = 1\,876$) produced live offspring.

Hatching size

At hatching, the mean DML of CM offspring (8.56 ± 0.75 mm, $n = 72$) was not significantly different from SM offspring (8.41 ± 0.92 mm, $n = 97$) (two-tailed independent *t*-test: $t = 1.13$, $p = 0.26$; Table 3).

Table 4. Hatching rate and mean hatching size (DML, mean ± *SD*) of eggs and offspring from the embryonic stress experiment.

	Control	Predator-exposed	Light-exposed	Comparison
Hatching rate	57.14% $n = 1\,876$	59.33% $n = 1\,876$	63.38% $n = 1\,876$	$p > 0.99$, $\chi^2 \geq 0.84$
Hatching size (mm)	8.56 ± 0.75	8.64 ± 0.73	8.71 ± 0.69	Stress: $p = 0.22$, $F = 1.54$,
	$n = 72$	$n = 85$	$n = 74$	Mother: $p < 0.001$, $F = 4.49$

Hatching rates were compared with a Cochran–Mantel–Haenszel chi-squared test, while hatching sizes were compared with a two-way ANOVA.

Likewise, no significant difference existed between C offspring (8.56 ± 0.75 mm, $n = 72$), PE offspring (8.64 ± 0.73 mm, $n = 85$), and LE offspring (8.71 ± 0.69 mm, $n = 74$) in hatching DML by stress treatment (two-way ANOVA: $F = 1.54$, $p = 0.22$; Table 4), although individuals were found to differ significantly by mother ($F = 4.49$; $p < 0.001$).

Discussion

Maternal stress clearly reduced egg-laying in cuttlefish. This difference could not be explained by female size or survival time: there was no difference in mean weight or DML between the two groups at death, and females from both groups survived for a little over 2 weeks after capture before they died naturally, with no significant difference in lifespan between groups (Table 2). One potential explanation for the reduction in the number of eggs laid by SM might be that stress responses depleted energy reserves necessary to sustain egg-laying activity. Since cuttlefish generally do not eat much while spawning (Boletzky, 1986), the energy for egg-laying and basic life processes is mostly derived from the set amount of body reserves remaining to the female. Reacting to stressors may accelerate energy consumption, and could therefore deplete the resources that females would otherwise use to lay eggs, and prevent the multiple bouts of laying over the course of weeks that is sometimes observed in captivity (Boletzky, 1987, 1988). Experimenting with various severities of stressors (i.e. a severe vs. mild stressor) and food rations (i.e. restricted vs. *ad libitum*) applied to reproducing females might reveal whether energy reserves do indeed limit spawning in female cuttlefish.

Normally, the egg membrane of *S. officinalis* is impregnated with ink secreted by the mother, resulting in black eggs that are opaque early in development. Unpigmented eggs are sometimes seen in aquaculture and in the wild (Sykes *et al.*, 2014), but the causes for this lack of pigmentation are unknown. In our experiment, a quarter of the laying SM produced white eggs that developed and hatched normally (Figure 3). (A few of the CM also produced white or greyish capsules, but these appeared to be unfertilized as they did not develop further, and were thus not included in our calculation.) Statistically, the ratio of SM (3/12) that laid viable white eggs did not differ significantly from CM (0/15) at our designated alpha (<0.05), but it constituted a statistical trend ($p = 0.08$). This suggests that stress during egg-laying has the potential to disrupt egg-laying in cuttlefish. Alternatively, white eggs could also be the result of acute ink depletion following an inking response to stress treatments, or perhaps females reduce egg pigmentation to conserve ink for use in escape responses. However, the females in our experiment did not appear to have depleted their ink supply during stress treatment, since all those examined (2016) still contained ink in their ink sacs at death. To determine if females temporarily run out of ink, actively suppress egg pigmentation or if there is a physical disruption to the egg-laying process which prevents membrane staining, the degree of egg pigmentation could be assessed after intentionally eliciting repeated strong inking responses in reproducing females. The adaptive effect of a lack of egg pigmentation for the eggs themselves is unclear. On the one hand, ink in the egg membrane is thought to play a protective role during embryogenesis by deterring consumption by predators and microbial attacks (Cornet *et al.*, 2015; Martins *et al.*, 2018) and by blocking excess light that could interfere with normal development (Bassaglia *et al.*, 2013). Therefore, a lack of ink might result in higher incidences of embryo damage, parasitism, and death. In addition, white eggs may be more conspicuous on many natural egg-laying substrates and attract predators that spot the visible embryo. On the other hand, a lack of pigmentation may have certain advantages. For instance, it could enable greater visual access to the surrounding environment, allowing embryos to develop predator- and prey-specific preferences and hunting strategies (see Darmaillacq *et al.*, 2008; Romagny *et al.*, 2012). It may also reflect a strategy to minimize time spent at the egg-laying site and maximize hatchlings' ability to disperse from that location at eclosion, since white *S. officinalis* eggs reared in captivity hatch sooner than their darker counterparts (Paulij *et al.*, 1991), resulting in smaller hatchlings with greater yolk reserves (Bouchaud and Daguzan, 1990). Finally, there is even the possibility that females modulate the amount of ink in their eggs to maximize camouflage by matching characteristics of the substrate (in this case, a featureless grey bin). Regardless of the reasons for this phenomenon and its consequences for embryo fitness, if the association between maternal stress and lack of pigmentation is substantiated by other experiments and observations, a lack of egg pigmentation could serve as a marker of the presence of stressors during the laying process, giving indirect indications of laying conditions in the wild or the suitability of a captive rearing environment.

In addition to laying a reduced number of eggs, fewer of the eggs laid by SM hatched. The hatching rate of CM (57.14%) fell mid-range of hatching rates reported in the aquaculture literature [e.g. 32—80% in Hanley *et al.* (1998); 30–85% in Sykes *et al.* (2013)], indicating that a basal level of stress existed in our facility, resulting in sub-optimal rearing conditions for both groups. The hatching rate of the SM group was even lower (22.27%) however, indicating that the maternal stress treatments reduced the viability of eggs even further. Such a difference in hatching rate was not seen between the control and embryonic stress groups, suggesting that stressors applied to mothers may have a stronger impact on reproductive output than stressors applied directly to offspring. Investigation into the mechanisms responsible for this reduction in the SM hatching rate is needed, including the potential for the disruption of fertilization of the eggs by stored sperm as oocytes are passed through the genital tract of the female.

Neither maternal nor embryonic stress was associated with significant differences in hatching size, suggesting that stress does not affect the rate of embryonic metabolism, which could potentially affect hatchling DML. Embryonic growth rate and metabolism are fuelled by a predetermined supply of yolk that is deposited at least 5 months before mating (Boucaud-Camou and Boismery, 1991). The proportion of those reserves that is converted into tissue is determined by the rate of embryonic development and metabolism (Bouchaud and Galois, 1990), which is likely affected by stress responses. We had expected stressors applied directly to developing embryos to accelerate growth or to speed metabolism, and thus reduce hatching size. Instead, we found no significant difference between treatment groups and control. The lack of difference between embryonic treatment groups might be explained by habituation to the stressors or may indicate that cuttlefish were simply not influenced by the stimuli we applied. In our experiment, embryos experienced several days of continuous or repeated exposure to stressors and it is possible that sensory habituation occurred. However, this seems unlikely in the case of predator odour since the ability to habituate to predator cues would be maladaptive, and continuous exposure to seabass odour during incubation has been shown to mediate traits like brain lateralization in cuttlefish embryos (Jozet-Alves and Hebert, 2012). Moreover, behavioural experiments conducted on these hatchlings revealed changes in predation behaviour after embryonic light exposure (O'Brien *et al.*, 2017).

In addition to testing for stress effects on reproduction, we also sought a simple indicator of stress in spawning females. In many other animals (e.g. birds, mammals, fish), cortisol and/or glucocorticoids are secreted in response to stress and mediate many of its effects (Moberg, 1991). Preliminary assays by our lab had detected corticosterone but not cortisol in the haemolymph of *S. officinalis* (C. Bellanger, unpublished data), so we selected this hormone for quantification by radioimmunoassay. To minimize disturbance to the animals, we collected and tested faecal samples rather than haemolymph. However, we were unable to detect significant differences between groups, despite the strong effect on egg-laying and hatching rate. The values detected were also larger and more variable than those observed in giant Pacific octopus: 0.146–3.28 ng mg^{-1} vs. 0.010–0.022 ng mg^{-1} dry faeces (Larson and Anderson, 2010). This suggests that faecal corticosterone levels may not be a particularly good indicator of stress in this species (at least during reproduction). Faecal cortisol has been found to be an unreliable indicator of stress in some other cases as well: in harbour seals (*Phoca vitulina richardii*) for instance, plasma cortisol concentration was strongly correlated with injection of adrenocorticotropin (the hormone stimulating cortisol release), but faecal cortisol levels were not (Gulland *et al.*, 1999). This may also be the case in cuttlefish, although more sensitive methods, such as ELISA Kits, High pressure liquid chromatography (HPLC), or Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS), should be tested on both faeces and embryos in the future. Alternatively, the endocrinology of the stress response in cephalopods may be more complex than a simple increase in corticosterone. Existing studies of the endocrine system in cephalopods suggest complex interactions with both the nervous and immune systems (Di Cosmo and Polese, 2016).

We also attempted to relate the amount of unused reproductive material in deceased females with stress treatments. Based on the observed reduction in the number of eggs spawned in 2015, we hypothesized that unused oocytes in the deceased females might be an indicator of unused reproductive potential and thus reflect maternal stress levels during egg-laying. We therefore examined the number of oocytes remaining in each female's pallial cavity at death, hypothesizing that there would be more in SM. However, we found no statistical difference between the two groups (Table 2, "remaining oocytes"). Both groups still possessed a large (and statistically equivalent) supply of oocytes in the pallial cavity, as well as an uncounted number (individual oocytes were difficult to distinguish) of additional oocytes in the ovary, suggesting that no individual had come anywhere close to reaching its potential fecundity (for further discussion, see Boletzky, 1987; Laptikhovsky *et al.*, 2003; Salman *et al.*, 2017). Since this trait did not correlate with stress treatments, it cannot serve as a proxy for stress levels in female cuttlefish. Other potential measures of maternal stress to be explored in this species include respiration rate, body patterning (e.g. use of the diematic display) and levels of locomotory activity.

As global demand for protein increases with human population, understanding the effects of rearing conditions and external factors on reproduction in cuttlefish and other farmed species is critical to optimizing yields and animal welfare (Villanueva *et al.*, 2014; Xavier *et al.*, 2015). Our results suggest that providing adequate tank space and minimizing handling of female cuttlefish may increase the number of eggs laid and the hatching rate in aquaculture. In particular, catching females well before copulation to allow them time to recover from the stress of capture and acclimate to an artificial setting may result in higher offspring yields. [However, it may interfere with other processes such as yolk reserve formation (which occurs months before) or even the proclivity to copulate.]

Conclusion

It appears that our stress treatment had a very strong impact on reproducing female cuttlefish, demonstrated by the total number of eggs and hatchlings produced. By contrast, there was no change in hatching rate when an artificial stressor (random bouts of bright LED light) and natural stressor (predator cues) were applied directly to developing eggs. Though the maternal stress treatment clearly affected reproductive output, we were unable to detect a measurable difference in faecal corticosterone concentration (a method used commonly in other species) or in unused reproductive material, nor did stress have any consistent effect on the hatching size of offspring. Unfortunately, this leaves us without any simple marker of maternal stress in cuttlefish, though a reduction in the number of eggs produced and hatched, as well as the absence of ink in eggs, might be used as *post hoc* indicators of stress in fisheries and aquaculture. The effects of prenatal stress on offspring behaviour in cuttlefish were explored in subsequent experiments (see O'Brien *et al.*, 2017). The results of these studies could be expanded by testing the combination of maternal and embryonic stress in cuttlefish—are the effects additive, synergistic or do they negate each other? It might also be worth more closely exploring the physiological mechanisms of prenatal stress effects. For instance, closer tracking of egg-laying could reveal to what degree the reduction in egg output is simply a direct, short-term effect of acute stress (i.e. the stress treatments reduce egg-laying by interrupting the process) vs. long-term, far-reaching physiological effects (e.g. hormonal cascades that suppress other life functions and deplete resources). Such experiments will improve our ability to care for cuttlefish in captivity, and inform our understanding of stress responses in animals.

