THESE

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Effects of Prenatal Stress on *Sepia officinalis*

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Posters

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O'Brien, C.E., Bellanger, C., Jozet-Alves, C., Mezrai, N., Darmaillacq A-S., Dickel L. "Reproductive Effects of Maternal and Prenatal Stress in the Cuttlefish, *Sepia officinalis*." **2017 L'Ecole doctorale Normande de Biologie Intégrative, Santé et Environnement (EdNBISE) conference** in Le Havre, France, March 16-17, 2017.

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Abbreviations

PReSTO'Cog – Effets de stress prénatals sur le développement précoce des comportements et des capacités cognitives

WM – Wild Mothers

SM – Stressed Mothers

UM – Unstressed Mothers

UM-C – Unstressed Mother-Control (eggs or offspring)

UM-PE – Unstressed Mother-Predator-Exposed (eggs or offspring)

UM-LE – Unstressed Mother-Light-Exposed (eggs or offspring)

HI – Heterogeneity Index, a measure of body patterning disruptiveness; higher values mean a more disruptive body pattern

PIT test – Prawn in a tube test (the standard method of measuring learning in cuttlefish)

DML – Dorsal mantle length (the standard measure of cuttlefish size); distance between forward edge of mantle and posterior tip of mantle (excludes head and tentacles)

PVF – Perivitelline Fluid (substance that surrounds developing cephalopod embryos)

CREC – Le Centre de Recherches en Environnement Côtier (marine station of the Université de Caen located in Luc sur Mer)

SMEL – Synergie Mer et Littoral (marine research facility located in Blainville sur Mer)

VL – Vertical Lobe

OL – Optic Lobe (Left (L) or Right (R))

HPA axis – Hypothalamic–Pituitary–Adrenal axis

5-HT – Serotonin (a monoamine)

5-HiAA – 5-Hydroxyindoleacetic Acid (the main metabolite of serotonin)

NA – Noradrenaline (a monoamine)

DA – Dopamine (a monoamine)

DOPAC – 3,4-Dihydroxyphenylacetic Acid (the main metabolite of dopamine)

LTP – Long Term Potentiation (the increase of synapse strength following stimulation)

ISAE – International Society for Applied Ethology (a pool of independent experts for governments, International bodies, industry organizations, and NGOs)

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General Introduction

I. General Introduction:

Ethology and Stress

In 1963, Niko Tinbergen outlined an investigative framework for behavioral analysis, identifying four guiding analytical perspectives: mechanistic (i.e. the physiological and molecular processes that cause a behavior), ontogenetic (i.e. the events during development that affect behavior), adaptive (i.e. the ways in which a behavior augments survival or reproduction) and phylogenetic (i.e. the degree to which behavior is shaped by ancestry). These four perspectives form the foundation of ethology, the study of animal behavior (Tinbergen 1963). Originally, ethologists were mainly interested in basic research documenting animal behavior. Since the 1970s, however, with the establishment of the International Society for Applied Ethology (ISAE), ethologists have become more and more interested in the overarching processes which can explain general trends in animal and human behavior. At the same time, one of the primary goals of ISAE and the ethological community is to improve the welfare of captive species in zoos, aquariums, laboratories and agricultural facilities by our increasing our ability to balance human needs with the needs of animals, whether physiological or behavioral. For this reason, a great deal of attention has lately been focused on the study of stress. Though the exact definition of stress is sometimes controversial, it here refers to a suite of physiological, morphological and behavioral changes that occur in the face of external challenges in an attempt to re-establish homeostasis or to lessen the impact of the offending stressor.

Stress can have both "positive" and "negative" effects on organisms. When the stressor is short-term and one that has been encountered during the evolutionary history of the species, the stress response should be able to mitigate its negative effects and increase overall fitness. However, when the stressor is chronic or novel, the organism's own stress response may actually have more of a detrimental impact on health and fitness than the stressor itself. This is well-illustrated in our own species: when facing immediate danger, such as a predator or an oncoming automobile, the hypothalamic–pituitary–adrenal (HPA) axis will initiate a suite of automatic physiological changes ("fight or flight response") that enable one to escape the situation as quickly as possible (Cannon 1939). Over the long term however, the continuous activation of the same HPA axis can damage various body systems (*e.g.* the immune system), degrade health (*e.g.* impaired sleep) and reduce quality of life (*e.g.* anxiety). Likewise, many health and societal ills result from a mismatch between our evolved stress responses and modern challenges. Such health issues have broad societal implications, resulting in huge expenditures on healthcare and social services, as well as lost productivity and lower workplace performance (Greenberg

et al. 1999). Growing awareness of these negative effects of stress has spawned a large body of work concerned with better-understanding these effects in ourselves and on the evolution of species (Seyle, 1976).

Prenatal stress

In the study of stress, the period of reproduction, spawning and embryonic development is particularly interesting due to its importance in establishing patterns of future physiology, morphology and behavior (Gottlieb and Wagner 1991; Bremner, Lewkowicz, and Spence 2012; Houdelier et al. 2013). Indeed, stress during this time (referred to as "prenatal stress") can have profound effects not seen when the same stimulus occurs elsewhere in the lifecycle. While prenatal stress often enables organisms to predict and adapt to challenges present in the postnatal environment (Gluckman and Hanson 2004), it can also result in a lifetime of problems. Prenatal stress in humans 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 et al. 2010).

There are three potential avenues by which prenatal stress can exert its effects: 1) on the mother herself (by affecting fecundity, mating behavior or egg-laying), 2) through the mother to the offspring (*e.g.* via hormone transmission or perhaps sperm selection) or 3) direct perception of and reaction to the stressor by the embryo (Fig. 1). Understanding the relative contribution of these three potential paths of stress and their interactions is necessary to comprehending the ways that stress can impact health, society, behavior and the evolution of organisms. For instance, stressors applied to females during the reproductive period ("maternal stress") have been shown to affect offspring survival, behavior, learning and anxiety in diverse groups such as primates, rodents, birds and fish (reviewed in Braastad 1998; Schreck, Contreras-Sanchez, and Fitzpatrick 2001; Henriksen, Rettenbacher, and Groothuis 2011). These effects could either result from a direct stress reaction by the mother herself (affecting the number of or the genetic composition of her embryos) or the result of transfer from mother to offspring via provisioning or hormones in the placenta or egg yolk (Groothuis et al. 2005; Hayward and Wingfield 2004; Lemaire et al. 2000; Weinstock 2008). Embryos themselves may also perceive and react to stressors ("embryonic stress") applied to spawning or brooding females. In order to distinguish between maternally-mediated effects and direct perception, embryos must be isolated from the female and have the stressors applied directly to them. However, it is currently difficult or impossible to separate a mother from her developing offspring in species with internal development (but see Roberts 2017) or maternal care of offspring after birth. However, oviparous species—those in

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which embryos develop outside the female—allow separation of mother from offspring as soon the eggs are laid.
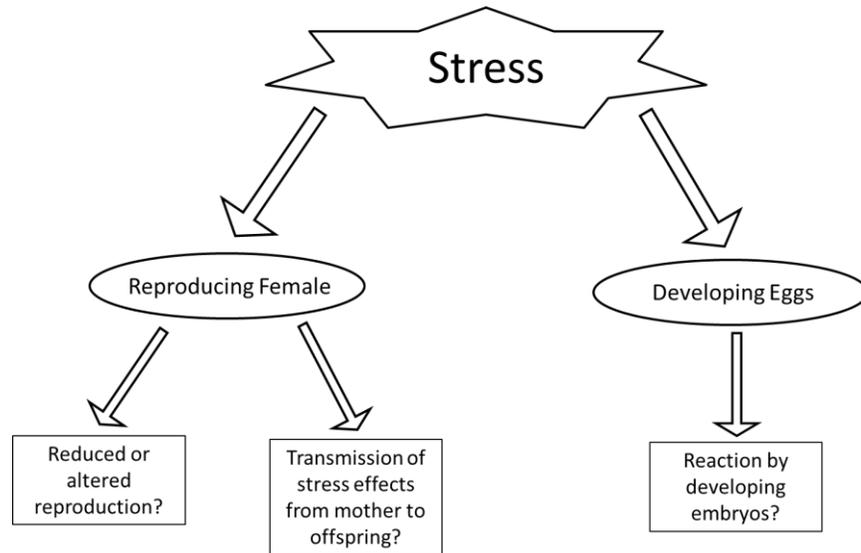
Figure 1. The three potential avenues of prenatal stress transmission.

There is also the question of the ecological relevance of particular stressors to the embryos. Naturally-occurring stressors, such as the odor of a predator, should, in theory, prompt an evolved, adaptive response. Indeed, when predator cues are applied to juvenile and adult animals, it often induces a change in phenotype or behavior that is adaptive in the face of this predator. A well-known example of this occurs in water fleas of the genus *Daphnia*; when a predator is detected, they develop spines and spikes that reduce the predator's ability to easily consume them (Walls and Ketola 1989). By contrast, an artificial stimulus not naturally encountered, such as bright light or loud noises, applied directly to developing embryos ("artificial stress") should confound the species ability to respond, resulting in maladaptive responses. For instance, exposure to high levels of anthropogenic noise has been linked to both stress responses and behavioral changes in cetaceans, which can have consequences as dire as stranding and death (Weilgart 2007).

PrestoCog, a comparative study of prenatal stress effects in oviparous species

This thesis is part of a larger ethological study called "Effets de stress prénatals sur le développement précoce des comportements et des capacités cognitives", or "PReSTO'Cog" for short. PReSTO'Cog is a collaboration between five labs across France. Each lab studies a different animal model: the domestic chicken, the Japanese quail, trout, the zebrafish and the European cuttlefish. These species represent a diverse range of animal groups: invertebrates (cuttlefish) and vertebrates (fish,

birds) as well as wild (cuttlefish), and domesticated species (chicken) and both poïkilotherms (cuttlefish, fish) and homeotherms (birds). All are oviparous and precocial, allowing offspring to be experimentally isolated from the female during embryonic development and free of the post-natal influence of maternal interaction. Finally, they are also relatively self-sufficient at birth, permitting immediate behavioral testing of the offspring. By comparing such phylogenetically-distant species, we address the topic of prenatal stress from Tinbergen's third and fourth perspectives—the adaptive and phylogenetic—uncovering clues to the evolutionary pressures and family history that led to the behavior we see in these species today. Ultimately, such insights could be applicable to other animal groups, including mammals, leading to improvements in human and animal welfare.

The unifying theme of this project is to determine if prenatal stress induces changes in offspring, and whether the type of prenatal stressor experienced affects the manner in which the offspring reacts. Do the effects of maternally-applied stressors on offspring differ from those of stressors applied directly to the embryos themselves? Does the response to an artificial stressor differ from that occurs in response to a naturally-occurring one? The effects of prenatal stress are assessed through a range of physiological, behavioral and learning tests of young offspring. We also search for clues as to the mechanisms of such effects, especially endocrinological evidence for the transfer of stress hormones from mother to offspring and changes in brain growth and morphology. These questions probe behavior from Tinbergen's first two perspectives—the mechanistic and ontogenetic—parsing the innate biological processes and external influences which interact to produce a particular behavioral repertoire.

Some definitions used in this thesis should be clarified. "Chronic stress" refers to stress induced by a stressors experienced continuously or repeatedly over an extended period of time. By contrast, "acute stress" is experienced after a single occurrence of a stressor. This thesis is predominantly focused on chronic stress, since it is generally associated with stronger and more long-term effects with greater implications for fitness. Note also that in the literature, many studies do not make the distinction between maternally-applied and direct embryonic stress, and refer simply to prenatal stress regardless of whether it was applied to the mother, to her offspring or to both.