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Annexe 4

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Maternal and Embryonic Stress Influence Offspring Behavior in the Cuttlefish *Sepia officinalis*

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Stress experienced during prenatal development—either applied to reproducing females (maternal stress), directly to developing offspring (embryonic stress) or in combination—is associated with a range of post-natal behavioral effects in numerous organisms. We conducted an experiment to discern if maternal and embryonic stressors affect the behavior of hatchlings of the cuttlefish *Sepia officinalis*, a species with features that allow for the examination of these stress types in isolation. Separating the impact of stress transmitted through the mother vs. stress experienced by the embryo itself will help clarify the behavioral findings in viviparous species for which it is impossible to disentangle these effects. We also compared the effect of a naturally-occurring (predator cue) and an "artificial" (bright, randomly-occurring LED light) embryonic stressor. This allowed us to test the hypothesis that a threat commonly faced by a species (natural threat) would be met with a genetically-programmed and adaptive response while a novel one would confound innate defense mechanisms and lead to maladaptive effects. We found that the maternal stressor was associated with significant differences in body patterning and activity patterns. By contrast, embryonic exposure to stressors increased the proportion of individuals that pursued prey. From these results, it appears that in cuttlefish, maternal and embryonic stressors affect different post-natal behavior in offspring. In addition, the effect of the artificial stressor suggests that organisms can sometimes react adaptively to a stressor even if it is not one that has been encountered during the evolutionary history of the species.

Keywords: body patterning, predation, visual lateralization, activity, threat response

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INTRODUCTION

Stress responses occur in reaction to any external or anticipated threat. In response to a predator, for instance, an animal may increase its metabolism and divert resources to its muscles and away from less critical functions like digestion and foraging behavior—the "fight or flight" stress response (Cannon, 1939). Other kinds of stressors will induce different reactions. In response to food scarcity, for instance, an animal may have the opposite reaction, prioritizing digestive processes to extract the maximum amount of energy from food items and even undertaking risky foraging behavior (Wang et al., 2006). While stress responses have presumably evolved to increase survival in the face of an immediate stressor, there is an increasing awareness that stress responses come with a host of negative fitness consequences. This often depends on whether the stressor causing the response is acute or chronic: A short, single experience of a stressor (e.g., a single encounter with a predator) often produces a short-term, adaptive response while long-term or repeated exposure to stressors (e.g., prolonged food shortage) can have lasting negative impacts on fitness (Jones, 1996; Miller et al., 2007). These costs come from the energetic tradeoffs involved in maintaining the response or in the form of missed opportunities (e.g., lost foraging time, mating opportunities). Chronic and repeated stressors are often associated with reductions in immune function, the advent of various diseases, negative impacts on psychological health and disruptions to normal biological functions (e.g., Katz et al., 1981; Miller et al., 2007; Favreau-Peigné et al., 2014). Thus, understanding the underlying causes and effects of stress responses has implications for medicine, psychology and developmental biology, and is studied in a number of animal models.

The long-term effects of stress that occurs during the embryonic development of an organism are known to be especially significant. Research in a number of vertebrate taxa demonstrates that stress responses in reproducing females can have a strong impact on the behavior of her offspring. In some cases, such stress may serve as an indicator of prospective environment, prompting adaptive changes to the offspring phenotype that help it cope with future challenges. Stress responses can also be associated with reduced offspring fitness; normal developmental processes can be disrupted and the animal may be more susceptible to disease (Gluckman and Hanson, 2004). While the effects of prenatal stress have been relatively well-documented in a number of taxa, it is often unclear if effects observed are the direct result of a stress response in the offspring or a maternally–transmitted effect. One potential mechanism for prenatal stress effects in offspring is the transfer of "stress hormones" (e.g., glucocorticoids, catecholamines) from mother to developing embryo. Such hormones are secreted by animals in response to stressors and affect physiology, behavior and metabolism. Their transfer to offspring via the placenta or egg yolk could explain many of the alterations to offspring phenotype that are sometimes observed (Hayward and Wingfield, 2004; Groothuis et al., 2005; Weinstock, 2008).

Alternatively or in parallel, embryos could be experiencing stressors directly and generating their own stress responses. Where most authors use the term "prenatal stress" to refer to an offspring's response to any stressor experienced during embryonic development, we distinguish between effects of stressors applied to the mother ("maternal stress") and those applied to the offspring themselves ("embryonic stress"). Investigations of stressors applied directly to developing embryos are much less numerous than studies of maternally-applied stress, largely for logistical reasons. By necessity, prenatal stressors must be applied to pregnant or brooding females in many behavioral models, since their embryos develop viviparously or ovoviviparously. Moreover, it has only recently become widely recognized that the embryos of many species are able to perceive and react to stimuli in the surrounding environment, and that this sensory input could provide essential information to prepare for challenges in the postnatal environment (e.g., Mathis et al., 2008). One way to gauge the relative contributions of maternal and embryonic stress responses is to compare their effects in experimental isolation using animal models that are oviparous and autonomous at birth (e.g., many fish, amphibians, precocial birds, and invertebrates). For example, experiments have demonstrated that rainbow trout eggs exposed to stress hormones (comparable to what a stressed mother might produce) result in offspring that are more fearful 5 months after hatching than control animals, although no differences were seen at 2 months (Colson et al., 2015). Likewise, when eggs of the same species were isolated from their mothers and subjected to conspecific alarms cues they demonstrated greater behavioral plasticity than non-stressed controls (Poisson et al., 2017). Therefore, it seems that both maternal and embryonic stressors affect behavior in this species. However, experiments with another species of trout failed to show any differences induced by prenatal stress, suggesting that susceptibility to prenatal stress is not universal across this subfamily (Ghio et al., 2016). By comparing these three studies, we can see that stress effects differ depending on stress type, species, context and age, a finding that likely holds true for other groups as well.

Despite their potential as good study models, there is an unfortunate lack of work with invertebrates, perhaps because invertebrates are sometimes considered unsophisticated and thus unworthy of behavioral study, and because experiments are complicated by the existence of larval phases in many species. The cuttlefish *Sepia officinalis* (Linnaeus, 1758) has neither of these issues. Like other coleoid cephalopods, it is neurologically and behaviorally sophisticated but unlike other coleoids and invertebrates, it has no pelagic larval stage, settling directly on the bottom after hatching (Hanlon and Messenger, 1998). Even more importantly for a potential model for the study of prenatal stress, this species is known to perceive and learn from within the egg (Romagny et al., 2012). A number of embryonic influences have already been identified in cuttlefish. For instance, embryos can develop post-hatching prey preferences and behavioral asymmetries from visual or odor cues (Darmaillacq et al., 2008; Jozet-Alves and Hebert, 2012) and habituate to repeated sensory stimuli, such as light, odor and tactile cues (Romagny et al., 2012). Documenting the effects of maternal and embryonic stress in this species may elucidate general principals about how animal offspring are affected by different types of stress, or indicate that the impact differs according to phylum. In addition, a better understanding of the effects of maternal and embryonic stress in *S. officinalis* would have direct implications for the welfare of cephalopods in aquaculture, laboratories and aquaria. This is important as cephalopods are increasingly recognized as advanced organisms capable of pain and suffering and were recently added to the list of protected animal groups covered by European welfare legislation (Directive 2010/63/EU).

In order to determine whether prenatal stress affects cuttlefish behavior, we subjected reproducing female cuttlefish and their eggs to stressful stimuli. Our primary goal was to determine if female cuttlefish transmit stress effects to their offspring.

Abbreviations: UM-C, Unstressed Mother Control eggs; SM, Stressed Mother eggs; WM, Wild Mother eggs; UM-PE, Unstressed Mother Predator-Exposed eggs; UM-LE, Unstressed Mother Light-Exposed eggs; HI, Heterogeneity Index.

To this end, we compared the offspring of "unstressed" and "stressed" captive females. We also included a group of "wild" eggs in order to assess whether captivity during egg-laying exerts any effects. Our secondary goal was to assess the relevance of stressor type to offspring. We tested the hypothesis that stress responses depend on stress type, particularly how "familiar" it is to the species. We predicted that a naturally-occurring stressor like odor cues from a co-occurring predator species would elicit an adaptive anti-predator response genetically programmed by natural selection. In contrast, we predicted that an artificial stressor would confound innate defense mechanisms and provoke behavioral responses with largely negative effects on fitness. We tested this hypothesis by comparing the effects of an artificial stressor (randomized bouts of bright LED light) to a naturally-occurring one (predator odor) applied to developing embryos. Experiments had already demonstrated that prenatal exposure to predator odor affect the post-natal behavioral lateralization of cuttlefish (Jozet-Alves and Hebert, 2012). LED light was selected as the artificial stressor since it can be detected by late-stage embryos (Romagny et al., 2012) and is likely to be present in aquacultural facilities and laboratories. Immediately after hatching, the offspring from each of these stress groups were tested in a battery of behavioral tests. These tests were chosen to assess a wide range of behaviors thought to be crucial to survival in the wild: body patterning, predation ability, brain lateralization, baseline activity and activity in response to an imminent threat. Behavior was tested during the first 10 days after hatching as this is thought to be the time of highest mortality in the lifecycle of cuttlefish (Bloor et al., 2013).

METHODS

Two different experiments were conducted, one testing for the potential transfer of the effects of captivity or stress from reproducing females to their offspring, and a second exploring the impact of stressors applied directly to developing embryos. In the first experiment, we exposed spawning female cuttlefish to daily removal from the water. We then compared the behavior of their offspring (SM) to that of offspring of a group of captive but unstressed mother controls (UM-C). We also compared both of these groups to offspring from naturally-spawned eggs collected from the wild (WM). While the maternal experience of these eggs was unknown and uncontrolled, their inclusion gives a sense of the effects of maternal capture and captivity (**Figure 1**).

In the second experiment testing embryonic stress, we subdivided eggs from the unstressed control mothers into three groups in order to investigate the effects of stimuli applied directly to embryos. We applied two kinds of stressors: a naturally-occurring stressor consisting of odor cues from common predatory fish (UM-PE) and an artificial stressor consisting of high intensity LED light timed randomly and unpredictably throughout the day and night (UM-LE). These two groups were compared to the unstressed mother control (UM-C) group used in the maternal stress comparisons (**Figure 1**).

After hatching, the effects of prenatal stress treatments on offspring were assessed with a battery of tests covering various aspects of the cuttlefish behavioral repertoire, including body patterning, visual lateralization, predation, activity patterns and fear response. These tests allowed us to make a broad assessment as to whether stressors affect offspring behavior and to make general comparisons between embryonic and maternal stress and between a natural and an artificial stressor. We predicted that the direct experience of an embryonic stressor would have a stronger effect on offspring behavior than maternal stress, which consists of information that must be transmitted indirectly to offspring through the mother. We also expected that cuttlefish would have evolved adaptive responses to the natural embryonic stressor (predator odor), but would demonstrate inappropriate and likely maladaptive responses to the artificial stressor since its response to this stimulus could not have been shaped by natural selection.

Animal Collection and Housing

Adult Females

Cuttlefish traps were set off the coast of France in the English Channel. Thirty seven adult female cuttlefish (*S. officinalis*) were captured between May and June, 2015 and 28 were captured in May, 2016 and transported to the Centre de Recherches en Environnement Côtier (CREC, marine station of the University of Caen, Luc-sur-Mer, France). These females were mated with males and then placed in treatment tanks in a semi-open flow-through seawater system (15 ± 1°C) under a 16:8 h light/dark cycle.

Captured females were split randomly into two groups, and eggs collected from them were designated as "unstressed mother—control" and "stressed mother." The females designated as unstressed mothers (six in 2015 and 12 in 2016) were maintained in large (1,000 L), round tanks enriched with stones, plastic algae, floating objects and plenty of shaded area. In 2015, these females were housed in these tanks singly, but in 2016, the capture of two dozen cuttlefish on a single day necessitated housing in groups of three. Those females assigned to be in the stressed mothers group were isolated in bare tanks (65 L) with a water depth of 19 cm and subjected to randomized 10-s removals from the water three times a day using a specially-made mesh platform. Eggs spawned by these stressed captive females after at least 1 week in these conditions were collected. Four unstressed mothers and four stressed mothers spawned between May 15 and June 9, 2015 and 11 unstressed mothers and eight stressed mothers spawned between May 14 and 29, 2016.

Eggs and Hatchlings

Wild mother eggs (WM) were collected by SCUBA divers from pre-placed tethers in the English Channel (49°19.667N-0°18.767W) in June, 2015 from a depth of 13.7 m. These, along with eggs collected from unstressed and stressed mothers in captivity, were moved to floating trays in 65L tanks (80 × 60 × 40 cm) after 8 h of steady temperature habituation (from 15° to 20°C). These were housed in a darkened room with exposure to the natural light cycle and supplied with seawater from a gently flowing open system and aerated by an airstone. A randomly-selected third of the control mother eggs, designated as controls (UM-C), along with WM and stressed mother SM eggs, were not treated any further. The other two thirds of the control mother

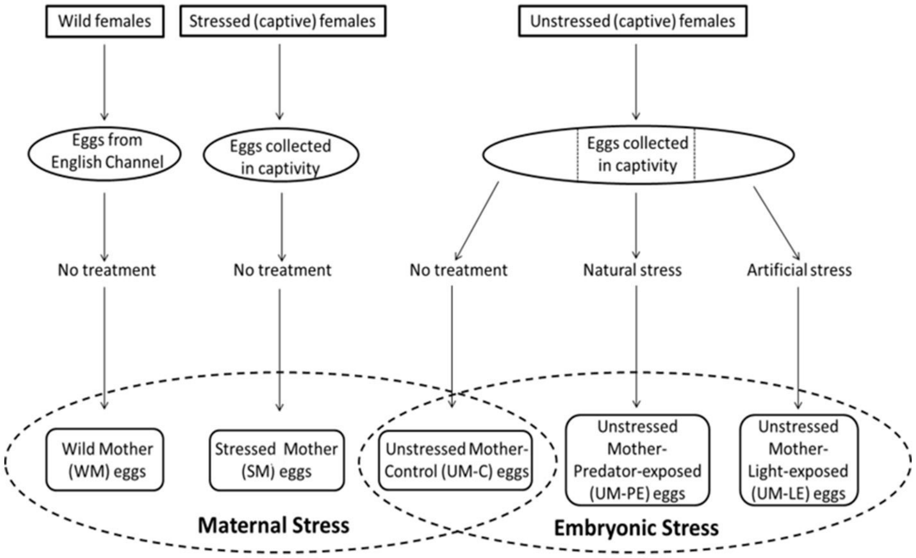

FIGURE 1 | Schematic representation of the experimental design.

eggs were divided randomly into predator-exposed (UM-PE) and light-exposed (UM-LE) groups. Three sea bass (Linnaeus, 1758; Dicentrarchus labrax; total length = 25–30 cm) were housed with UM-PE eggs, separated by a mesh barrier that allowed the eggs chemosensory and visual exposure to the predatory fish. Light-exposed eggs experienced strong LED illumination (20.7klux, approximately 10 cm from surface of water) for 90 min a day (six randomly-timed periods of 15 min). All eggs were gently agitated once a day to remove detritus and discourage parasite growth.

Hatchlings were recorded and collected at 08:00 each morning between June 29 and August 5, 2015, and July 2–24, 2016, and then transferred to a new tank to remove them from any further exposure to the stress treatments. Between experiments, hatchlings and juveniles were maintained in individually-labeled compartments to preserve identity. These compartments were situated in an aerated open seawater system (19–23°C) with a water depth of 7 cm. Sex determination was not possible at this age. All hatchlings born on a single day comprised a daily cohort. A total of 22 cohorts (numbering up to 12 individuals each) were hatched and tested daily between July and August. In 2015, after the predation experiment on Day 4, individuals were fed a single shrimp (Crangon crangon; Linnaeus, 1758) per day. In 2016, hatchlings were fed *ad libitum* starting on Day 4.

Behavioral Experiments

Following the 2 months of prenatal stress treatments described in the previous section, the resulting offspring were subjected to a battery of tests conducted during the first 10 days after hatching (**Figure 2**). These behavioral tests were selected to determine whether the stress treatments had affected certain key aspects of the behavioral phenotype—body patterning, predation ability, brain lateralization, activity level and response to a threat. The data resulting from these tests were analyzed in R, GraphPad (Prism®) and StatXact®7 (Cytel Inc.). All *p*-values are two-tailed and alpha was set at 0.05.

Body Patterning

In 2015, on the day of hatching (Day 1), between 9:00 and 10:30, up to 12 cuttlefish at a time were placed in randomized order in small uniform gray ("uniform background") circular compartments with slanted sides to minimize shadows (radius = 2.9 cm bottom, 3.35 cm top, length of sides = 2.5 cm; mean gray value = 101 ± 3.9) under white LED light (0.63 to 0.88 klux) and photographed at 0, 5, 15, and 30 min after placement on the background with a Panasonic HDC-SD60 camera. On Day 2, between 10:30-12:00, cuttlefish order was re-randomized and each was photographed four times (0, 5, 15, and 30 min after placement) against a checkered pattern ("disruptive background"). The check size of the disruptive background was selected to be approximately the size of a hatchling's main body-patterning component, the dorsal mantle square (3 × 3 mm), since previous studies have shown that this usually elicits a disruptive pattern in cuttlefish (Chiao et al., 2015).

ImageJ was used to assess the heterogeneity index (HI), a measure of body pattern disruptiveness, of individuals from the photographs. By selecting the outline of the mantle by hand and measuring the "standard deviation," HI was calculated from the standard deviation between the mean gray values of every individual pixel (x) comprising the dorsal mantle ($\bar{x}$), and the total number of pixels (N) selected, with higher values indicating higher overall disruptiveness of body patterning (see methodological description in Di Poi et al., 2014).

$$HI = \sqrt{\frac{1}{N}\sum(x-\bar{x})^2} \quad (1)$$

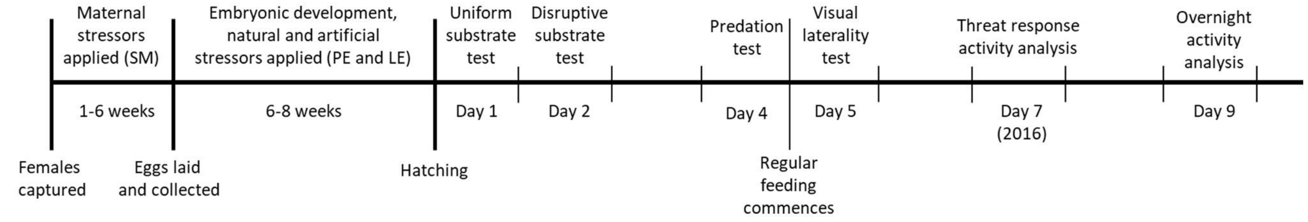

FIGURE 2 | Timeline of stress treatments and behavioral tests. All tests except for the threat response activity analysis occurred in 2015.

Only photographs in which cuttlefish had settled and remained motionless were used for these measurements. Because there was little variation over time in individuals' HI, the values from the four time points were averaged and used to calculate group means for each background type. In total, 55 WM, 41 UM-C, 43 SM, 44 UM-PE, and 39 UM-LE offspring were measured. HI values conformed to parametric assumptions as determined by visual inspection of histograms and normality plot, and were compared with the "anova" function in the "nlme" R package. *Post hoc* comparisons were made using the "glht" function in the "multcomp" R package.

Initial Prey Encounter

Food was withheld until Day 4, when individuals were gently moved from their compartments and placed in circular open-field arenas (radius = 5.9 cm, 250 mL) between 21:00 and 23:00, corresponding to peak feeding time (twilight) for this species (Quintela and Andrade, 2002). Each cuttlefish was allowed 15 min to habituate to the new environment, after which time filming commenced for 15 min (Panasonic HDC-SD60) and a single shrimp (C. crangon, total length 0.7—1.4 cm) was introduced. Videos were analyzed using VLC Media Player and ImageJ to collect data. The moment that cuttlefish orientated toward shrimp with their body was defined as the "time of detection" while the moment that tentacles touched the shrimp and subdued it successfully was defined as the "time of capture." Most caught shrimp on the first attempt, although any tentacle extensions without successful capture of the shrimp were recorded as a "failed capture attempt." Seven variables were calculated from this information: latency to detection (time between prey introduction and detection), latency to attack (time between detection and first strike at prey), latency to capture (time between detection and capture), distance of detection (distance between nearest cuttlefish eye and shrimp at time of detection), attempted capture rate (percentage of cuttlefish that attempted capture), capture rate (percentage of cuttlefish attempting capture that succeeded in capturing the shrimp) and success rate (percentage of attempted captures that were successful). In total, 56 WM, 37 UM-C, 40 SM, 38 UM-PE, and 42 UM-LE offspring were tested. Latencies and distance of detection did not meet parametric assumptions, so groups were compared with exact Kruskal-Wallis tests by Monte Carlo sampling followed by *post hoc* exact permutation tests (with sequential Bonferroni correction). The variables "attempted capture rate," "capture rate," and "success rate" were compared with chi square exact tests.

Visual Laterality Test

These tests were conducted between 10:00 and 22:00 5 days after hatching. The testing apparatus consisted of a start box (3.5 × 5 cm), a movable transparent barrier and two darkened shelters (3.5 × 4 cm) located 15 cm apart (see Jozet-Alves et al., 2012). Each shelter contained blue aquarium gravel and was shaded with a plastic cover. The apparatus was filled with seawater (renewed between trials) and placed under a bright fluorescent lamp (5.5 lux at the surface of the arena). In order to determine if stress induced a population-level eye-use preference, individuals were tested for shelter choice (in randomized order) by gently positioning them in the start box in such a way that it could view both shelters. Once the cuttlefish was in position, the transparent barrier was removed and the cuttlefish was allowed free access to the entire arena. Bright light is unpleasant to cuttlefish, and thus they were highly motivated to exit the start box and seek one of the darkened shelters. In total, 43 WM, 40 UM-C, 43 SM, 42 UM-PE, and 41 UM-LE offspring were tested. Within-group comparisons (the proportion turning right vs. left) were made with binomial tests and between-group comparisons (whether the proportion of those turning left differed between maternal or embryonic stress groups) were analyzed with chi square exact tests.

Overnight Activity Analysis

At midnight of Day 9, four cuttlefish from each daily cohort were randomly selected and placed in a circular open-field arena (radius = 5.9 cm, depth = 2.3 cm, 250 mL) made of opaque white plastic (sides) and a glass base. Illuminated from below by infrared light (which is not visible to the cuttlefish but is recorded by the camera), each individual was filmed from overhead for 6 h with a software-specific camera in a darkened room. This period corresponds with the times at which cuttlefish have been found to be most active (Denton and Gilpin-Brown, 1961; Jäckel et al., 2007; Frank et al., 2012; Oliveira et al., 2017). Videos were analyzed with Ethovision (Noldus®), a software package for behavioral tracking. The total distance traveled, time spent moving, and mean meander were recorded for each individual. Some individuals were unusable due to poor lighting and were excluded. In total, 20 WM, 10 UM-C, 15 SM, 8 UM-PE and eight UM-LE offspring were analyzed. These data did not conform to parametric assumptions, so were analyzed with exact Kruskal-Wallis tests followed by *post hoc* exact permutation tests (sequential Bonferroni correction).

Threat Response Activity Analysis

At noon on Day 7, two pairs of treatment- and age-matched cuttlefish were randomly selected from the daily cohort. They were placed in the open-field arena described in the previous paragraph and recorded and tracked in the same manner. After 1 h of filming, 50 ml of "blank" water from the UM-C egg tank was added to the arena of one member of each pair and 50 ml of "predator odor" water from the UM-PE egg tank containing the three seabass (*D. labrax*) was added to their counterparts' arenas. This was accomplished using tubes already present beneath the waterline of each arena in order to minimize the disturbance of the addition of water. The total distance traveled and time spent moving were recorded for each individual in the same manner as described above. To control for individual differences, post treatment values are expressed as a percentage of the initial hour for each individual (baseline). In total, groups of 10 UM-C, SM, UM-PE, and UM-LE offspring were divided into "blank" ($n = 5$ per stress group) and "predator odor" treatments ($n = 5$ per stress group). These data did not conform to parametric assumptions, so were analyzed with a non-parametric analysis of longitudinal data (R package "nparLD") followed by *post hoc* exact permutation tests (sequential Bonferroni correction).