Presentation of the study animal

Many invertebrates are both oviparous and precocial, making them potential candidates to study the ways that stress can affect a species as discussed above. Moreover, invertebrates represent 97% of the species on earth, live in nearly every part of the planet and demonstrate an awesome diversity in modes of life and behavior. Next to arthropods, the invertebrate molluscs are the second most populous phylum, and like arthropods, have successfully colonized sea, freshwater and terrestrial

habitats. Certain molluscs have also evolved in many ways that are convergent with vertebrates (e.g. the "lung" of terrestrial gastropods), making them good comparative models. The cephalopoda is a group of molluscs comprised of about 700 extant species (Hanlon and Messenger 1998), divided into four main groups: octopus, squid, cuttlefish and nautilus (Fig. 2A-D). They are exceptionally sophisticated, demonstrating advanced perceptual abilities, learning, memory, problem-solving and plasticity (Hochner, Shomrat, and Fiorito 2006). Cephalopods have nervous systems that are highly-centralized (Budelmann 1995) and uncharacteristically large, with brain to body weight ratios exceeding those of fish and reptiles and approaching those of mammals and birds. The nervous system has been well-studied over the last century, with specific cognitive tasks localized to specific brain lobes (summarized in (Dickel et al. 2013). Cephalopods display both convergent (e.g. the cephalopod and vertebrate eye) and divergent (e.g. cephalopod jet propulsion versus the muscle-powered propulsion of fish) adaptations to evolutionary challenges as other animal groups (Packard 1972; Hochner, Shomrat, and Fiorito 2006). Their sophistication and their position as invertebrates means that cephalopods have much insight to offer as counterpoints to more traditional vertebrate models, and their unique and extraordinary behaviors often challenge our notions of the general principles underlying animal behavior. It has even been posited that competition between fish and cephalopods shaped the evolution of numerous traits in both groups during the Mesozoic (Packard 1972).
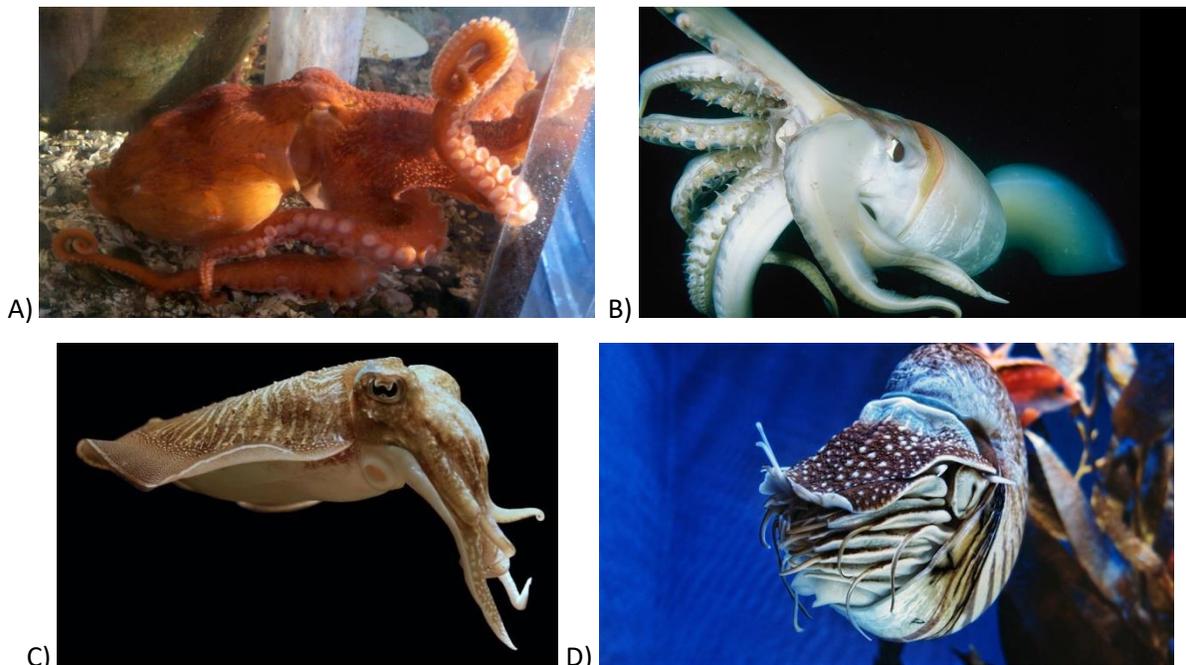
Figure 2. A) The giant Pacific octopus, *Enteroctopus dolfleini* (photo by C.E. O'Brien)*;* B) the giant squid (Architeuthis dux (photo by Brian J. Skerry, National Geographic); C) the common cuttlefish, *Sepia officinalis* (photo by Hans Hillewaert); D) the chambered nautilus, *Nautilus pompilius* (photo by William Cho).

Like the other PReSTO'Cog models, the common cuttlefish *Sepia officinalis* (Linnaeus 1758) (Fig. 2C), is an excellent model for studying the effects of prenatal stress because it is oviparous and precocial. *S. officinalis* is also a species that is important both commercially and scientifically: fisheries exist in both the Atlantic and Mediterranean (Dunn 1999) and it is cultured in several laboratories and aquaculture facilities (Pascual 1978; Forsythe, DeRusha, and Hanlon 1994; Pedro M. Domingues, Sykes, and Andrade 2002). Indeed, along with *Octopus vulgaris*, it is one of the most commonly-studied species of cephalopod. Most importantly, cuttlefish and other cephalopods have the advantage of being phylogenetically-distant from more typical animal models like rats and monkeys: they are an invertebrate group separated from vertebrates by hundreds of millions of years of distinct evolution, allowing them to serve as a reference point to determine whether the stress effects that we observe in different species are products of shared ancestry or separate evolutionary developments (Tinbergen's third and fourth questions). Ultimately, a better understanding of the effects of prenatal stress in *S. officinalis* will yield general insight into the processes and strategies by which organisms survive.

Additionally, this research will help fill gaps in knowledge about the specific biological needs of cuttlefish, particularly those regarding housing, reproduction and behavioral markers of welfare. Hopefully, insight from this work will improve the ability of aquaculturists and researchers to set standards of care and standard practice. This is particularly necessary due to the recent inclusion of cuttlefish and other cephalopods in European animal welfare legislation (Directive 2010/63/EU) governing the use of animals in experimental procedures. It may also help with future captive-rearing and release efforts, which sadly may become increasingly necessary with growing food demands and climate change. For instance, *Sepia apama*, the giant Australian cuttlefish, was recently designated as "near threatened" due to intensive fishing of breeding aggregations in specific locations and is facing a projected 20% decrease in population levels if current catch rates continue (IUCN 2017). And alarmingly, local British newspapers report that fishermen are taking advantage of lax regulations and are harvesting cuttlefish in the south west of the United Kingdom at unprecedented rates in response to demand in Asia (Jones 2017; Payne 2017). If this trend continues, we may soon have greater ecological and economic incentives to augment natural stocks artificially. One particularly economic strategy would be to recover and culture the eggs laid on cuttlefish traps and normally lost when the gear is cleaned at the end of the harvest season (Blanc and Daguzan 1998). Already, pilot efforts to culture cuttlefish eggs and hatchlings in large, outdoor ponds have been undertaken with moderate success (Roussel and Basuyaux, 2016). The work presented here will hopefully inform these efforts.

Thesis overview

In this thesis, prenatal stress is investigated from the four ethological perspectives that (Tinbergen 1963) outlined through a broad spectrum of physiological, behavioral, cognitive and neurobiological tests to determine if and how prenatal stress affects cuttlefish. Two major kinds of stress were investigated: stressors were applied to both reproducing females (maternal stress) and developing embryos (embryonic stress). Two types of embryonic stressors were applied: a naturally-occurring one and an artificial one (Fig. 3).
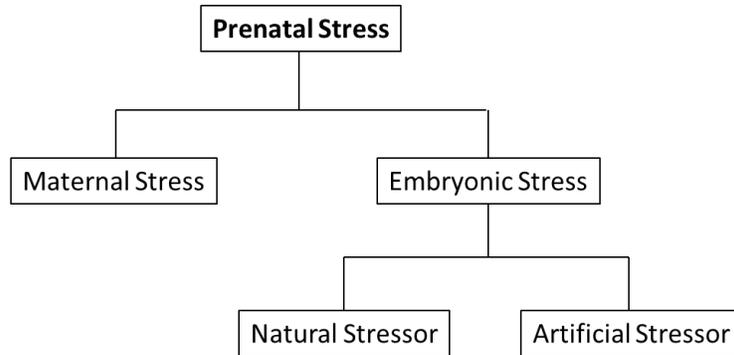
Figure 3. Schematic representation of the categories of stress investigated.

In Chapter 1, data is reported showing that stress affects the quantity and quality of the eggs of reproducing females. Chapter 2 examines the two other potential means of stress effects: the transfer of non-genetic material (e.g. hormones) from mother to offspring and direct embryonic perception. First, it provides detailed background information on the early development and sensory abilities of this species in two review papers. Next, it overviews the effects of maternal and embryonic stress on offspring behavior and learning observed in a number of experiments. Chapter 3 reports some related experiments concerned with some of the practical implications of prenatal experience and its effect on behavioral research. Finally, the main findings are reviewed in the discussion and conclusion, with a prospective on the future of cephalopod research and suggestions for further research.

Chapter 1: Stress and Reproduction in Cuttlefish

Chapter 1: Stress and Reproduction in Cuttlefish

        This chapter presents a brief overview of reproduction, followed by an article outlining the effects of stress on cuttlefish reproduction and finally, an account of a "mesocosm" experiment which provides anecdotal support for the conclusions of the article.

I. Article #1: "Effects of Maternal and Embryonic Stress on Egg Production and Offspring in the Cuttlefish, *Sepia officinalis*"

In the English Channel, Sepia officinalis has a one to two year life cycle (Gauvrit, Goff, and Daguzan 1997) spent mostly living in deeper offshore waters. Towards the end of their life, they migrate to shallower coastal waters to mate, lay eggs and die soon thereafter (Boletzky 1987). Each female can lay dozens to thousands of eggs fertilized by stored sperm from one or more males (Hanlon, Ament, and Gabr 1999). Depending on the temperature, these eggs hatch 2-3 months later as autonomous benthic hatchlings about 1cm in total length (O'Brien, Mezrai, et al. 2016). While we were primarily interested in quantifying the effects of prenatal stress on cuttlefish offspring, the data collected in the course of testing the effects of maternal stress on hatchling cuttlefish also demonstrated the effects of stress on cuttlefish egg production. These results are reported in the following manuscript.

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

Stress and cuttlefish reproduction

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Summary Statement:

Handling stress applied during egg-laying significantly reduces reproductive output of the cuttlefish *Sepia officinalis*, while stressors applied directly to developing embryos (predator cues or bright LED light) had little effect.

List of symbols and abbreviations:

DML: Dorsal Mantle Length, the standard measure of cuttlefish size, defined as the distance between the posterior tip of the mantle and the edge of the mantle just behind the eyes.
CM: Control (unstressed) Mother
SM: Stressed Mother
C: Control eggs
PE: Predator-Exposed eggs
LE: Light-Exposed eggs

Author Contributions

Caitlin E. O'Brien (CEO): Collected data, analyzed results and wrote the manuscript.
Cécile Bellanger (CB): Planned and oversaw assays of corticosterone in embryos and feces, assisted with manuscript preparation.
Christelle Jozet-Alves (CJA): Assisted with statistical analyses and manuscript preparation.
Nawel Mezrai (NM): Collected data and assisted with manuscript preparation.
Anne-Sophie Darmaillacq (ASD): Contributed to experimental design and assisted with manuscript preparation.
Ludovic Dickel (LD): Designed and supervised the experiment, provided editorial guidance for manuscript preparation.

Competing Interests

No competing interests declared.

Abstract

Stress has been shown to have profound effects on animals, particularly if it occurs during reproduction or embryonic development. Invertebrate mollusc cephalopods offer unique points of comparison to typical vertebrate models in the study of stress. Over the course of two summers, we investigated the effects of stressors applied to reproducing and developing cuttlefish, *Sepia officinalis*, by comparing the number of eggs produced by 20 females in a typical captive setting with 19 females subjected to moderate stress during egg-laying (confined space and repeated removal from the water). We also subjected the eggs of 15 unstressed females to naturally-occurring (predator cues) and artificial (random bouts of bright LED light) stressors during development in order to gauge the impact of direct stress on embryos. We found that stressed females produced significantly fewer eggs per female (152.58 ± 96.93 versus 505.23 ± 373.30 eggs/female) and that fewer of those eggs hatched (22.27% versus 57.14%). Simultaneous attempts to identify a simple proxy for female stress levels (fecal corticosterone and unused reproductive material) in reproducing females were unsuccessful in detecting differences between stress treatments, although we found that 25% of stressed mothers laid mostly transparent eggs lacking the dark pigment typical of this species, constituting a statistical trend. In contrast to maternal stress, stressors applied directly to developing embryos had no effect on hatching rate. Neither maternal nor embryonic stress was associated with differences in hatching size. Our results suggest that reducing stress during egg-laying may increase aquacultural egg yields in *S. officinalis* and that developing embryos are less affected by stress applied directly to them than to their mother.

Introduction

Organisms have evolved to react to unexpected phenomena in their environment (e.g. predators, changes in environmental parameters, unfamiliar sensory stimuli) or to anticipated pain or suffering with a physiological or behavioral response. 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, Contreras-Sanchez, and Fitzpatrick 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 (Appleton and Palmer 1988; Palmer 1985). However, while these responses have evolved to enhance fitness in the face of predators, they divert resources away from other life functions. In *Littorina obtusata* for example, the shell-thickening in response to a crab predator (*Carcinus maenus*) 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 increase reproductive output or offspring success by inducing adaptive responses on the part of the mother or her offspring. For instance, when spawning female sticklebacks (*Gasterosteus aculeatus*) sense predators, their offspring exhibit stronger anti-predator behavior (tighter shoaling) than offspring from unstressed females (Giesing et al. 2010). But 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 the stress response (Moberg 1991). When exposed to stressors, animals secrete these hormones, which induce changes in behavior, metabolism and physiology. There are numerous studies associating maternal stress with changes in offspring, and it seems likely that stress hormones are being transferred from mother to her offspring via the placenta or egg yolk and mediating some of these changes (Braastad 1998; Henriksen, Rettenbacher, and Groothuis 2011; Welberg and Seckl 2001). However, the embryos of many of these species are capable of sensing the environment outside the egg or placenta to some degree (G. Gottlieb 1976) and could potentially experience stress directly from environmental stimuli during development (we refer to this here as "embryonic stress"). Thus, it is

sometimes unclear if stress reactions observed in offspring are due to the transfer of maternal hormones or to a stress response by the embryos themselves, especially in animals in which fetuses or eggs develop within the female. Comparing the effects of maternal and embryonic stressors is necessary, but since this separation is not possible in viviparous and ovoviviparous animals, inferences must be drawn from animals with different reproductive habits.

Along with fish, reptiles and amphibians, many invertebrate species are good candidates for this kind of investigation since they are oviparous (the embryos typically develop separately from the mother), allowing them to be experimentally isolated from the female for testing the effects of embryonic stress. Testing both maternal and embryonic stress in a complementary and concurrent way allows general comparisons to be made between the stress types. Among invertebrates, cephalopod molluscs are particularly good comparative models, since their physiology and behavior converge with that of vertebrates in many instances (Grasso and Basil 2009). Comparisons of phylogenetically distant species that share features due to convergent evolution allow us to infer the shared evolutionary pressures that led to their development. 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 feces 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 hemolymph 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).

The cuttlefish *Sepia officinalis* is a cephalopod mollusc inhabiting the Mediterranean and the Atlantic coasts of Norway, the United Kingdom, Ireland, Europe and North Africa (ANGEL Guerra 2006). A well-established fishery and aquaculture industry make it economically important, and it is a model species in biological research (Bloor et al. 2013). *S. officinalis* reproduces only once at the end of life and dies very soon after, though egg-laying may last for several days or weeks (Sigurd v Boletzky 1987). In captivity, typically lays a few hundred to a few thousand eggs each (Correia et al., 2005; Domingues et al., 2002, 2001, Guerra et al., 2015, Sykes et al., 2013, 2009, 2006). Embryonic development lasts between 40 and 90 days, with higher temperatures accelerating embryogenesis (Olivier Bouchaud 1991a; O. Bouchaud and Galois 1990) but usually yielding smaller hatchlings (Gauvrit, GOFF, and Daguzan 1997). Eggs develop outside the mother and there is no direct parental care during embryonic development or after (Bloor et al. 2013).

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 crowding by conspecifics (Correia et al. 2005; António V. 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 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 in order 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, 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 timed randomly throughout the day over the course of embryonic development. 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 odor 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 maturation (Dickel, Boal, and Budelmann 2000a), 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 in order 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. Since measurement of fecal glucocorticoids is used in many species as a non-invasive way to quantify stress (e.g. (Metrione and Harder 2011; Tempel and Gutiérrez 2003), we tested whether or not fecal corticosterone could be used

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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.) We also 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.

Materials and Methods
2.1 Females

2.1.1 Broodstock Conditions:

Throughout May, 2015, and on May 10, 2016, 39 female cuttlefish (*S. officinalis*) approximately two years old were captured 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 14:10h) with a mean temperature of 15±1°C. Upon capture, female cuttlefish were mated with males (12hrs 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 five months (Hanlon et al., 1999). Each female was fed one large or two medium-sized *Carcinus maenas* (Linnaeus 1758) per day.

2.1.2 Stress Treatments:

Females were partitioned into two groups. "Control mothers" (CM, n = 19) were maintained in large (1000L, 168cm diameter, 61.5cm height), round, blue tanks enriched with stones, artificial algae, floating objects and shaded areas. In 2015, seven CM were housed individually in these conditions, and in 2016, due to a large number of females captured in one 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 singly in small, unenriched square grey bins (65 L, 80x60x40 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 15min blocks between 09h00 and 18h00. During removal from the water, females would usually eject water from their siphon several times in an attempt to reenter 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 translucent 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 next 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 one week of treatment were used in assessments of hatching success and size in order to ensure sufficient representation of each mother or group of mothers (CM n = 8; SM n = 9) manifestation of any treatment effects (Table 1).

Table 1) Composition of female stress groups by year. All data from 2015 and 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.

	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*	1 Non-Layer*	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*	

*Directly observed.

2.1.3 Fecal Corticosterone Assays:

Fecal collection and extraction methods were adapted from Larson and Anderson, 2010. In May and June of 2016, feces from the spawning females were collected daily from the bottom of every tank using a hand net. Fecal strands were removed separated from other detritus with tweezers and placed in dry 1ml vials. These were frozen and stored at -80°C for five to six months (depending on collection date) while various extraction protocols were tested and the assay was validated. In November 2016, fecal samples were thawed, weighed (0.3-1.2 g wet feces) 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 feces of two to four individuals) and into six time points (feces from two subsequent days) in order 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 feces), followed by vortexing (20 min) and centrifugation (20 min, 2800 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 (37kHz, 15min), an ImmuChem Double Antibody CorticosteroneTM 125I RIA kit (MP Biomedicals, LLC) was used to quantify the corticosterone present in the feces of each treatment group. A gamma counter measured relative radioactivity of the samples and corticosterone concentrations were calculated via comparison to a standardized curve.

2.1.4 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 in order to count the number of oocytes remaining in the pallial cavity.

2.2 Eggs

2.2.1 Egg Collection:

For the first three weeks of incubation, eggs were maintained in floating, circular baskets (3.8L) 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 three 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. In order to ensure that any potential stress effects had time to manifest and that they were represented by adequate sample sizes, only eggs from mothers that had laid at least 50 eggs after one week of treatment were used to calculate hatching success and hatching size (see Table 1). Eggs were maintained until hatching in floating mesh trays (55x70x7cm) in 65L (80x60x40 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 (15:9-16:8h 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.

2.2.2 Stress Treatments:

Eggs from the nine most fecund SM were allowed to develop without any further treatment (Fig. 1a), 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 (Fig. 1b). "Predator-exposed" (PE) eggs were placed in

a tank with three mature European sea bass (*Dicentrarchus labrax*, approximately 25cm-35cm 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, 20.7klux, approximately 10 cm from surface of water) in 15 min bursts six times every 24h (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" (C) group in this phase of the experiment as well. Hatching occurred between June 29 and August 5, 2015 and from July 2-24, 2016.
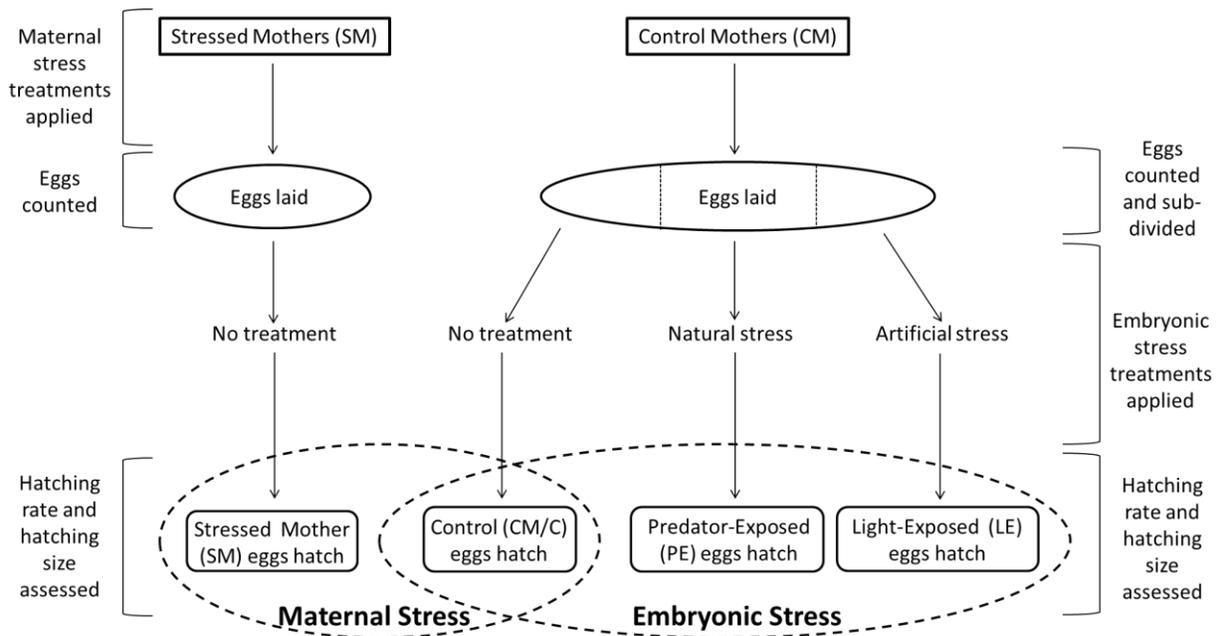
Fig. 4) **Schematic representation of experimental design.** Note that the eggs of the unstressed control females were subdivided to create the three embryonic treatment groups and that both maternal stress and embryonic stress were compared to the same control group of eggs and hatchlings (CM in the maternal stress comparisons, C in embryonic stress comparisons) separately. Adapted from (O'Brien et al., 2017).

2.3 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 were excluded from size assessments. Sex discrimination is not possible at this age.

2.4 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).

2.5 Statistics

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

To compare the number of females that laid eggs, as well as the number of females that laid translucent 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. We also tested for a correlation between the number of eggs laid and lifespan after capture of CM and SM with canonical correlation analysis. The sample sizes used to calculate these values varied since some measurements were not possible in certain individuals.