Ethical Note

This research followed the guidance given by Directive 2010/63/EU, and French regulations regarding the use of animals for experimental procedures, and was approved by the Regional Ethical Committee Cenomexa (Committee agreement number: 54; project agreement number: A14384001). The experiment was designed to decrease animal distress by minimizing the number of animals. Enrichment was provided to unstressed captive adult cuttlefish. After spawning, adult females died naturally following senescence (June/July). After the completion of behavioral experiments, juvenile cuttlefish were anesthetized in 17.5g/L MgCl2 and euthanized with an overdose of ethanol (2%) for neurobiological testing (results not detailed here).

RESULTS

Body Patterning

In the maternal stress groups, a repeated measures ANOVA revealed a significant effect of the background type (i.e., uniform vs. disruptive: $p = 0.001$; $F = 11.299$), and of the treatment groups ($p < 0.001$; $F = 15.66$). As no interaction was found ($p = 0.915$; $F = 0.089$), this analysis showed that mean HI are higher on the disruptive background whatever the group considered (**Figure 3**). Pairwise *post hoc* comparisons showed that mean HI values are lower in UM-C eggs than in WM eggs ($p < 0.001$) and SM eggs ($p = 0.034$). There was no significant difference between WM and SM HI scores ($p = 0.021$).

In the embryonic stress groups, a repeated measures ANOVA revealed a significant effect of the background type (i.e., uniform vs. disruptive: $p = 0.007$; $F = 7.493$), but not of stress treatment groups ($p = 0.066$; $F = 2.733$). As no interaction was found ($p = 0.893$), this analysis indicates that mean HIs are higher on the disruptive background in all groups (data not shown).

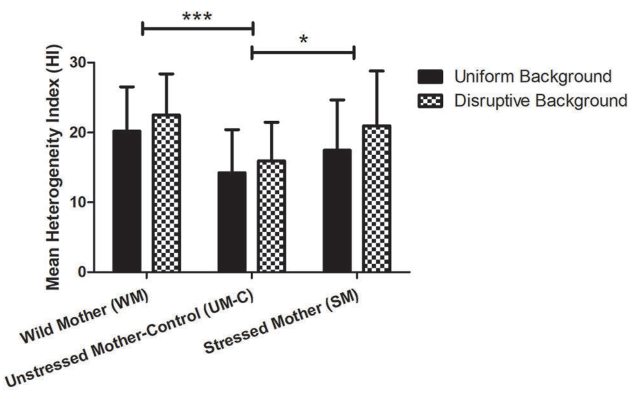

FIGURE 3 | Heterogeneity Index (HI) ± s.d. of maternal stress groups on uniform and disruptive backgrounds. Between groups, WM offspring ($n = 55$) and SM ($n = 43$) had significantly higher HI than UM-C ($n = 41$; $p < 0.001$ and $= 0.034$). Significant differences between groups are indicated by connecting brackets. $^{*}p < 0.05$; $^{***}p < 0.001$.

Initial Prey Encounter

In the maternal stress groups, there were no significant differences between groups for any of the variables measured (data not included).

Among the embryonic stress groups, there were no significant differences between groups in latency of detection, latency to attack, latency to capture or success rate (data not included). However, distance of detection was significantly different among the treatment groups (exact Kruskal-Wallis test: $p = 0.0178$; $H = 7.636$). Pairwise *post hoc* tests showed that this distance was significantly lower in UM-PE than in UM-LE (exact permutation test, sequential Bonferroni correction: $p = 0.008$; see **Table 1**). Attempted capture rate was also significantly different among the treatment groups (chi-square test: $p < 0.001$; $X^2 = 18.795$). Pairwise *post hoc* T-tests showed that this rate was higher in UM-LE than in UM-C and UM-PE groups (**Table 1**).

Visual Laterality Test

In the maternal stress groups, 72.1% of WM ($n = 43$), 47.5% of UM-C ($n = 40$) and 60.5% of SM ($n = 43$) offspring chose the shelter viewed in their left visual field (**Figure 4**). This group-level bias was only significant in WM group (exact binomial tests: $p = 0.005$). The proportion of individuals choosing the shelter located in their left or their right visual field was not significantly different between groups (chi square exact test: $p = 0.083$; $X^2 = 5.237$).

In the embryonic stress groups, 47.5% of UM-C ($n = 40$), 59.5% of UM-PE ($n = 42$) and 61.0% of UM-LE ($n = 41$) offspring chose the shelter perceived in their left visual field (data not included). No group-level bias was found, whatever the group considered (binomial tests). The proportion of individuals choosing the shelter located in their left or their right visual field was not significantly different between groups (chi square exact test: $p = 0.434$; $X^2 = 1.797$).

Overnight Activity Analysis

In the maternal stress groups, the distance traveled and time spent moving (**Figures 5A,B**) were significantly different

TABLE 1 | Attempted capture rate (percentage of cuttlefish that attempted captured), capture rate (percentage of cuttlefish that captured shrimp), success rate (the percentage of successful captures) of embryonic stress groups during the initial prey encounter.

	UM-Control $n = 35$	**UM-Predator Exposed (natural stressor) $n = 37$**	**UM-Light Exposed (artificial stressor) $n = 34$**	**Group comparisons**	***Post hoc* tests**
Attempted capture rate (%)	40.0	48.65	88.24	$p < 0.001$, $X^2 = 18.795$	UM-C vs. UM-LE: $p < 0.001$ UM-LE vs. UM-PE: $p = 0.008$
Capture rate (%)	85.71	88.89	96.67	$p = 0.492$, $X^2 = 1.862$	
Success rate (%)	85.71	84.21	96.67	$p = 0.333$, $X^2 = 2.568$	

Both group comparisons and post hocs are chi squared exact tests (sequential Bonferroni correction).

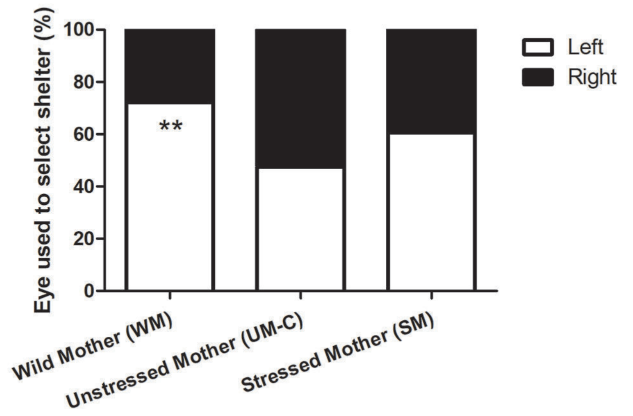

FIGURE 4 | Eye used to select shelter in maternal stress groups. More WM ($n = 43$) chose the shelter in their left visual field (binomial test; $p = 0.005$, signified by asterisks) while no preference was found in UM-C ($n = 40$) or SM ($n = 43$). The proportions were not significantly different between groups ($p = 0.08$).

between groups (Kruskal-Wallis tests: distance: $p = 0.009$; $H = 8.982$; time moving: $p = 0.028$; $H = 7.036$). Pairwise *post hoc* comparisons showed that both variables were significantly greater in SM ($n = 15$) than in UM-C offspring ($n = 10$) (exact permutation tests: distance: $p = 0.002$; time: $p = 0.005$). Finally, no significant differences existed between groups in mean meander (Kruskal-Wallis test: $p = 0.374$; $H = 1.965$; **Figure 5C**). In addition, WM showed a statistical trend for higher distance traveled than UM-C (exact permutation tests: $p = 0.058$).

In the embryonic stress groups, there were no significant differences between groups for any of the variables measured (Kruskal-Wallis tests; data not included).

Threat Response Activity Analysis

In the maternal stress groups, the non-parametric analysis for longitudinal data revealed a significant difference within groups according to time (i.e., before vs. after water addition), but not according to treatment groups (i.e., WM, SM, and UM-C) or cue type (i.e., blank water vs. predator odor), for both distance traveled ($p < 0.001$; $F = 32.666$; **Figure 6A**) and time moving ($p < 0.001$; $F = 25.284$; **Figure 6B**). As no interaction was found, this analysis showed that mean distance traveled and time spent moving are decreasing after adding water whatever the treatment group and the cue type considered.

In the embryonic stress groups, the non-parametric analysis for longitudinal data revealed a significant difference within groups according to time (i.e., before vs. after water addition), but not according to treatment groups (i.e., UM-C, UM-PE, and UM-LE) or cue type (i.e., blank water vs. predator odor), for both distance traveled ($p < 0.001$; $F = 37.982$; **Figure 7A**) and time moving ($p < 0.001$; $F = 32.437$; **Figure 7B**). As no interaction was found, this analysis showed that mean distance traveled and time spent moving decrease after adding water whatever the treatment group and the cue type considered.

DISCUSSION

We conducted this experiment with the aim of determining if prenatal stress affects cuttlefish behavior, and to compare various stressor types. We found that maternal stress was associated with differences in offspring body patterning and activity patterns. By contrast, offspring exposed to a natural stressor, predator odor, showed no differences from controls, while embryos exposed to an artificial stressor, bright light, differed in their predation behavior. In addition, we found that maternal captivity during spawning may affect visual laterality (summarized in **Table 2**).

Body Patterning

In all groups, the mean HI (disruptiveness) on the disruptive background was consistently higher than that of the uniform one, suggesting that all cuttlefish adjusted their body patterns to the background. Significant differences were also seen between groups: In our experiment, maternal stress increased the mean disruptiveness of the body pattern displayed. Our results also suggest that female captivity during egg-laying can induce a group bias for higher disruptiveness in her offspring, since the offspring of wild mothers had the highest HI overall. Previous experiments with cuttlefish hatchlings have detected similar differences in body patterning between groups incubated in different environments (O'Brien et al., 2016a) and exposed to certain pharmaceuticals during development (Di Poi et al., 2014;

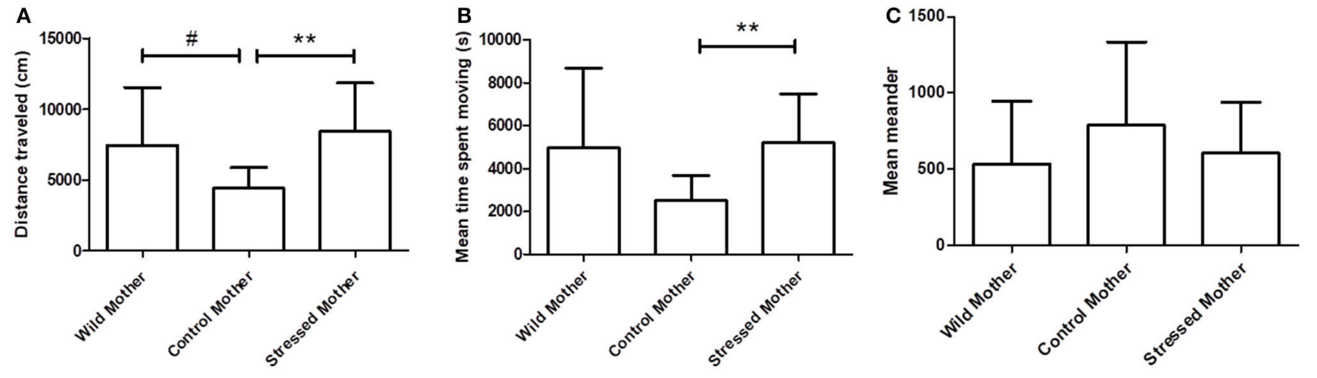

FIGURE 5 | The total distance traveled **(A)**, time spent moving **(B)** and mean meander (turn angle/distance traveled; **(C)** ± s.d. of maternal stress groups in the overnight activity analysis test. Significant differences (indicated by connecting brackets with asterisks) exist between UM-C ($n = 10$) and SM ($n = 15$) in both distance traveled and time spent moving ($p = 0.009$ and 0.005; *post hoc* asymptotic permutation tests with sequential Bonferroni correction. WM $n = 20$. $^{**}p < 0.01$; #indicates a statistical tendency ($p < 0.08$).