Fecal 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 one week of treatment (one

CM in 2015 and two SM in 2016) were excluded. 2x2 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

3.1 Females

3.1.1 Egg-laying:

The proportion of SM (60%) that produced eggs did not differ from CM (78.95%) (two-tailed chi-squared exact test: X^2 = 1.64, CM n = 19, SM n = 20, p = 0.3; Table 2). 15 CM laid a total of 6567 eggs while 12 SM laid a total of 1831 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). For CM, there was a strong correlation between lifespan after capture and eggs per female (canonical correlation test: R = 0.90, n =12, p < 0.0001; Table 2), and only a weak correlation for SM (canonical correlation test: R = 0.34, n = 20, p = 0.14).

Table 2) Proportion of egg layers, size (DML and weight), lifespan after capture (days), the correlation between lifespan after capture and number of eggs laid and remaining oocytes (mean ± s.d.) of female cuttlefish. 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 Fisher exact test, all others with exact permutation tests (these calculations include both egg-layers and non-layers).

	Control Mothers	Stressed Mothers	Comparison
Proportion of Egg Layers	78.95% n = 19	60.0% n = 20	p = 0.3 X^2 = 1.642
DML (cm)	23.29 ± 3.25, n = 17*	23.03 ± 1.89, n = 17*	p = 0.81 t ≥ 396
Weight at death (kg)	1.29 ± 0.30, n = 17*	1.31 ± 0.25, n = 16*	p = 0.84 t ≥ 21.85
Lifespan after Capture (days)	15.63 ± 7.21, n = 19	14 ± 5.0, n = 20	p = 0.38 t ≥ 297
Correlation: Number of Eggs Laid and Lifespan after Capture	p < 0.0001 n = 12	p = 0.14 n = 20	
Remaining Oocytes (2016 only)	108.33 ± 33.26, n = 12**	117.5 ± 48.64, n = 12**	p = 0.65 t ≥ 1300

*Accurate body measurements were not possible for some specimens due to scavenging activity by crabs.
**Due to poor preservation, dissection and was not possible for some specimens.

3.1.2 Fecal Corticosterone:

The mean corticosterone concentration over six days in CM was 0.70 ± 0.36 ng mg^{-1} dry feces and 0.79 ± 0.56 ng cort mg^{-1} dry feces in SM. No significant difference existed between treatment groups (GLMM: X^2 ≥ 0.07, n = 4 (means calculated from the pooled feces of one to four individuals over two days), p = 0.79; Fig. 2).
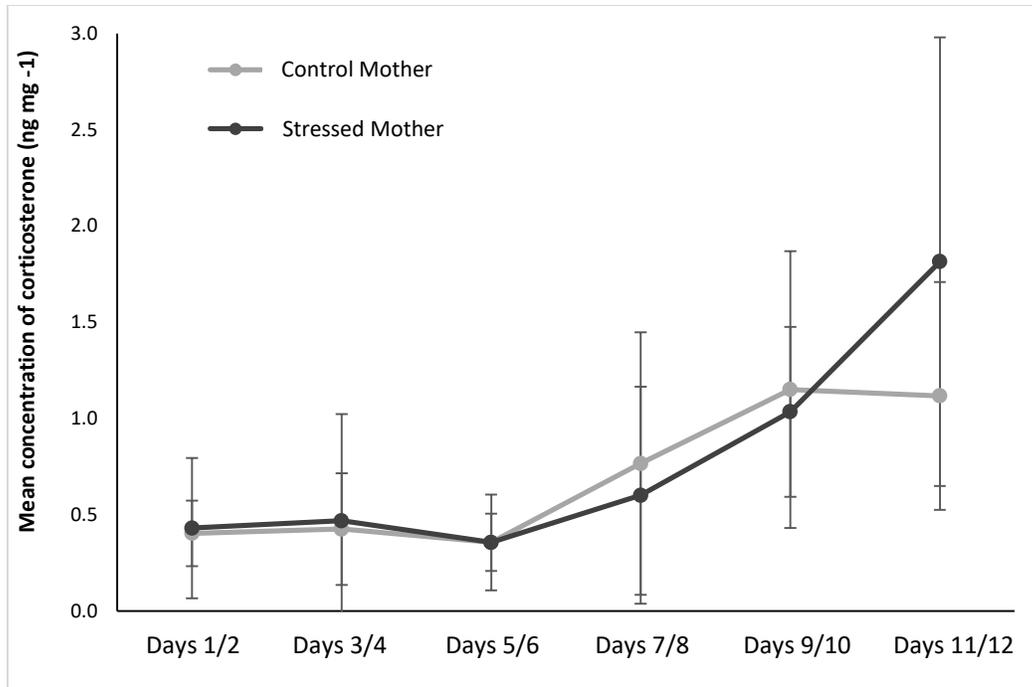
Fig. 5) Fecal corticosterone concentration (mean ng corticosterone mg^{-1} dry feces ± s.d.) over time, measured from fecal samples collected in 2016. No significant difference exists between groups (GLMM: $X^2 \geq 0.07$, n per data point = 4 (means calculated from the pooled feces of one to four individuals over two days), p = 0.79). Data are displayed as group means (dots) ± standard deviation (whiskers).

3.2 Eggs

Egg-laying occurred from May 15 to June 9, 2015 and from May 14 to 29, 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—see Table 1 for details); SM = 152.58 ± 96.93 per female; n = 12; two-tailed exact permutation test: t ≥ 4042, p < 0.001). Among CM, there was no difference in eggs per female between years (exact permutation test: t ≥ 2675, 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 25% of laying SM (n = 12) produced eggs with little or no ink in the egg capsule (Fig. 3). This was not significantly different from laying CM (n = 15) which did not produce any viable translucent eggs (a few (<25) translucent capsules were laid, but these did not develop any further) but constitutes a statistical trend (two-tailed chi-squared exact test: $X^2 = 4.22$, p = 0.08).

Fig. 6) A normal (ink-stained) _S. officinalis_ egg (left) and a translucent 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.

3.3 Hatchlings

3.3.1 Hatching Rate:

Significantly more (two-tailed chi-squared test: $X^2 \geq 453.50$, $p < 0.0001$; Table 3) CM eggs (57.14%, n = 1876) produced live offspring than SM eggs (22.27%, n = 1724).

Among the embryonic treatment groups, there was no significant difference in hatching rates between groups (Cochran-Mantel-Haenszel chi-squared test: $X^2 \geq 0.84$, $p > 0.99$; Table 4): 57.14% of C eggs (n = 1876), 59.33% of PE eggs (n = 1876), and 63.38% of LE eggs (n = 1876) produced live offspring.

3.3.2 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).

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).

Table 3) Hatching rate and mean hatching size (dorsal mantle length (mm), mean ± s.d.) of eggs and offspring from the maternal stress experiment. Hatching rates were compared with a chi-squared test, while hatching sizes were compared with a two-tailed independent T-test.

	Control Mother	Stressed Mother	Comparison
Hatching Rate	57.14% n = 1876	22.27% n = 1724	p < 0.0001, $X^2 \geq 453.50$
Hatching Size (mm)	8.56 ± 0.75 n = 72	8.41 ± 0.92 n = 97	p = 0.26, t = 1.13

Table 4) Hatching rate and mean hatching size (DML, mean ± s.d.) of eggs and offspring from the embryonic stress experiment. Hatching rates were compared with a Cochran-Mantel-Haenszel chi-squared test, while hatching sizes were compared with a two-way ANOVA.

	Control	Predator-Exposed	Light-Exposed	Comparison
Hatching Rate	57.14% n = 1876	59.33% n = 1876	63.38% n = 1876	p > 0.99, $X^2 \geq 0.84$
Hatching Size (mm)	8.56 ± 0.75 n = 72	8.64 ± 0.73 n = 85	8.71 ± 0.69 n = 74	Stress: p = 0.22, F = 1.54, Mother: p < 0.001, F = 4.49

Discussion

The purpose of this study was to determine whether maternal ~~and embryonic~~ stress~~ors~~ affects cuttlefish egg production, and whether maternal or embryonic stress affect hatching rate and hatching size. Using an oviparous species also allowed us to compare the relative impact of maternal versus embryonic stress~~ors~~. In parallel, we attempted to determine whether fecal corticosterone levels (a commonly-used and noninvasive measurement of stress levels in many animals) or the number of unused oocytes remaining at death (a potential proxy for unused reproductive potential), would correspond to any reproductive effects of maternal stress observed in cuttlefish.

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 stress groups at death, and females from both groups survived for a little over two weeks after capture before they died naturally, with no 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 (S. v 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 ~~or even~~ that is typically observed (Sigurd v Boletzky 1987; S. von Boletzky 1988). This hypothesis is supported by the positive correlation between the lifespan after capture and eggs per female for CM (Table 2), suggesting that in this group, the number of eggs produced may largely be a function of how long a female survived. By contrast, the weaker correlation between these factors for SM suggests that another factor may be responsible for the reduced egg output. Experimenting with various severities of stressors (i.e. a severe versus mild stressor) and food rations (i.e. restricted *versus ad libitum*) applied to reproducing females might reveal whether energy reserves limit spawning.

In addition to the drastic reduction in egg output, 25% of SM produced viable partially or entirely translucent eggs, while CM did not (Fig. 3). In most cases, the egg membrane of *S. officinalis* is impregnated with dark ink from the mother, although translucent eggs are occasionally seen in both aquaculture and in the wild (António V. Sykes, Domingues, and Andrade 2014). In our experiment, the ratio of SM displaying this trait did not differ significantly from CM, but it constitutes a statistical trend and we believe that it may be related to the stress treatment. It is possible that these transparent eggs were the result of acute ink depletion following an inking response to stress treatments. However,

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females did not appear to deplete their ink supply during stress treatment, since all those examined (2016) still contained ink in their ink sacs at death. Ink staining occurs during the secretion of oviducal jelly around the eggs by the oviductal and nidamental glands, and our results suggest that stress can somehow disrupt this process (S. v Boletzky 1986; Cornet et al. 2015). To determine if females temporarily run out of ink or if there is a physical disruption to the egg-laying process which prevents membrane staining, the degree of egg ink-staining could be assessed after intentionally eliciting strong inking responses in reproducing females. Replenishment of the ink sac after depletion by stress responses may even contribute to the hypothesized energy depletion discussed in the previous paragraph.

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) and by blocking excess light that could interfere with normal development (Bassaglia et al. 2013). Since *S. officinalis* lays large clutches in the open, it seems likely that ink is important for egg survival in this species as camouflage or protection from light. It is known that transparent *S. officinalis* eggs reared in captivity hatch earlier than their darker counterparts (Paulij et al. 1991), and this could result in smaller hatchlings (O. Bouchaud and Daguzan 1990). Still, the lack of ink may be a severe disadvantage in the natural environment due to the presence of predators and parasites. Alternatively, it could be adaptive by enabling greater access to visual information about the surrounding environment (e.g. what predator and prey species are present at the hatching site). Regardless of the reasons for this phenomenon, if the trend is substantiated by other experiments and observations, the presence of translucent eggs in a clutch 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 reducing the number of eggs laid by SM, fewer of the SM eggs 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 (António V. Sykes et al. 2013)), while the SM rate was very low (22.27%). Such a difference in hatching rate was not seen in embryonic stress groups, suggesting that stressors applied to mothers may have a stronger impact on reproductive output than stressors applied directly to offspring. More investigation is needed into the mechanisms responsible for reducing the hatching rate in SM, 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

have affected hatchling DML. Since the amount of yolk fueling embryonic growth is determined at least five months before mating (Boucaud-Camou and Boismery 1991), growth rate and metabolism are fueled by a predetermined supply of yolk. The proportion of those reserves that are converted into tissue is determined by the rate of embryonic development and metabolism (O. Bouchaud and Galois 1990) , which is likely affected by stress responses. We had ~~also~~ 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 odor since the ability to habituate to predator cues would be adaptive, and continuous exposure to seabass odor during incubation has been shown to mediate traits like brain lateralization in cuttlefish embryos (C. Jozet-Alves and Hebert 2012). Moreover, behavioral experiments conducted on these hatchlings revealed changes in predation behavior 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 laying 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 hemolymph of *S. officinalis* (C. Bellanger, unpub. data), so we selected this hormone for quantification by radioimmunoassay. In order to minimize disturbance to the animals, we collected and tested fecal samples rather than hemolymph. 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 to 3.28 ng mg^{-1} vs. 0.010 to 0.022 ng mg $^{-1}$ dry feces (Larson and Anderson 2010). This suggests that fecal corticosterone levels may not be a particularly good indicator of stress in this species (at least during reproduction). Fecal cortisol has been found to be an unreliable indicator of stress in some other cases as well: in harbor seals (*Phoca vitulina richardii*) for instance, plasma cortisol concentration was strongly correlated with injection of adrenocorticotropin (the hormone stimulating cortisol release), but fecal 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 feces 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 levels. We had hypothesized that unused oocytes in the pallial cavity of deceased females might be a potential indicator of unused reproductive potential, and so we examined the number remaining at death, hypothesizing that there would be more in SM. However, there was no difference between the two groups (Table 2, "remaining oocytes"). Our new hypothesis based on these data is that females' ovaries still contained a large supply of immature oocytes that was not exceeded in this experiment and that the number remaining at death simply reflects the volume of the pallial cavity (the ovary periodically passes batches of oocytes to the pallial cavity for laying (Sigurd v Boletzky 1987) and our specimens contained an uncountable (individual oocytes could not be distinguished) number of additional immature oocytes at death). Regardless, since this trait did not correlate with stress treatments, it cannot be used as a proxy for stress levels in deceased female cuttlefish. Potential measures of stress that remain to be explored 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 in order to allow them time to recover from the stress of capture and acclimate to an artificial setting may result in higher offspring yields. (On the other hand, 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 stressors (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 fecal corticosterone

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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 ~~occasional~~ 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 behavior 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) versus 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.

II. Supplementary data

The following experiment provides further evidence that maternal stress reduces reproductive output in cuttlefish. It was conducted in collaboration with Dr. Olivier Basuyaux, and with the assistance of the SMEL.

Laying Site Choice Experiment

As the goal of PReSTO'Cog is to assess the effects of prenatal stress, we wanted to test the relevance of this topic in the real world. In May and June of 2017, we conducted a pilot experiment addressing the question of whether or not females make choices about where they lay their eggs. If given the opportunity, would females select a site that would be more advantageous for their offspring? Hatchling cuttlefish are small and highly vulnerable to predation, especially fish, so it seems logical that females would select sites with lower predator density if at all possible. In this experiment, we provided females with two potential laying sites consisting of old fishing traps with six ropes attached. We thought that these would offer attractive egg-laying substrate to females, since cuttlefish eggs are often found laid on cuttlefish traps and since similar ropes lashed to the seabed can be used to collect cuttlefish eggs. Conveniently, the traps could also be easily modified to encage a predatory fish, in this case, a gilt-head sea bream (*Sparus aurata*). The experiment was conducted in large (600m^3— 75x10x0.8m) unused oyster ponds (Fig. 9) at the Cabanor (Cooperative Aquacole de Basse Normandie) for aquaculture and shellfish in Blainville sur Mer. These old ponds were ideal "mesocosms" in which to conduct experiments, being refreshed by high tides twice per month through channels connecting to the sea and naturally containing numerous crustaceans, fish, algae and small invertebrates.

a)   b)

Figure 7. Large ponds once used for oyster aquaculture at the CABaNor Aquaculture Cooperative (Blainville sur Mer, Normandy). a) Overhead view, note the channels connecting the ponds to the sea at high tide (photo by O. Basuyaux). b) Close-up view of an experimental pond (photo by C. E. O'Brien).

In these ponds, we created a two-way choice test, placing a laying site with the predatory sea bream in one half of the pond, and an identical but predator-free laying site on the opposing end (see Fig. 10). We hypothesized that if females make a choice in where to lay their eggs, they would choose the laying site least dangerous to their offspring, the one without the potential predator. Alternatively, if they may simply lay at the first minimally-suitable location, and in that case, the distribution of eggs should be evenly divided between laying site type. Five ponds were used, and the placement of the laying sites was randomized. Cuttlefish were introduced to the pond by placing them in the water close to the middle of one side. Only one female was housed in each pond at a time, and was kept there until it died naturally. The laying sites were visually inspected daily for the presence of eggs.
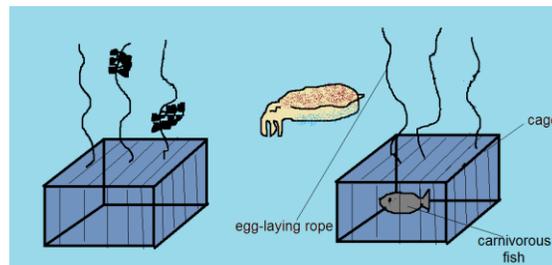
Figure 8. Schematic of the egg-laying site choice test: two modified cuttlefish traps were provided. One contained a potential hatchling predator, and both had six ropes, of a type and diameter known to "attract" cuttlefish eggs in the wild, affixed to them.

Unfortunately, a paucity of gravid females and unforeseen behavior resulted in a sample size too low for analysis. Of the eight females placed in the oyster ponds, only three eventually laid eggs. Moreover, only one of these females actually laid eggs on a designated laying site. The two other females, instead of laying eggs on the modified cuttlefish traps as we had hoped, laid eggs on plants that encircled the edge of the ponds.

While the intended goal of this experiment was not achieved, it did provide some anecdotal support for the proposition of the preceding manuscript that stress to spawning females results in egg reduction and white eggs. The two females that laid eggs on plants each laid hundreds of black eggs (662 and 756). By contrast, the single female that laid on a designated laying site produced only 71 eggs, all of which were transparent. In retrospect, it seems likely that an inadvertent difference between experimental ponds might be linked to the explanation for this. The pond containing the two more prolific egg-layers had many square meters of floating yellow filamentous algae (*Enteromorpha* sp.) at both ends, while the single more modest egg-layer was in a pond without any algae. Because of the algae, the females in these ponds were able to lay their eggs from a well-concealed vantage point, while the other female laid without the same level of cover. It may be that laying while exposed was

"stressful", resulting in the lower number of eggs and their transparent membranes. Methodologically, if this particular experiment is repeated, it should be conducted in ponds with the algae removed.

III. Chapter Summary

   The work presented here demonstrates that stress can influence cuttlefish via at least one of the three potential pathways by reducing females' total reproductive output and the quality of the eggs produced (Fig. 12). This hints at the existence of tradeoffs between the female stress response and her reproductive output. More importantly, it shows that stress may impact cuttlefish before their eggs are even laid, a factor we must be aware of when formulating mechanistic and adaptive (Tinbergen's first and third perspectives) explanations for cuttlefish behavior. It also suggests a very specific guideline for cuttlefish aquaculture: minimize the handling of reproducing females in order to maximize reproductive output and egg quality.
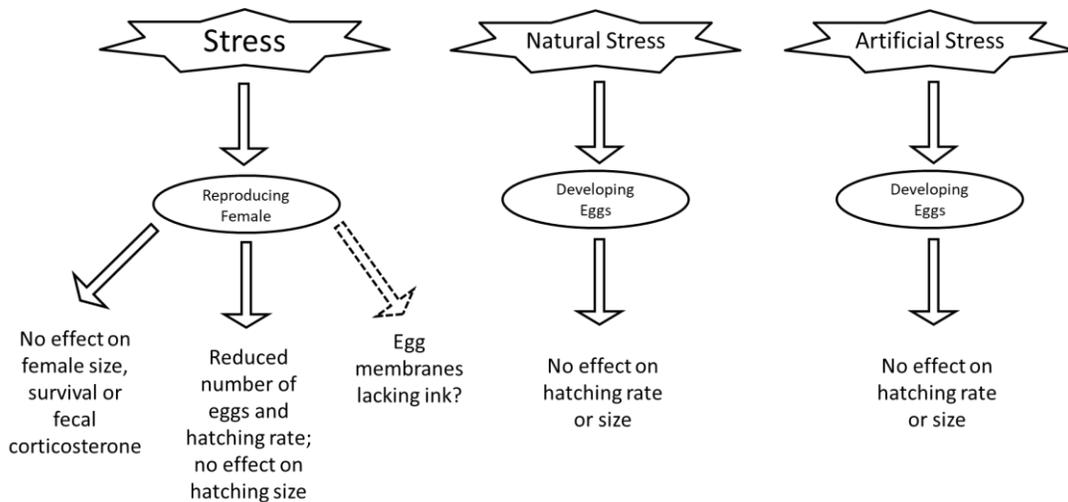
Fig. 9. Schematic summary of Chapter 1. Stress to the mother during egg-laying reduces her reproductive output and likely the quality of her eggs as well. At the same time, a naturally-occurring and an artificial stressor applied directly to developing embryos had no effect on hatching rate or size. The dashed arrow indicates a statistical tendency.

Chapter 2: Prenatal Stress Effects on Offspring

Chapter 2: Prenatal Stress Effects on Offspring

This chapter explores two potential avenues of stress influence: from mother to offspring and direct perception by the embryo itself. It is composed of two extensive reviews of cuttlefish development and sensory abilities, followed by reports from numerous behavioral and learning tests and neurobiological assays.

I. Article #2: "Behavioral development in embryonic and early juvenile cuttlefish (*Sepia officinalis*)"

This manuscript offers an in-depth overview of cuttlefish development, providing context to the experiments that follow.

Behavioral Development in Embryonic and Early Juvenile Cuttlefish *(Sepia officinalis)*

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Though a marine 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.

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 (Packard 1972; Hanlon and Messenger 1998). The life history and habits of cuttlefish enables 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 them exceptional sensory access to the surrounding environment during the final stages of embryonic development (Romagny et al. 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 (<1week 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.

Embryonic Development

Embryogenesis and Yolk Reserves

Spawning of *S. officinalis* eggs occurs in the English Channel in shallow (5-60m), well-lit coastal waters throughout the spring and summer (Nixon and Mangold 1998); Basuyaux & Legrand, 2013). 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 (Boletzky 1983; Nixon and Mangold 1998; Nixon and Young 2003; Blanc and Daguzan 1998). 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 towards the very end, accomplishing the majority of growth and differentiation during the last few stages (Fioroni 1990; Domingues, Bettencourt, and Guerra 2006). 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 (Richard 1971; Cronin and Seymour 2000). The embryo within becomes visible, giving unprecedented access to late prenatal stages (Fig. 12).

Fig. 10. Stage 30 *Sepia officinalis* embryo (approx. mantle length 6mm) seen through the transparent egg membranes. Photo by Nawel Mezrai.

Cuttlefish eggs are able to tolerate limited episodes of stress (*e.g.* prolonged emersion, handling) and still hatch normally (Jones, Ridgway, and Richardson 2009), but otherwise have a fairly narrow range of physical requirements in terms of temperature and salinity (Boletzky 1983; Nixon and Mangold 1998). Within the tolerated temperature range, higher temperatures accelerate growth and result in shorter development times. Because of this, eggs laid 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 (S. V. Boletzky 1983; Olivier Bouchaud 1991b).