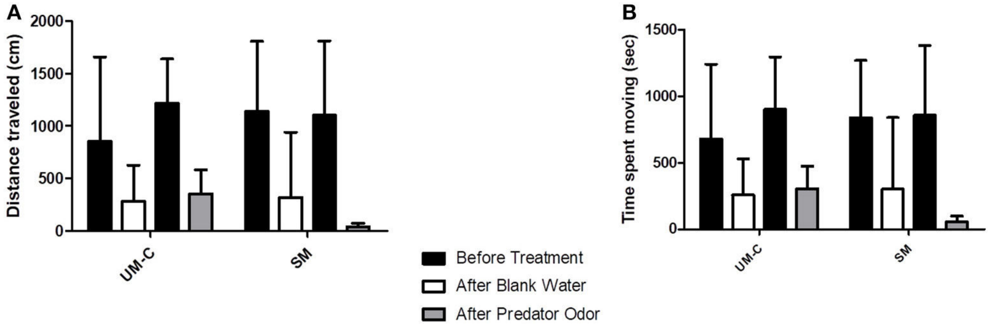

FIGURE 6 | The total distance traveled **(A)** and time moving **(B)** ± s.d. for maternal stress groups in the threat response activity analysis. Differences within groups are indicated by connecting bars; $n = 5$ for all bars.

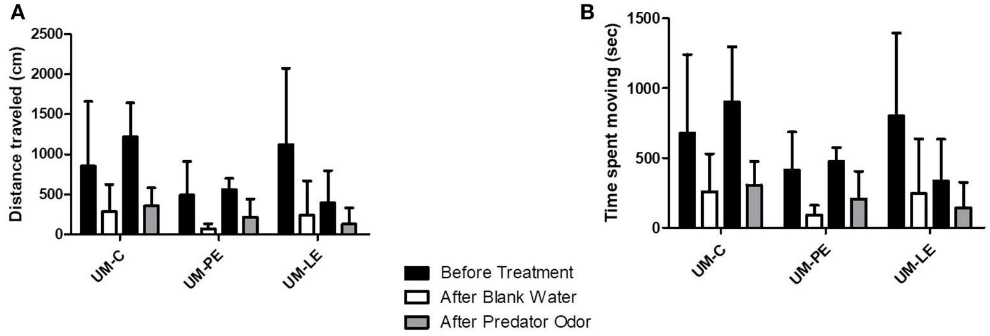

FIGURE 7 | The total distance traveled **(A)** and time moving **(B)** ± s.d. for embryonic stress groups in the threat response activity analysis. Differences within groups are indicated by connecting bars; $n = 5$ for all bars.

Bidel et al., 2016). The existence of similar differences between maternal stress groups in this experiment indicates that maternal experience can also affect this behavior, and may be adaptive for their offspring—higher disruptiveness could potentially improve camouflage on the variegated backgrounds often present in the natural environment.

Where the tactic of adult cuttlefish is often to match the background by expressing more uniform patterns in response to uniform backgrounds and more disruptive patterns in response to disruptive ones (Mathger et al., 2007; Barbosa et al., 2008), young cuttlefish usually display a fairly chronic body pattern that often clashes with the background (Hanlon and Messenger,

TABLE 2 | Summary of behavioral test results in comparison to the unstressed control mothers.

	Body Patterning	**Predatory Behavior**	**Visual Laterality**	**Activity Patterns**	**Threat Response**
	2015 data	**2015 data**	**2015 data**	**2015 data**	**2016 data**
Wild Mother offspring (WM)	Higher disruptiveness	No effect	Group-level left bias not observed in control group	Statistical tendency for higher distance traveled	Not tested
Stressed Mother offspring (SM)	Higher disruptiveness	No effect	No effect	Greater distance traveled and time spent moving	No effect
Natural stressor: Predator-exposed as eggs (UM-PE)	No effect	No effect	No effect	No effect	No effect
Artificial stressor: Light exposed as eggs (UM-LE)	No effect	Higher attempted capture rate	No effect	No effect	No effect

1988; Poirier et al., 2005). The ability to produce a uniform body pattern emerges during the first few months of life (see O'Brien et al., 2016b), and the results of the present experiments suggest that maternal stress and environment may delay the emergence of this ability.

Predation

Almost twice as many UM-LE offspring attempted capture than UM-C or UM-PE. Light is known to influence the timing of hatching (Paulij et al., 1991), and it is possible that these offspring had higher feeding motivation at the same age than other hatchlings because of increased energetic needs due to accelerated embryonic development. Faster development could also have accelerated visual maturation, leading UM-LE hatchlings to be better than their siblings at detecting prey. Indeed, UM-LE were able to detect prey at a significantly greater distance than UM-PE. It is worth noting however, that although a greater proportion of UM-LE captured shrimp, they were not better predators than the other groups, since the capture and success did not differ significantly between groups (close to 100%). This is in accordance with early experiments suggesting that prey capture operates using a highly-stereotyped program that improves little with age or experience (Wells, 1958). Despite not being better at hunting, young cuttlefish with higher feeding motivation would likely grow faster from consuming more prey.

Visual Laterality

In our experiment, no group-level bias was found in the control group. This is in accordance with previous experiments showing that a left eye-use preference for shelter seeking is not fully developed until a month after hatching (Jozet-Alves et al., 2012). Among all other groups, only WM group displayed a group-level preference toward the left side on Day 5. These results do conflict somewhat with the findings of Jozet-Alves and Hebert (2012); in that study, the authors showed that prenatal exposure to predator odor induced a left preference 3 days after hatching. However, this preference was slight, and it was necessary to test each cuttlefish more than once to detect it. Our experiment used a single trial per individual, a method formerly utilized in birds (Pittet et al., 2009), and it is possible that running only one trial did not allow us to detect the presence of the fledgling eye-use preference seen in the other groups.

The fact that eye-use preference did exist in the WM group suggests that when egg-laying and early development occur in the wild, the maturation of the left eye use preference is faster. Being lateralized from hatching may have an adaptive advantage by rendering WM offspring able to dual task (Vallortigara and Rogers, 2005). For example, while using their right eye for hunting (Schnell et al., 2016) they can simultaneously "keep an eye out" for shelter with their left should the need for a rapid escape arise.

Overnight Activity

In our assessment of baseline activity level, we found no differences between embryonic stress groups, while stressed mother offspring were associated with greater activity than control mother offspring, and similar to that of WM. We also observed a statistical tendency for WM hatchlings to travel a greater distance than UM-C. Activity levels and open field behavior have been used in behavioral research as a means of quantifying the impacts of various prenatal stressors in a variety of animals. No previously-published studies have measured this behavior in cuttlefish hatchlings, but we can draw insight from other species.

Some species, including rhesus monkeys and salmon, demonstrate decreases in overall activity after maternal or embryonic stress (Schneider, 1992; Clarke et al., 1996; Espmark et al., 2008), while others, including blue foxes and Japanese quails, show increases in activity and steps taken in open field tests (Braastad, 1998; Guibert et al., 2011). The effects of prenatal stress on activity have been studied most extensively in rodents, especially rats, and results are mixed. Some authors (Masterpasqua et al., 1976; Peters, 1986; Hilakivi et al., 1989; Sandi et al., 1996; Wilson et al., 2013) report increases in exploration and open field activity. Others report no or little effect of stress (Chapman and Stern, 1979; Van den Hove et al., 2005), or even opposite effects according to sex (Alonso et al., 1991). The majority of studies however, find decreases in movement and "exploration" in the offspring of females subjected to a variety of stressors during pregnancy (Hockman, 1961; Fride et al., 1986; Suchecki and Neto, 1991; Poltyrev et al., 1996; Vallee et al., 1997; Fujioka et al., 2001; Patin et al., 2004). Thus it seems that cuttlefish may differ in this respect from most vertebrate models and could therefore serve as a means to explore the factors driving the evolution of this response in different animal groups.

Based on insight from the studies in other animals that do show activity increases (cited above), the greater activity level observed in SM may reflect a search for shelter or food or an urge to escape. This could be advantageous by allowing young cuttlefish to avoid predation and to grow more quickly. It is also worth noting that an open field test conducted under laboratory conditions may not reflect "natural" behavior that would be seen in the wild. Indeed, a study in lab mice that compared open field behavior in the lab to the same test conducted in an outdoor grassy field found marked limitation in the number of behaviors expressed in the artificial setting (Fiore et al., 1995).

The group differences observed suggests that the stress experienced by the females during egg-laying was transmitted to their offspring and altered behavioral patterns. Physiologically, such an increase might be the result of slower vertical lobe maturation. This is the area of the brain potentially responsible for behavioral inhibition (Dickel et al., 2001, 2006), and a less mature VL would permit a higher level of basal activity. This experiment provides a starting point for future activity analyses with hatchling cuttlefish.

Threat Response

Many animals strongly alter their activity patterns in response to predator odor, especially in aquatic ecosystems. In particular, there is an extensive amount of literature documenting the behavioral responses of numerous aquatic gastropod and bivalve species (the extant molluscan groups most closely related to cephalopods) to waterborne predator odors, including escape responses such as crawling out of the water or burying (e.g., Snyder and Snyder, 1971; Jacobsen and Stabell, 2004; Dalesman et al., 2006), as well as reductions in movement such as cessation of filter feeding or decreases in foraging and migration (e.g., Reimer and Tedengren, 1997; Smee and Weissburg, 2006). Adult cuttlefish are known to react to predators with increases in escape behavior (Staudinger et al., 2013) and numerous body pattering displays (Adamo et al., 2006). Cuttlefish embryos are able to detect odors starting during the final third of embryonic development and respond to it in various ways, including embryonic increases in breathing rate (Romagny et al., 2012; Mezrai et al., in preparation), as well as post-natal behavioral lateralization (Jozet-Alves and Hebert, 2012) and changes in prey preference (Guibé et al., 2010). Thus, the ability to detect waterborne predator cues is present before hatching. The existence of odor-induced anti-predator responses in other molluscs, coupled with chemosensory abilities of embryonic cuttlefish, led us to predict that a change in activity pattern would be observable in response to predator odor in young hatchlings. The predator cue we utilized came from sea bass, which are known to prey on hatchling cuttlefish in the wild (Blanc and Daguzan, 1999), and thus represent an imminent threat to survival which should elicit a change in movement.

A reduction in activity was observed in all groups after the addition of either predator odor or blank water. This was a continuation of a pattern of progressively decreasing activity over time, and no group's reaction to predator odor differed from that of their response to blank water. Thus, it seems that unlike many other molluscs and adult cuttlefish, hatchling cuttlefish do not possess a marked locomotory threat response. Perhaps they rely exclusively on burying and/or body patterning to avoid predation. Unfortunately, the video quality and lack of sand necessary for the behavioral tracking software to function optimally prevented us from observing any burying or body patterning response. Researchers should take advantage of evolving video analysis technology to incorporate these possibilities into future tests of activity and threat response.

Maternal vs. Embryonic Stress

Body patterning and activity levels were both affected by maternal stress, while embryonic stress only affected one aspect of predatory behavior. Additionally, the differences between WM and UM-C in activity and turning bias suggest that the environment in which eggs are laid can also affect offspring behavior. In sum, maternal stress and spawning environment resulted in more post-natal behavioral changes than the direct experience of stressors in the egg. The greater post-natal reaction to the maternal stimuli suggests that mothers' experience might be a more reliable indicator of future prospects than stressors experienced by the embryos directly.