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 (S. v Boletzky 1975; O. Bouchaud and Daguzan 1990; Olivier Bouchaud 1991b; Dickel, Chichery, and Chichery 1997a). However, this more rapid development results in smaller hatchlings (Roger T. Hanlon and Messenger 1988; O. Bouchaud and Daguzan 1990), and this has implications for survival and the onset of predatory behavior (S. von Boletzky 1994; Olivier Bouchaud 1991b). Water temperature varies with location, depth, season, currents, tide and weather, so the timing and position of an egg can strongly affect its later prospects (Bloor et al. 2013).

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 et al. 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 and 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.)

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 et al. 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). 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 et al. 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 (Paulij et al. 1990; Paulij, Bogaards, and Denucé 1990; Bloor et al. 2013; Di Poi et al. 2014a; Bidel, Di Poi, et al. 2016). 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 an appropriate location, at a suitable time and in appropriate densities (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 (Fig. 13). 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 (Anne-Sophie Darmaillacq, Lesimple, and Dickel 2008a; Mathieu Guibé, Boal, and Dickel 2010a; Mathieu Guibé et al. 2012; C. Jozet-Alves and Hebert 2012; Christelle Jozet-Alves and 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.
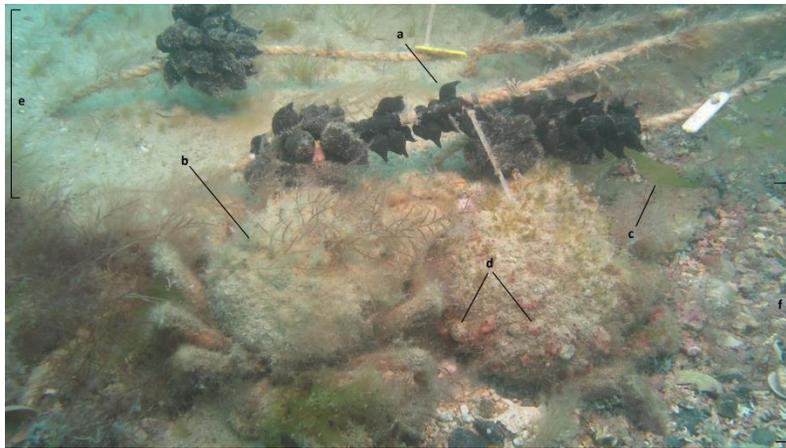
Fig. 11. Cuttlefish eggs (a) *in situ*, 26 June, 2014 at an artificial laying-site (pre-placed tethers) in the vicinity of Blainville sur Mer, France. Note the crab, *Maja squinado* (b) in the foreground as well as the presence of numerous algaes, including *Ulva* sp. (c), *Lithophyllum incrustans* (d), various unidentified epibionts and a diversity of surrounding substrate types including sand (e), rocks and shell debris (f). Eggs are approximately 2.5-3.0cm in length (Boletzky 1983). Photo by Olivier Basuyaux, of the Synergie Mer et Littoral (SMEL).

One intriguing difference between *S. officinalis* and many other cephalopods is their dark egg capsule. Where most cuttlefish, squid and octopus have translucent eggs, the egg membrane in *S. officinalis* is stained with a layer of ink from the mother (but see next paragraph). It is possible that this ink-staining aids in defense by camouflaging the eggs themselves or by masking the movement of the embryo within (A. Guerra and Gonzalez 2011). The fact that other cuttlefish species with translucent eggs 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, and Mather 2014), while *Sepia esculenta* has a sticky exterior that accumulates a layer of camouflaging detritus (Hanlon and Messenger 1998). 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 lefish eggs may also play a protective role (Hanlon and Messenger 1998; Boletzky 2003; Derby et al. 2007). 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, and Scholl 1976). It is hypothesized to

reduce the likelihood of detection and predation by reducing embryo movement and preventing premature hatching (Marthy, Hauser, and Scholl 1976; Weischer and 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 and Marthy 1983).

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 2013a). These recurring episodes are analogous to REM sleep in vertebrates and are referred to as "motorically active sleep" (MAS) (Corner 2013a). This behavior starts sometime before stage 29, and continues into postnatal life (Corner 2013a). Other cephalopods (squid and octopus) and invertebrates (*e.g.* nematodes, annelids, cnidarians, and insects) also exhibit similar prenatal or larval behavior (Corner 2013b).

Non-Associative Learning

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*. Goldkrand & Litvack, 1991; Smotherman & Robinson, 1992; van Heteren*, Boekkooi, Schiphorst, Jongsma, & Nijhuis, 2001; Colombelli-Négrel, Hauber, & Kleindorfer, 2014). 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, Abrams, Barry, Bhatnagar, Clayton, Colombo, Coppolag, Geyerh, Glanzmani, Marslandj, McSweeney, Wilsonl, Wum, & Thompsonn 2009).

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 *et al.*, 2006a; Darmaillacq , Chichery, Shashar, & Dickel 2006; Guibé *et al.*, 2012) hatchlings have an "innate" preference for shrimp or shrimp-shaped objects (but see below). Darmaillacq (*et al.*, 2004a; 2006a; 2006b) demonstrated that this preference could be overridden by chemical and/or visual exposure to crabs shortly after hatching. 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 *et al.*, 2004a; 2006a; 2006b; 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 seven days later (Darmaillacq *et al.*, 2008). Other experiments demonstrated that this sensitive period closes about 6 hours after sunrise on the day of hatching (Darmaillacq *et al.*, 2006a).

Imprinting and perceptual learning allow cuttlefish to learn the characteristics of available prey in their environment during the perinatal period (Healy, 2006; Darmaillacq *et al.*, 2014). 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, Calandreau, Arnould, Nowak, Levy, Noirot, Bouvarel, & Leterrier, 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 the shrimp 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 in *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 that 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.

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 towards 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.

HATCHLINGS and EARLY JUVENILES

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, 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.

Sepia *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 (Nixon & Mangold, 1996; Jereb & Roper, 2005; Bloor *et al*., 2013) which give hatchlings numerous opportunities to conceal themselves (Fig. 13). 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 (Fig. 14) and thereafter as juveniles (Fig. 15) until they reach 90 days of age (Hanlon & Messenger, 1988).

Fig. 12. Hatchling cuttlefish (approx. mantle length 10mm) displaying a disruptive body pattern on a uniform substrate. Photo by Anne-Sophie Darmaillacq.

Fig. 13. Three-month old juvenile cuttlefish (approx. mantle length 65mm). Photo by Caitlin E. O'Brien.

Sensory Abilities

Cuttlefish are thought to rely on ocular vision as their primary sense (Hanlon & Shashar, 2003). Basic visual perception is possible well 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*., 2013b). This apparatus has been used to demonstrate that PS appears in cuttlefish around seven 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*., 2013b). 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, Josef, Lerner, McCusker, Darmaillacq, Dickel, & Shashar, 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 both the suckers of the arms and tentacles, as well as the region surrounding the mouth (Sundermann, 1983; Nixon & Mangold, 1998). 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 moving, although not at every frequency in that range (Komak,

Boal, Dickel, & Budelmann, 2005). Interestingly, juvenile cuttlefish (one and three 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 one and three months, juveniles show a decrease in responsiveness to vibrational stimuli. At three 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).

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 (Hanlon & Messenger, 1988; Ferguson & Messenger, 1991; Ferguson, Messenger, & Budelmann, 1994; Hanlon & Messenger, 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 can't produce full body patterns (Hanlon and 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 juvenile's own white square, while the disruptive pattern is induced by squares 40 to 120% of the cuttlefish's own white square and small rocks (Barbosa*,* Mäthger, Chubb, Florio, Chiao, & Hanlon, 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 (Messenger, 1977; Mäthger, Barbosa, Miner, & Hanlon, 2006) and without any visual feedback from cuttlefish's 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 (Fig. 3; Hanlon & Messenger, 1988; Poirier*,* Chichery, & Dickel, 2005; Dickel*,* Darmaillacq, Poirier, Agin, Bellanger, & Chichery, 2006). 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 and deceptive resemblance (Cott, 1941; Hanlon & Messenger, 1988). 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*,* Bowie, Billard, Darmaillacq, Jozet-Alves, Benhaïm, Basuyaux, & Dickel, 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, Broom, & Osorio, 2007; Langridge, 2009; Staudinger*,* Buresch, Mäthger, Fry, McAnulty, Ulmer, & Hanlon, 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 (Poirier *et al.,* 2004; Lee, Yan, & Chiao, 2012). 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 (Hanlon & Messenger, 1988; Poirier *et al.,* 2005; Allen*,* Mäthger, Barbosa, & Hanlon, 2009). 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; Poirier *et al.,* 2004; Poirier *et al.,* 2005; Lee *et al*., 2010).

Juveniles face a diverse set of potential predators with varying sensory acuity and attack strategies, especially teleosts (Le Mao, 1985; Hanlon & Messenger, 1988; Blanc & Daguzan, 1999; Langridge *et al.,* 2007). 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 3.5cm 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; Hanlon & Messenger, 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.

Other Defensive Behavior

In addition to their camouflage abilities, hatchlings and juveniles possess several defensive behaviors that do not involve body-patterning. From the moment of hatching, they are capable of burying themselves using their funnel and fins to dig a shallow depression and cover themselves with sand (Boletzky, 1974; Mather, 1986), although not all hatchlings do this immediately. Burrowing entails a three-step sequence that lasts about five seconds, 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 two 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 a "freeze" response employed by cuttlefish may be able to counter the electrical detection by sharks and other non-visual predators (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.

Predation

In the late stages of embryonic development, yolk is transported from the outer sac to the inner via the yolk collar (Boucher-Rodoni, Boucaud-Camou, & Mangold, 1987; Boletzky, 1983). 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 two to five days after hatching, hatchlings do not hunt, subsist on internal reserves and grow relatively slowly (Wells, 1958; Richard, 1971; Messenger, 1973; Boucaud-Camou*,* Yim, & Tresgot, 1985; Nixon, 1985).

Hatchlings usually begin feeding on prey before their yolk is entirely exhausted (Wells, 1958; Boletzky, 1975; Boletzky, 1987; Dickel *et al.,* 1997). 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 prompts maturation (Yim, 1979; Boucher-Rodoni *et al*., 1987). 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 towards 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 towards 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 (50s or more) than a tentacle attack (<300ms), leaving cuttlefish more vulnerable to their own predators (Chichery, 1992). In addition to personal risk, prey size and form, the choice between these two strategies may also be influenced 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, 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 (Dickel, 1997; Boal, Wittenberg, & Hanlon, 2000).

Juvenile cuttlefish capture and digest prey items rapidly (Yim, 1979), 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). Like embryos, growth in juveniles can be strongly affected by environmental factors: cooler water temperatures can slow the rate of metabolism (Forsythe, 1994) while the use of dark rearing tanks has been shown to increase growth in hatchlings and juveniles (Sykes, Domingues, Márquez, & Andrade, 2010). 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 (Le Mao, 1985; Blanc *et al.,* 1998). Notably, this is concurrent with the maturation of the digestive gland (Yim, 1979), but may also reflect neural maturation and the refinement of attack strategies.

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 hours, the cuttlefish spends more time resting in the subsequent 24 hours, 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.

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 (M. J. Wells 1968; Young 1961), gastropods (*e.g.* (Sahley, Rudy, and Gelperin 1981; Walters, Carew, and Kandel 1981), bees (*e.g.* Couvillon and Bitterman 1980), insects (*e.g.* (Dukas 1999) and worms (*e.g.* (Rankin, Beck, and Chiba 1990; Avarguès-Weber et al. 2010).

      In cuttlefish, this phenomenon was first demonstrated in adultsand subadults by (Darmaillacq, Dickel, et al. 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 2006a; 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, and 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 et al. 2013).

      After several unsuccessful attacks on the inaccessible prawn in the tube, adult cuttlefish are able to remember the association for several minutes (Wells 1958, 1962; J. B. Messenger 1973). If presented a shrimp in a tube between 20min and 60min 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 1973, 1971). This pattern is thought to result from separate short-term and long-term memory (STM and LTM) processes (Dickel, Chichery, and Chichery 1998a).