Maternal experience is known to "program" offspring in many other species; most commonly, the offspring of mothers exposed to a particular predator showed adaptive responses when encountering that predator itself (reviewed in Agrawal et al., 1999; Storm and Lima, 2010). In birds and mammals, such maternal stress effects are likely mediated by the transfer of stress hormones in the egg or placenta (Hayward and Wingfield, 2004; Groothuis et al., 2005; Weinstock, 2008). Since cuttlefish lack a planktonic larval phase and their dispersal abilities are likely limited by their size, any dangers present at or near the spawning site are likely to be a threat to cuttlefish at hatching. Anticipating and preparing for these threats makes adaptive sense. The higher disruptiveness and greater activity levels of stressed mother offspring and the higher disruptiveness and left turning bias of wild mothers could be advantageous to hatchlings by improving camouflage and facilitating escape from predators.

The effects of maternal environment and stress should be taken into account when planning, conducting and interpreting future laboratory experiments with cuttlefish—the behaviors observed may differ depending on how subjects were obtained (i.e., bred in captivity or collected from the wild) and handled, and experimenters should carefully consider their experimental priorities (i.e., whether they are trying to assess natural behavior) before they source cuttlefish eggs for experiments. More broadly, further experimentation in other oviparous species is important in understanding the results obtained in viviparous and ovoviviparous species for which maternal and embryonic effects cannot be disassociated.

Artificial vs. Natural Embryonic Stressors

Sea bass (*D. labrax*) are a particularly relevant stressor to cuttlefish since they have long co-existed in the English Channel and readily predate on hatchling and juvenile cuttlefish (Blanc and Daguzan, 1999). Sensing sea bass odor in the natal environment is a direct signal of post-natal threat for hatchling cuttlefish. Because of this, selective pressure for embryos to detect and prepare for this threat is presumably strong. Indeed, embryonic exposure to seabass odor is associated with increased lateralization in cuttlefish hatchlings, a behavioral adaptation which can facilitate rapid escape (Jozet-Alves and Hebert, 2012). In these five experiments however, the predator cues had no discernable behavioral effect.

It is possible that embryos habituated to the predator odor. In our experiment, UM-PE embryos were housed in tanks with seabass for most of development, and had the ability to sense odor cues for the last seven (of 30) stages of embryonic development (Romagny et al., 2012). Thus, they had at least several weeks of chemosensory exposure to these predators. Post-natal studies in other animals, including fish, rats and lizards, have shown that while acute stress exposure can result in adaptive changes (e.g., increased predator avoidance behavior or HPA-axis sensitivity), long-term or repeated exposure can actually reduce or eliminate the adaptive response (Dielenberg and McGregor, 1999; Weinberg et al., 2009). On the other hand, some studies show a lack of habituation to predator odor applied long-term (e.g., Epple et al., 1993). If habituation to predator odor is indeed occurring in cuttlefish, the evolutionary reason for this merits further scrutiny. One possibility is that because the predator odor was not paired with alarm cues from injured conspecifics in our experiment, the cuttlefish embryos learned to regard it as benign. Such a phenomenon occurs in harbor seals, which learn to distinguish between the calls of fish-eating and seal-eating orca populations and behave accordingly (Deecke et al., 2002).

In parallel, we tested an "artificial" stressor that could be compared to the effect of predator odor. We selected an artificial light source (LED panels) at a high intensity to penetrate the opaque egg membrane. The timing of the light regime was randomized and mimicked what might occur in some artificial settings. Though this stressor was a completely artificial stimulus and not indicative of a threat, it was associated with a strong, seemingly adaptive effect on predation behavior. Thus, our prediction of positive effects in response to predator odor and of disruptive effects in response to LED light was not supported by these results. This suggests that the evolutionary "familiarity" of a stressor (i.e., whether the species has encountered it before) is not the only explanation for fitness differences in the stressor response. The fact that we found an effect of light (increased predation) and no effect of predator odor may instead be explained by the relevance of the sensory modalities engaged by each stressor. While both odor and light can be perceived and responded to by embryos, cuttlefish are highly visual animals (Darmaillacq et al., 2017), and thus visual cues are likely to be more relevant to them than odor cues. Alternatively, this behavior may simply reflect a physiological improvement in visual acuity due to the wider ranges of light intensity experienced during embryonic development. Further testing exploring the role of different cues and sensory modalities are ongoing (Mezrai, in preparation).

CONCLUSION

The results reported here can serve as a basis for future behavioral tests examining prenatal stress and other embryonic influences. The tests utilized were non-invasive methods and, when employed as a battery, cover a broad range of behaviors critical to survival that give a rough measure of offspring fitness and treatment group differences. In particular, the activity analyses and threat response test were the first to be conducted with hatchling cuttlefish, and should offer valuable baseline data for researchers hoping to utilize such tests in the future. Further experimentation with other sources of prenatal stress will elaborate on the results reported here and could reveal previously-unknown prenatal pressures driving offspring behavior.

At the same time, greater effort should be made to account for the effects of spawning environment and early stimulation when planning and interpreting laboratory experiments and in the welfare of this regulated species. It is well-established that environmental enrichment is crucial to early cognitive development in cuttlefish (Dickel et al., 2000) and is recommended for the welfare of adults (Fiorito et al., 2015). The results presented here underscore the importance of maintaining a stimulating environment for reproducing females and even potentially their eggs. Researchers should strive to maintain at least a basic level of sensory enrichment for captive adults, and carefully consider the environmental cues experienced by developing eggs. Future guidelines will hopefully standardize a basic level of enrichment for all European cuttlefish research. It may even be beneficial to include predator cues and other mild stressors to encourage the development of certain aspects of the behavioral phenotype (e.g., hunting ability). Carefully adapting captive enclosures to cuttlefish needs will ensure the psychological well-being of individuals and the reliability of experimental results, promote growth in aquaculture and yield more savvy offspring for future hatch and release programs.

SUMMARY STATEMENT

The effects of several chronic prenatal stressors (maternal stress, embryonic exposure to predator odor or bright light) on hatchling cuttlefish are compared in five tests.

AUTHOR CONTRIBUTIONS

CO: Primary data collector and author of article; CJ-A: Assisted with data analysis, experimental design and editing of manuscript; NM: Data collector, editor of manuscript; CB: Data collector, editor of manuscript; A-SD: Supervisor, editor of manuscript; LD; Primary supervisor, editor of manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Annexe 5

Mezrai, N., Houdelier, C., & Lumineau, S. (in preparation). Prenatal auditory stimulation influence emotivity and social behaviour of quail chicks.

TITLE:

Prenatal auditory stimulation influence emotivity and social behaviour of quail chicks.

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ABSTRACT:

Behavioural development in young birds is influenced both by genetics and environmental factors. Prenatally, embryos are able to perceive and respond to a variety of stimuli. These stimuli (tactile, chemical, auditory or visual) can influence behavioural development and their effects may persist into the postnatal period. Some prenatal stimuli may induce prenatal stress in the embryo. This purpose of this experiment is to analyse the effects of auditory stress (predator vocalization or metallic noise) on the emotional reactivity and social behaviour of young Japanese quail (*Corturnix coturnix japonica*). Our results suggest that prenatal stress impacts the emotional and social responsiveness of young. The quails that have experienced predator vocalizations are less emotionally reactive than other individuals in the open field and in new-object tests. Conversely, young birds that have experienced metallic noise are much more sensitive to social separation. Thus, we conclude that prenatal stress influences postnatal development and learning abilities in Japanese quail.

Keywords: auditory stimulation; behavioural development; emotivity; social behaviour; Japanese quail

Introduction

Ontogeny refers to the development and behavioural maturation that occurs over the course of life [1]. This dynamic and complex process involves genetic and environmental factors present before and after birth [2]. These factors, including abiotic parameters (such as photoperiod) and biotic (such as social environment) may modulate a number of behavioural traits [3,4]. During the postnatal period, parental care can influence the emotional, social and feeding behaviours of young [5]. Similarly, during the prenatal period, even if the embryo develops in an enclosed environment, it may perceive tactile, vestibular, olfactory, gustatory and hormonal stimuli [6]. The timing of these stimuli will determine how they will affect ontogeny. The development of sensory systems in mammals and birds has a definite chronological order with some overlap in the development of different sensory modalities. The tactile sensory system differentiates first, then the gustatory and olfactory system, followed by the auditory system, and finally the visual system [7]. In most altricial species such as rats or domestic dogs, only tactile and chemical sensory systems are completely developed during the second half of gestation. The development of the auditory and visual systems is completed after birth [7]. Conversely, in precocial species such as sheep and domestic fowl, all of the sensory systems develop prenatally [7].

Prenatal stimulation of the embryos can have beneficial and/or deleterious effects on behavioural development [4,6]. We are interested in the auditory sensory modality, which is paramount to the bird communication. It has been shown that prenatal auditory experience influences the postnatal auditory preferences in some species. Indeed, embryos of Pekin ducklings (*Anas platyrhynchos*) and domestic chicken (*Gallus gallus domesticus*), are able to learn the vocalizations of their species, which allows them to discriminate after hatching [4,8]. However, certain repeated auditory stimuli such as distress calls can disrupt the establishment of postnatal visual preferences in bobwhite quail (*Colinus virginianus*: [9]). Similarly, some chemical stimuli of maternal origin can influence the ontogeny of young birds [10]. In mammals, maternal glucocorticoids can enter the embryo through the amniotic fluid and modulate behavioural development [11,12]. Similarly, in birds, the composition of the eggs may be affected by stressful living conditions of the laying female which can influence the development of the young. Thus, females stressed during their laying phase can produce higher levels of egg corticosterone and sex steroids (testosterone, androstenedione) and have more emotionally reactive young [13–15]. The chemical prenatal environment can influence a wide range of behaviours such as social, emotional or sexual behaviour of the individual [13,15–17].

Analysing the effects of stressful stimuli applied directly to the embryo is difficult to implement in mammals because the effects will be confounded with prenatal maternal influences (the mother may also react to these events). The bird model is an ideal alternative model in which to address this, since the embryo develops outside the mother's body, and the prenatal environment can be controlled. We therefore plan to analyse the effects of repeated auditory perturbations during the prenatal period on the ontogeny of young birds. After exposing experimental subjects to natural or artificial auditory stimuli, we will determine the impact of the stimuli on emotional reactivity (the individual's response into experimental fear-eliciting situations) and on the social behaviour of stimulated and unstimulated chicks.

Methods

Eggs incubation

Japanese quail (Galliformes, Phasianidae) are precocial birds, young are born with well-developed motor and sensory systems. We used quails from an industrial breeding: Les cailles de Chanteloup (Corps-Nuds, France). 294 fertilized quail eggs were placed in three identical artificial incubators (Incubator Ducat Version © TU models 140, N = 61 per incubator). Each incubator was placed in a soundproof room. Egg incubation typically lasts 17 days [18]. During the first 14 days, the eggs were maintained at a humidity of 45% and a temperature of 37.7 °C and rotated 45° every 30 minutes. On the 15th day of incubation (ED15), humidity was increased to 70% in order to induce hatching. Throughout the period of incubation, the eggs developed in constant darkness so as not to induce motor lateralization [19]. A red light (60W) was used to monitor the progress of incubation (this light not induce functional asymmetry contrary to white light; [20]).

Chicks and housing conditions

At hatching, chicks were individually identified by using coloured leg rings. Then, they were placed in collective cages (101x65x35cm) in experimental groups of ten individuals. The young were reared on wood shavings, with water and food provided *ad libitum*. A warming bulb (38±1°C) was placed in each cage to ensure proper thermoregulation until the chicks were 10 days old. When chicks became able to regulate their own temperature, the warming bulbs were switched off and the temperature in the room was kept at 20 ± 1°C. Chicks were exposed to a 12:12h light: dark cycle. The general development of chicks was followed by weighing them weekly, from hatching until they were 6 weeks old, using electronic scales.

Auditory stimulation

To study the impact of auditory stimulation on behavioural development, three batches were created. An unstressed control group (C) (N = 13); a group exposed to natural stress (NS) during the prenatal period (N = 11) and a group exposed to artificial stress (AS) during the prenatal period (N = 23). We characterized their emotional reactivity and social behaviour between the 4th and 16th day of life (D4 to D16) (cf. figure 1).

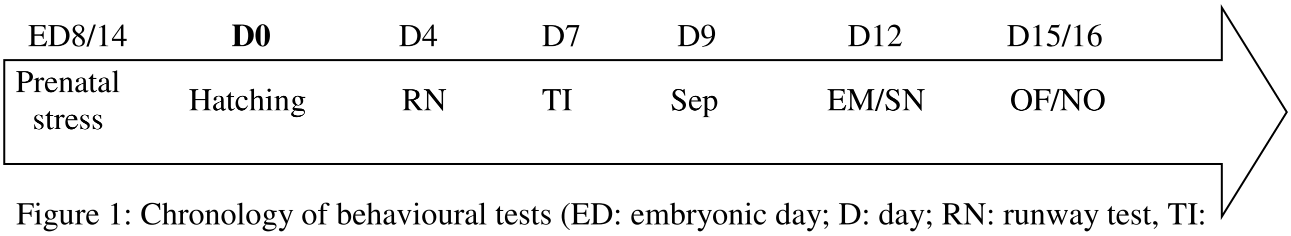

Figure 1: Chronology of behavioural tests (ED: embryonic day; D: day; RN: runway test, TI: tonic immobility test; Sep: separation from siblings; EM: emergence test; SN: sudden noise test; OF: open field test, NO: novel object test).

AS and NS chicks were exposed to sound stimuli from ED8 to ED14, early in the development of the auditory sensory system [21]. NS embryos were subjected to vocalizations from the predatory hawk *Accipiter nisus* [22]. AS embryos were subjected to recording of a metal dish falling on the floor. Between ED8 and ED11, embryos were subjected to 13 minutes of auditory stress per hour (100 sequences stress of 4.7 seconds ± 0.1) (312 minutes per day). Then, to avoid habituation to this stimulus, the transmission frequency was doubled (624 minutes per day) from ED11 to ED14. Similarly, to avoid habituation, each sequence included breaks of random intervals of 1 to 5 seconds. The stimuli were broadcast through speakers placed in the incubator at a maximum intensity of 65 dB (measured on the surface of the eggs).

Finally, body weight, an indicator of somatic growth, was measured weekly from birth (D0) until the 37th postnatal day (D37) for the three experimental groups. The width of the cloacal slit, an indicator of sexual development, was also measured between D30 to D37 using a digital caliper (Mitutoyo®, Japan).

Behavioural tests

Emotional reactivity

1. Tonic Immobility

Tonic immobility (TI) is a natural antipredator reaction characterized by a catatonic state of the animal. Its duration is a good index of inherent fearfulness [23]. Tonic immobility can be easily induced by placing a bird on its back and by restraining it in this position for 5 seconds prior to release. Each individual was removed from its cage and placed on its back in a U-shaped device and held in this position for 10 seconds prior to release. The experimenter, placed out of the subject's sight, recorded both number of induction(s) required to obtain a TI lasting at least 10 seconds (with a maximum of 5 inductions) and the duration of tonic immobility (with a maximum of 300s). Instances in which the subject did not remain in TI duration for longer than 10 seconds were scored as 0s.

2. Emergence test and sudden noise test

This test measures the willingness of individuals leave a small and dark environment, considered a safe haven, to explore an unfamiliar environment (Laurence, 2012). The experimental arena is a soundproof cage with one transparent face whose floor is covered with wood chips (83x60x35cm). Quail were placed in an opaque wooden box (18x18x18cm) and positioned at the entrance of the experimental arena. The transport box was kept closed for 1 min and then opened until chick exited (maximum of 5 min). The experimenter noted latency of emergence from the wooden box into the experimental box. When a quail had not emerged, a maximum score of 180 s was recorded. Once the animal was in the test cage, the transport box was closed and the chick was observed for 3 minutes. The experimenter noted its comfort behaviours (grooming, stretching, and scratching) as well as its active and passive fear behaviours (high observation, runs, jumps, defecate). Then, the experimental noise was broadcast for 5 seconds (60dB measured in the center of the box). The immediate reaction of the subject and its behaviour during the subsequent 3 minutes were recorded.

3. Open-field test

This test allowed us to observe behavioural responses expressed by an animal placed suddenly in an unknown and open environment. The animal was placed at the center of a darkened heptagonal arena marked by white walls 30cm long and 60cm high. The experiment began when the light was switched on. The latency and the frequency of each individual behaviour was recorded continuously for 5 minutes. Low activity in this environment was considered to reflect strong emotion in the individual [24].

5. Novel object test

This test was performed immediately after the open field test. The animal was placed at one end of the darkened experimental arena. During this time, an unknown yellow T-shaped object (20 cm high) was placed at the opposite end of the device (cf. Figure 2). Once the light was switched on, all behaviours and the latency were recorded. Additionally, the latency to leave the starting box D1 and D2 as well as the latency to reach areas near the object (O2 and O1) were noted.

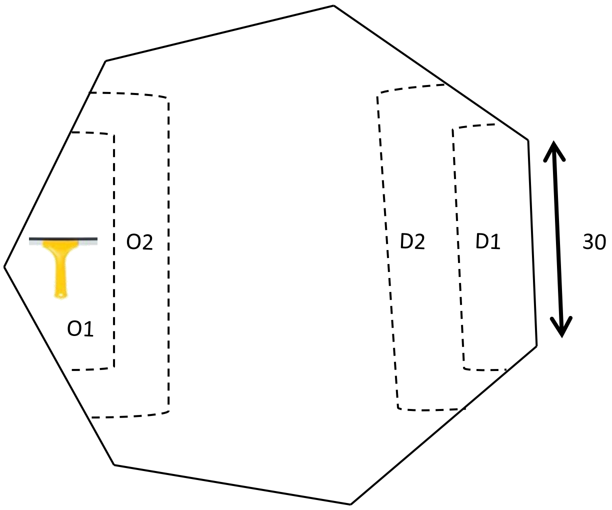

Figure 2: Experimental arena for the novel object test. Area D1: starting area; D2 area: adjacent to the starting area; O2 area: adjacent to the object area; O1 area: object area. Dimensions are indicated in cm.

Social behaviour

1. Runway test

The runway test allowed us to measure the social motivation of individuals, we measured their latency to join social stimuli and the time spent near conspecifics of the same age. The device consisted of a corridor of 90x20x20cm with a starting area at one end, and a cage with 4 unfamiliar conspecifics at the other end. The corridor was divided into four zones: a starting area located in the most distal zone (A: 32cm long), an intermediate zone (B: 32cm long), a proximate region (C: 32cm long) and an area for contact with the social stimulus (P: 4cm long). To perform this test, the individual was placed in a box (18x18x18cm) at the entrance of the tunnel for 30 seconds. The door of the box was opened allowing the chick free access to the device. Once the chick entered the runway, it was observed for 5 minutes. The latency and frequency of each behaviour and the time spent in each zone of the corridor was continuously recorded.

2. Separation from siblings

This test measured the responsiveness of chicks in social isolation. To perform this test, one chick was removed from its home cage with siblings and placed in a similar cage alone. Latency and frequency of each behaviour was recorded for 3min. The number of calls and the number of steps were considered to be an indication of the chick's motivation to join conspecifics (i.e. social motivation).

Statistical analyses

Most of our data were not normally distributed, so we used non-parametric statistical tests to compare behaviour between C, AS and NS chicks. Mann-Whitney tests, computed with R(3.0.2), were used to compare morpho-physiological measures. A principal component analysis (PCA) followed by varimax rotations was used to assess the effect of prenatal stimulation on the general behavioural phenotype of chicks. This PCA was computed using Excelstat® (2014). For all of these tests, the significance level was fixed at 5%. In the figures, the P values associated with tests are symbolized by: * for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$ and # when only a tendency is observed ($0.05 < p < 0.1$).Data presented in the figures are mean and standard error (m ± SE).

Results

Somatic and sexual development

At hatching, AS chicks were lighter than the NS and C chicks (cf. figure 3; Mann-Whitney: C *vs.* AS: z=86.5 p=0.03; C *vs.* NS: z=72.5 p=0.76; AS *vs.* NS: z=81.5 p=0.04). Thereafter, somatic and sexual development are similar in the three experimental groups (Somatic development: D19: C *vs.* AS: z=144.5 p=0.87; C *vs.* NS: z=67.0 p=0.79; AS *vs.* NS: z=108.0 p=0.5; D30: C *vs.* AS: z=141.5 p=0.96; C *vs.* NS: z=63.0 p=0.62; AS *vs.* NS: z=104.0

p=0.52; D37: C *vs.* AS: z=128.5 p=0.62; C *vs.* NS: z=67.0 p=0.79; AS *vs.* NS: z=117.5 p=0.89: Sexual development: D30: C *vs.* AS: z=111.0 p=0.27; C *vs.* NS: z=53.0 p=0.28; AS *vs.* NS: z=117.0 p=0.88; D37: C *vs.* AS: z=119.0 p=0.41; C *vs.* NS: z=69.0 p=0.88; AS *vs.* NS: z=89.0 p=0.22).

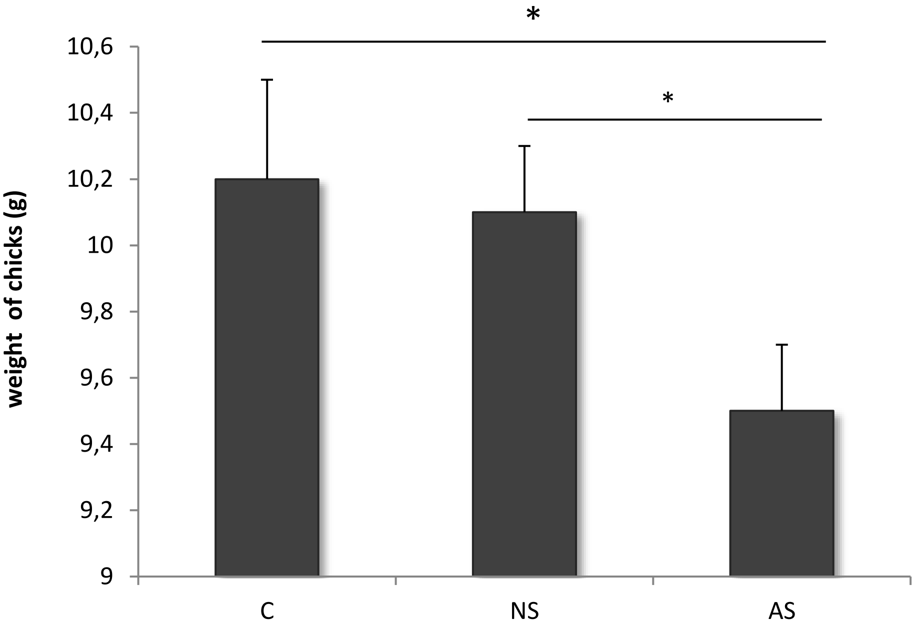

Figure 3: weight (g) at hatching of C, NS and AS chicks. Mann-Whitney test: * p≤0.05.

Impact on emotivity and social behaviour

The principal component analysis identified three factors that explain 55.9% of the total variance between variables. The first axis, called "**emotivity 1**" (19.5%), was characterized on one side by long latency for the first step in the open field test and a long latency to approach a new object, and on the other side by a high frequency of grooming in open field test. The second axis called axis of "**sociality**" (20.8%), was characterized by a lot of call cries in response to a sudden noise, a long latency to join congeners in the runway test, fear behaviour and call cries in the separation test. The third axis, "**emotivity 2**" (15.6%), was characterized by the duration of the tonic immobility, latency in the emergence test and frequency of fear behaviour in the sudden noise test.

The coordinates of C, NS and AS barycenters are illustrated in figure 4. Regarding axis 1, NS chicks exhibit lower emotivity as their coordinates are below those of C and AS chicks (Mann-Whitney test: NS *vs*. C: Z=-2.3; p=0.02; NS *vs.* AS: Z=-2.3; p=0.02). On axis 2, the coordinates of AS chicks are higher than those of C chicks and tend to be higher than NS chicks (AS *vs.* C: Z=-3.0; p=0.003; NS *vs.* AS: Z=-1.8; p=0.07). AS chicks exhibit more distress behaviour in social isolation and have a greater latency to join congeners than C chicks. Finally, on axis 3, the coordinates of NS and AS chicks tend to be higher than C chicks (C *vs.* AS: Z=-1.7; p=0.08; NS *vs.* C: Z=-1.8; p=0.08) which may indicate that the stressed chicks are more emotionally reactive. Finally, NS chicks display significantly more fear behaviours in sudden noise test (associated with the axis 3) than C chicks (Mann-Whitney test: NS vs. C: Z=-1.9; p=0.05).