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 (Dickel, Chichery, and Chichery 1998; Agin 2006b). After this age, cuttlefish display fully-operational STM (retention for <5min) with either a 5 or 20min training session (Agin et al. 1998; Dickel, Chichery, and Chichery 1998). By contrast, a separate LTM (retention for 1hr or more) emerges about 15 days after hatching and continues to improve over the next several weeks: the ability to retain learning for1hr retention reaches maximum levels around 60 days (Dickel *et al.,* 1998), while 24hr retention matures around 90 days of age (Dickel, Chichery, and Chichery 2001a). 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, and Budelmann 2000b). 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 et al. 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 two 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 (Grant et al. 1995; Dickel, Chichery, and Chichery 1997a, 2001b; Dickel et al. 2006; Nixon and Mangold 1998; Véronique Agin et al. 2006); Agin *et al*., 2006b). Additionally, staining with phosphorylated neurofilament 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, Chichery, and Chichery 1997a). 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 its visual field only develops later (Sanders and 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, Boal, and Budelmann 2000b; Dickel, Chichery, and Chichery 1997a). Prey pursuit requires STM, which occurs around 8 days (Dickel, Chichery, and Chichery 1998a) and is correlated with the advent of the VL/sub-VL tract (Dickel, Chichery, and Chichery 1997a).

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, Dickel, and Mather 2014). Early predation experiences probably have a strong effect on developing cuttlefish (primacy effect—Burghardt and 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.

Conclusion

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, Chichery, and Dickel 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 2006; Langridge 2009; Staudinger et al. 2013). In the related *S. pharaonis,* exposure to a contrasted substrate as a young juvenile changes later body patterning expression (Lee, Yan, and Chiao 2010) and substrate preference (Lee, Yan, and Chiao 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 et al. 2004, 2006; Darmaillacq, Chichery, and Dickel 2006), the expansion of the diet around one month (M. J. Wells 1962), the increasing range of body patterns (Hanlon and Messenger 1988), increasing learning and memory abilities (Dickel, Chichery, and Chichery 1998b, 2001b) and use of multiple predation strategies (Dickel, Chichery, and Chichery 1997a), 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.

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, Bowie, 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 two 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, Bowie, et al. 2016).

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 (Shepherdson, Mellen, and Hutchins 2012). It is generally recognized that enrichment and other such measures aimed at reducing animal distress enhance not only animal welfare, but 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, Chichery, and Dickel 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, Chichery, and Dickel 2005). In experiments with a related species, *S. pharaonis,* juveniles were raised with a varied (checkerboard) or uniform substrates. At two and a half and three 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, Yan, and Chiao 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 et al. 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, Boal, and Budelmann 2000b). Social enrichment alone was also associated with some memory improvements, but not to the same degree as the combination of enrichment types (Dickel, Boal, and Budelmann 2000b). 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, Boal, and Budelmann 2000b; Lee, Yan, and Chiao 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, Boal, and Budelmann 2000b). These results demonstrate three things: 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.

In addition to being good humane and experimental practice, 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 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 (Andrews et al. 2013; Fiorito et al. 2014; Fiorito et al. 2015). Such inquiry will have practical benefits for aquaculture, restocking, comparative and developmental psychology and the general study of behavior.

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 5. 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

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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.

Table 5. A summary of development in *Sepia officinalis*. Modified from Dickel et al. 2006. Sources are cited throughout the text.

	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

ACKNOWLEDGMENTS

II. Article #3: "Visual ecology and the development of visually guided behavior in the cuttlefish"

Among all of the cuttlefish's sensory systems, the visual system is thought to be of primary importance. The following is an in-depth review of the development of this system during the early life stages involved in this project.

Visual ecology and the development of visually guided behavior in the cuttlefish

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Abstract

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

This study was carried out in accordance with the recommendations of 'name of guidelines, name of committee'. The protocol was approved by the 'name of committee'.

Word count: 3855 / number of figures: 4

1. 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 16). 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 17). 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 avoidance and visually-guided behavior (e.g. predation, Darmaillacq, Chichery, et al. 2004; camouflage, Zylinski, Darmaillacq, and Shashar 2012; navigation, (Lelia 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, Milbury, and Hanlon 2002), and that of visual lateralization. Lastly, more recent data regarding the development and plasticity of defensive behavior in juveniles will be presented.

Figure 14. Eyes of the cuttlefish *Sepia elongata* caught off the coast of Eilat (Gulf of Aqaba, Israel; photo AS Darmaillacq).
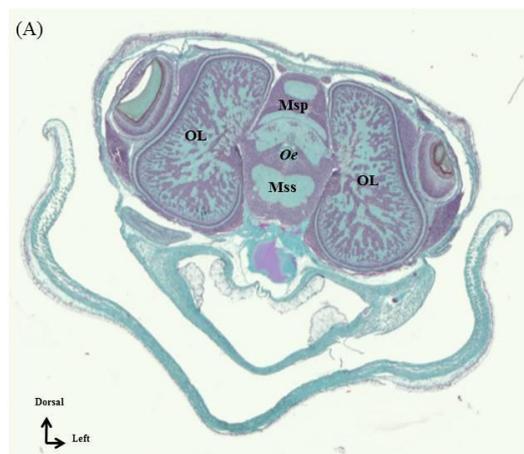

Figure 15. Central nervous system of 3-month-old *Sepia officinalis* cuttlefish. Frontal section. Prenant-Gabe trichrome stain. Abbreviations: OL = optic lobe; SpM = supra-esophageal mass; SbM = sub-esophageal mass; Oe = oesophagus. Modified from Jozet-Alves et al. 2012a.

2. Embryonic development of the visual system and embryos' responses to visual stimuli

2.1 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 to 30; Boletzky, Andouche, and Bonnaud-Ponticelli 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 behavioural 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, Lewkowicz, and Spence 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. 2017 this volume).

2.2 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, Jaisson, and Lenoir 1992), amphibians (Mathis et al. 2008), rats (Hepper 1988), dogs (Wells and Hepper 2006), precocial birds (Sneddon, Hadden, and Hepper 1998), altricial birds (Colombelli-Négrel

et al. 2012; Colombelli-Négrel, Hauber, and Kleindorfer 2014), and humans (Moon, Lagercrantz, and Kuhl 2013).

Studies showed that embryonic visual experience affects both feeding and defensive behaviours. Cuttlefish embryos visually exposed to juvenile crabs for the last week before hatching will prefer crabs to their innately preferred shrimp prey (Darmaillacq, Lesimple, and Dickel 2008a). 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 18A). 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 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 18B). Juvenile cuttlefish, that spontaneously prefer dark shelters, lose this bias when they have been exposed embryonically to white ones (M. Guibé and Dickel 2011). Lee, Yan, and Chiao (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.
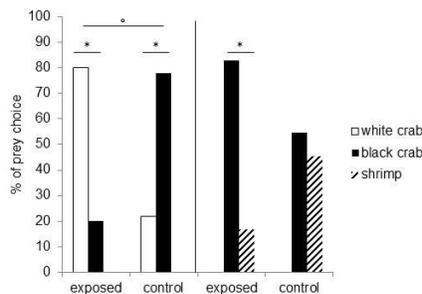
Figure 16. 7-day-old cuttlefish's prey choice depending on whether they have been exposed to white crabs during embryonic development ('exposed') or not ('control'). *Significant prey preference within groups (chi-square exact test: $p < 0.05$) and °significant difference in prey choice between groups (Fisher's exact test: $P < 0.05$). Modified from (Mathieu Guibé et al. 2012).

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, Boal, and Dickel 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.

3. 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, Milbury, and Hanlon 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.

3.1. 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, Cotton, and Williamson (2005) showed that visual acuity improves as cuttlefish grow, ranging from a minimum separable angle

of 2.5° to 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, Hanlon, and deM Petz 1998; Shashar et al. 2000); in cuttlefish, it may also play a role in communication between adults (Shashar, Rutledge, and Cronin 1996; Boal et al. 2004a) and in navigation (Cartron et al. 2012). PS matures gradually after hatching. Cartron et al. (2013) 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 19). However, a choice test with fully polarized or depolarized mysids (transparent shrimps) showed that one week-old cuttlefish detect polarized shrimp faster than non-polarized, suggesting an earlier maturation of PS (Cartron et al. 2013). 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; Stubbs and Stubbs 2016), cuttlefish are sensitive to contrast. Indeed, most hatchling cuttlefish (75%) showed an OMR to the black, white and grey striped pattern rotating at the lowest velocity, with the proportion reaching 100% by the age of one 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, Kinoshita, and Homberg 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 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.
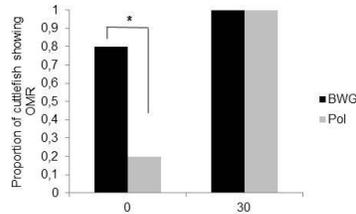
Figure 17. Proportion of the cuttlefish (N=10 per group) that showed an optomotor response (OMR) to BWG (luminance only; black) or Pol (polarization; grey) patterns rotating at a velocity of 30 deg s–1, at hatching (0) and at the age of 30 days. Asterisks indicate a significant difference in the percentage of cuttlefish showing an OMR between the BWG and Pol patterns McNemar's test, P<0.05). Modified from (Cartron et al. 2013).

3.2. Ontogenesis of visual lateralization

Cerebral lateralization, a trait that is widespread in animal kingdom (Vallortigara and Rogers 2005; Frasnelli, Vallortigara, and Rogers 2012), is often revealed behaviourally 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. 2006) which can be the result of an eye use preference as in octopus (Byrne, Kuba, and Griebel 2002; Byrne, Kuba, and Meisel 2004). In juveniles, Jozet-Alves et al. (2012) 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 odour before hatching, they preferentially turn to the left in the simple T-maze (Jozet-Alves and Hebert 2012); 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.

3.3. Influence of environmental constraints on PS and visual lateralization

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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. 2013; Cartron et al. 2013), 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. 2013). 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.