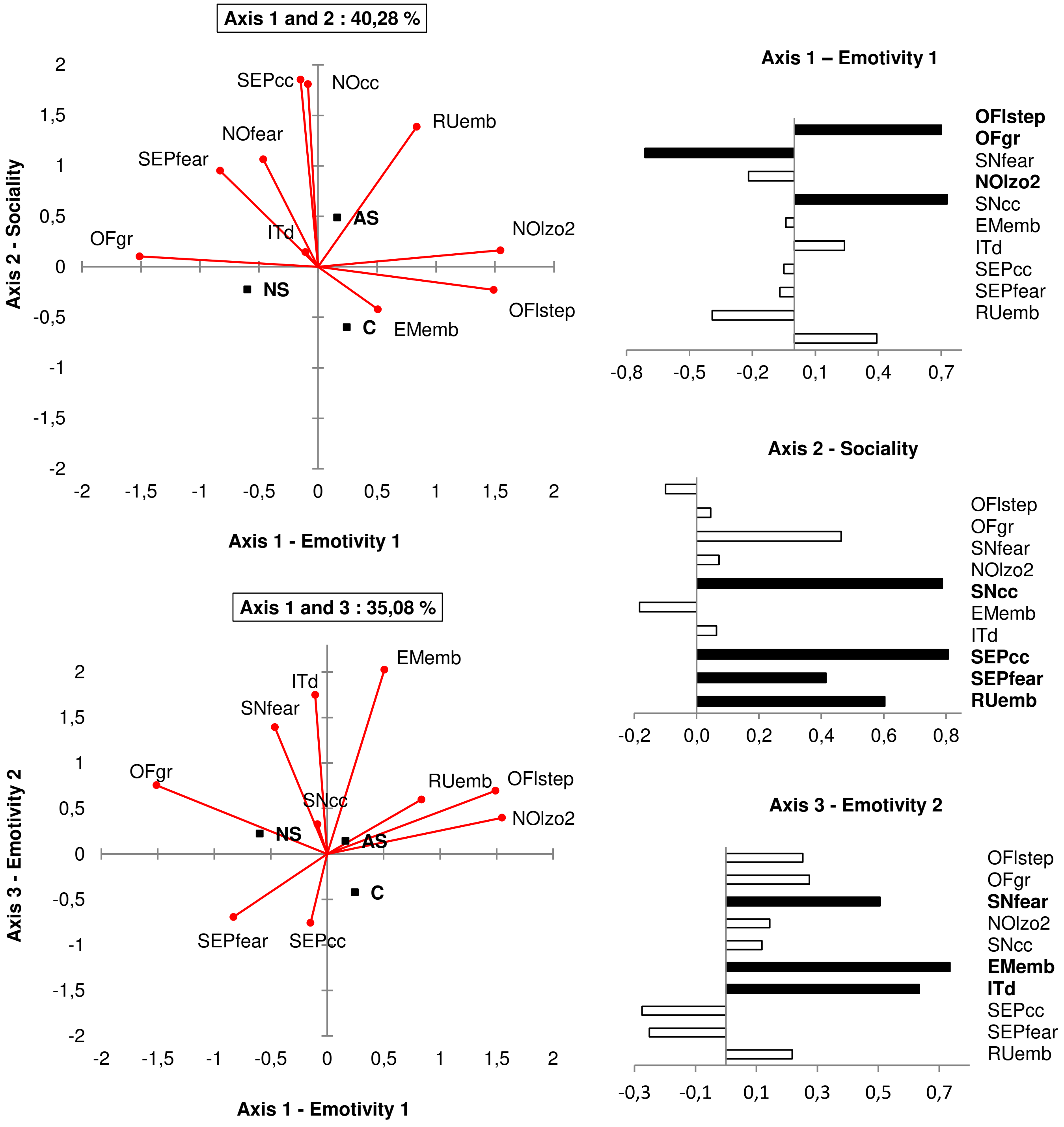

Figure 4 : Principal Component Analysis performed on relevant behaviours in emotionality and sociability tests (OFlstep: Latency until first step in open field test; OFgr: frequency of grooming in open field test; SNfear: frequency of fear behaviors in sudden noise test; NOlzo2 : latency to come in close proximity to a new object in the new object test; SNcc: Frequency of call cries during the sudden noise test; EMemb; latency until the emergence of chick; ITd:

duration of tonic immobility; SEPcc: frequency of cries during the separation test; SEPfear: frequency of fear behaviors in the separation test; RUemb: latency until emergence in the runway test). **Left:** Representation of variables and observations of the center of gravity (lot AS, NS and C) after Varimax rotation. **Right**: contribution (black) of the different variables to the three axes.

Discussion

In this study, we analysed the effect of prenatal stress on quail chicks, we first find that chicks stressed with artificial stress (AS) were lighter than chicks stressed with natural stress (NS) and control chicks (C). It is now well admitted, prenatal stress decrease the body weight of young at birth or hatching (mammals : [25]; [26] ; birds:[27,28]). For this quail chicks, artificial stress is probably more stressful than natural stress used during our study. However, after hatching, there is no significant difference between each group. The effects of prenatal stress can be diminished and/or disappear during their postnatal life.

Although the effects on growth are not visible after birth, we have been able to show strong effects on the development of emotivity and sociality. We have identified different behavioural profiles among our experimental groups, and these profiles vary according to the type of auditory stimulation. AS chicks were more social than controls: they were more responsive to social isolation and appeared less motivated to join unfamiliar individuals. NS chicks, meanwhile, were less emotionally reactive than C and AS chicks in an open field situation and in the presence of a new object.

Influence of prenatal stress on social behaviour

In mammals and birds, breaking the link between social partners induces emotional distress, resulting in calls cries and jumping [29]. In our study, AS individuals were much more responsive to social separation, emitting more distress calls and exhibiting more fear behaviours than C individuals. However, AS chicks also showed a greater latency to join unknown congeners in the runway test. These two results appear contradictory because individuals more vulnerable to social isolation normally have greater social motivation [30,31]. Nevertheless, it appears that social motivation, measured during the runway test, was influenced by particular characteristics of individual stimuli. Indeed, it appears that quail selected for their high latency to join unknown congeners reduce this latency when unknown congeners are replaced by familiar congeners [29]. We can therefore hypothesize that the AS quail may be more attached to familiar partners and that the artificial prenatal stress has influenced the development of social behaviour. It would be interesting to continue our investigation by analysing the social behaviour of SA individuals in their daily interactions.

Influences of prenatal stress on emotional reactivity

Emotional reactivity depends on both specific characteristics of the individual, the individual's experience and on the situation [17,24]. In our study, NS chicks were less emotional than C and AS chicks in the open field and new object tests. Indeed, NS chicks take their first steps earlier in the arena and they express more well-being behaviours.

NS chicks also approach unknown objects much faster than other individuals. This result contradicts studies showing a positive correlation between prenatal stress and emotion in mammals [11]. However, in birds, some types of prenatal stress (hypothermia, injection androgens) do not always result in any increase in emotionality (higher level of activity or development of a proactive behavioural phenotype [13,16]). Thus, prenatal stress may affect the development of emotional reactivity in birds differently. Note, however, that the NS individuals also demonstrate a stronger reaction to sudden noise than controls, reflecting a stronger emotional response during this test. These results reveal the multidimensional aspect of emotional reactivity [32] and that the emotional response of an individual can depend on the situation with which it is confronted. For example, two lines of Japanese quail selected to have either high or low emotional reactivity, will certainly differ in the duration of tonic immobility (test based on the selection), but have similar responses when a new object is introduced into their environment [33]. It is therefore necessary to improve our assessment of the effects of prenatal stress on the emotional reactivity of the individual.

Effect of acoustic stimulation type

In our study, the behavioural development of young chicks differed depending on the type of acoustic stimulation. While a natural stressor influenced emotional reactivity, an artificial stressor affected social behaviour. These differential effects show that the impact of prenatal auditory stimulation depends on characteristics of the stimulus and on the processes involved. Two hypotheses could explain this: (1) the structure of the sound could play a role in these prenatal effects. Indeed, even if we controlled the intensity and duration of the two stimuli, they would still differ in frequency and sequential organization, and these parameters can be seen at different times of embryonic development [34]. (2) These two stimuli may also be "integrated/interpreted" different ways by different individuals, inducing distinct behavioural effects. This phenomenon can be more easily identified. It would however be instructive to search for other sings of embryonic response to both stimuli including physiological and behavioural markers. For example, measuring the vocal response of quail eggs following the application of the two auditory stimuli to determine if the response differs depending on the situation could help us decide between these two hypotheses.

Our study showed, for the first time, the effect of negative prenatal auditory stimulation on the behavioural development of a young bird. This influence, however, appears to depend on the characteristics of the stimulation. In order to continue our line of investigation in this area, it would be useful to analyse the effects of prenatal auditory stress on a wild breed of chicks, such as the quail *Coturnix coturnix coturnix*. We should determine if, on the one hand, behavioural development of these individuals depends on stimulus type and, on the other hand, if their influence is effected by the breed's degree of domestication. Finally, our work has revealed an effect of prenatal stress on the behavioural development of individuals, even 20 days after the procedure. It is thus necessary to consider the potential consequence of auditory stress on subsequent generations, since the intergenerational transmission of maternal stress has been demonstrated recently in quail [17].

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Perception et apprentissage périnatal chez la seiche : Approche comparative et effet du stress prénatal

Cette thèse est centrée sur les capacités sensorielles, cognitives et sur les effets du stress chez deux espèces de seiche : *Sepia officinalis* et *Sepia pharaonis*. Nous avons d'abord démontré que les embryons répondent à différents stimuli environnementaux (lumière, proies, prédateurs, encre de seiche) mettant en évidence que l'information sensorielle passe à travers la capsule de l'œuf, ce qui permet une continuité sensorielle transnatale. De telles réponses sont possibles puisque leur système chimiosensoriel et visuel sont fonctionnels avant l'éclosion. Nous avons également montré que les embryons des deux espèces sont capables d'apprentissage simple (empreinte alimentaire) et associatif (conditionnement classique) et que ces capacités précoces pourraient augmenter leurs chances de survie avant et après l'éclosion en permettant la reconnaissance des proies et des prédateurs. Enfin, nous avons montré que le stress embryonnaire naturel (odeur de prédateur) et artificiel (lumière) ont des effets modérés voire nuls sur les capacités d'apprentissage périnatal. Ces résultats comportementaux ont été observés sans grande différence entre les deux espèces qui vivent pourtant dans des environnements très éloignés. Pris ensemble, ces résultats démontrent que les embryons de seiche ne sont pas isolés de leur environnement mais détectent et traitent les informations environnementales qui modulent leur comportement après l'éclosion.

Mots clés : *S. officinalis*, *S. pharaonis*, reconnaissance et apprentissage prénatal, stress prénatal.

Perception abilities and perinatal learning in cuttlefish: comparative approach and effect of stress

The focus of this thesis centres on the sensory, cognitive abilities and stress effect of two cuttlefish species: *Sepia officinalis* and *Sepia pharaonis*. First, we demonstrated that embryos respond to different environmental stimuli (i.e. light, prey, predators, ink) showing that sensory information passes through the egg capsule which allows a sensory transnatal continuity. Such responses to external stimuli are likely facilitated through their visual and chemosensory systems that are functional prior to hatching. We also demonstrated that the embryos of these two species are capable of simple learning (food imprinting) and associative learning (classical conditioning). These early abilities might increase their survival chance before and after hatching because they allow prey and predator recognition event before hatching. Finally, we showed that both natural embryonic stress (predatory odour) and artificial stress (light) have moderate or no effects on perinatal learning abilities. The same behavior was observed on the two species whereas they live in different continent. Taken together, these results demonstrate that cuttlefish embryos are not isolated from their environment, but rather detect and process external information which shapes their behaviors after hatching.

Key-words: *S. officinalis*, *S. pharaonis*, prenatal recognition, prenatal learning, prenatal stress.