4. 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 1998; 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, Chubb, and Hanlon 2007). More, several authors (Chiao and Hanlon 2001; Barbosa et al. 2008; Barbosa et al. 2007) 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, Kelman, and Hanlon 2005; Zylinski, Osorio, and Shohet 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 20a) while disruptive coloration occurs on more patchy/contrasted substrates (Figure 20b,d). Uniform, mottle and disruptive patterns are usually mixed to varying degrees (Hanlon et al. 2009; Figures 20b,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 1998), 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. 2004b).
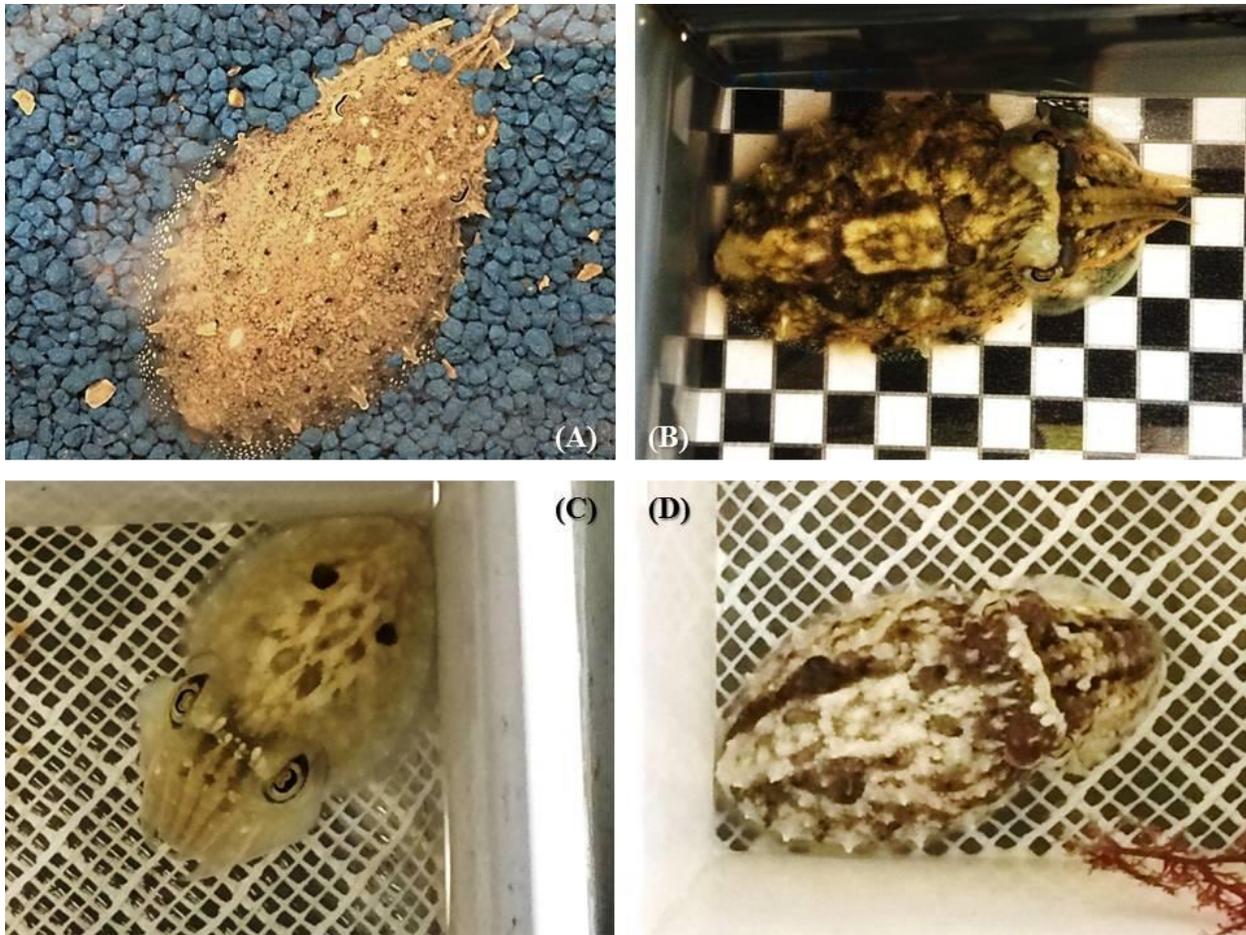
Figure 18. The diversity of body patterns displayed by 2-month-old cuttlefish (ca. 3-4 cm dorsal mantle length). a) stipple-uniform pattern elicited on uniform blue gravel; b) disruptive pattern elicited on a black and white checkerboard combined with mottle pattern; c) deimatic pattern following exposure 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.

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 400-500/mm² at hatching to 35-50/mm² 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 20c). In younger animals, this pattern appears very rarely (Thorpe 1956; 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 two months (Poirier, Chichery, and Dickel 2005). Later, individuals from each group were tested on either a uniform grey 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 towards small, low-threat teleost fish but not toward larger more dangerous predators such as sea bass or small sharks (Langridge, Broom, and Osorio 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 (Darmaillacq, Chichery, 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-Eberhard 1989), possibly even before hatching (Figure 21). 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 2013; Skelhorn and Rowe 2016).

Figure 19. Stage 30 embryo (less than 1 cm) showing a mottle-disruptive coloration inside the egg. It has also squirted ink; note the cloud ink in the perivitellin fluid. Note that the embryo is seen from under through a peeled *S. officinalis* egg (photo C.E. O'Brien).

5. 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, Boal, and Budelmann 2000c), 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, Lewkowicz, and Spence 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.

III. Article #4: "Maternal and Embryonic Stress Influence Offspring Behavior in the Cuttlefish *Sepia officinalis*"

This manuscript reports data from a number of behavioral experiments conducted with

cuttlefish hatchlings after maternal or embryonic stress treatments.

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

Prenatal Stress affects Cuttlefish Behavior

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Abstract
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 versus 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

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.

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, Hung, & Randall, 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, Chen, & Zhou, 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.* Favreau-Peigné et al., 2014; Katz, Roth, & Carroll, 1981; Miller et al., 2007). 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 (Groothuis et al. 2005; Hayward and Wingfield 2004; 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, Ferrari, Windel, Messier, & Chivers, 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 five months after hatching than control animals, although no differences were seen at two 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, Lesimple, & Dickel, 2008; Jozet-Alves & 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. 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 & 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 ten 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 (Fig. 1).
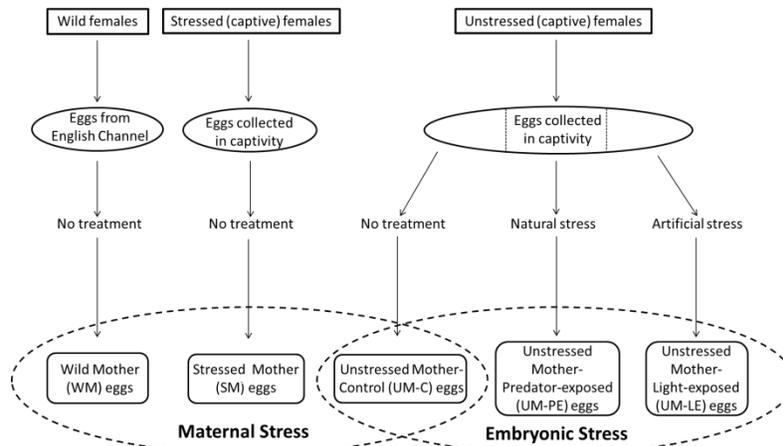
Fig 20.) Schematic representation of the experimental design.

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 (Fig. 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 (*Sepia 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 16:8h 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 (1000 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 19cm and subjected to randomized 10-second removals from the water three times a day using a specially-made mesh platform. Eggs spawned by these stressed captive females after at least one 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 x 60 x 40cm) after eight hours of steady temperature habituation (from 15°C 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 eggs were divided randomly into predator-exposed (UM-PE) and light-exposed (UM-LE) groups. Three sea bass (*Dicentrarchus labrax*; Linnaeus, 1758; 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 10cm 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 7cm. 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 two months of prenatal stress treatments described in the previous section, the resulting offspring were subjected to a battery of tests conducted during the first ten days after hatching (Fig. 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.
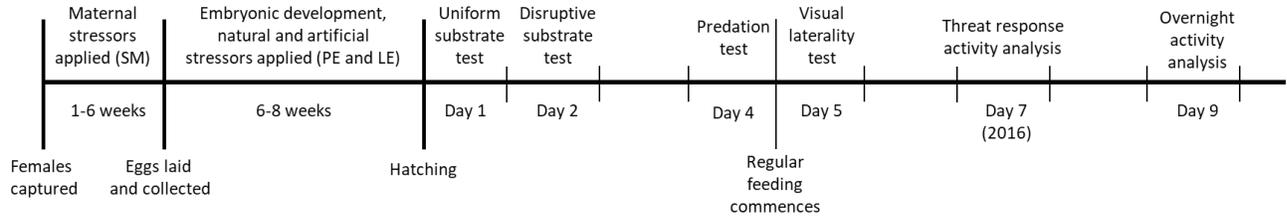
Maternal stressors applied (SM)	Embryonic development, natural and artificial stressors applied (PE and LE)	Uniform substrate test	Disruptive substrate test	Predation test	Visual laterality test	Threat response activity analysis	Overnight activity analysis
1-6 weeks	6-8 weeks	Day 1	Day 2	Day 4	Day 5	Day 7 (2016)	Day 9

Females captured   Eggs laid and collected   Hatching   Regular feeding commences

Fig. 21) Timeline of stress treatments and behavioral tests. All tests except for the threat response activity analysis occurred in 2015.

Body Patterning

In 2015, on the day of hatching (Day 1), between 9:00-10:30, up to 12 cuttlefish at a time were placed in randomized order in small uniform grey ("uniform background") circular compartments with slanted sides to minimize shadows (radius = 2.9cm bottom, 3.35cm top, length of sides = 2.5cm; mean grey value = 101 +/-3.9) under white LED light (0.63 to 0.88 klux) and photographed at 0, 5, 15 and 30min 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, Chubb, and Hanlon 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 grey 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, Bidel, Dickel, & Bellanger (2014).

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

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 15min (Panasonic HDC-SD60) and a single shrimp (*C. crangon*, total length 0.7 cm—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 five days after hatching. The testing apparatus consisted of a start box (3.5 cm x 5 cm), a movable transparent barrier and two darkened shelters (3.5 cm x 4 cm) located 15cm 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 versus 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 6hrs 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; Frank et al. 2012; Jäckel et al. 2007; 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 one hour 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 anaesthetized in 17.5g/L $MgCl_2$ 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 *versus* 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 (Fig. 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).
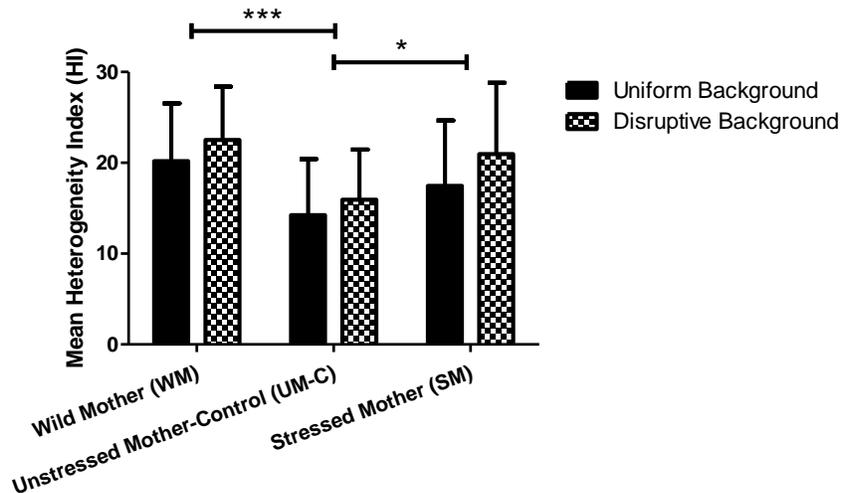
Fig. 22) Heterogeneity Index (HI) ± s.d. of maternal stress groups on uniform and disruptive backgrounds. Between groups, WM offspring (n = 55) and SM (n = 43) had significantly higher HI than UM-C (n = 41; p < 0.001 and = 0.034). Significant differences between groups are indicated by connecting brackets. * indicates p < 0.05; *** indicates p < 0.001.

In the embryonic stress groups, a repeated measures ANOVA revealed a significant effect of the background type (*i.e.* uniform versus 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).

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).

Table 6. 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. Both group comparisons and post hocs are chi squared exact tests (sequential Bonferroni correction).

	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%	$p = 0.333$, $X^2 = 2.568$	

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 (Fig. 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: $p = 0.083$; $X^2 = 5.237$).
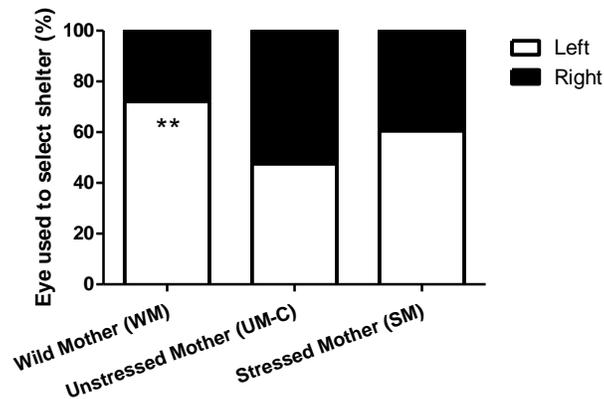
Fig. 23) Eye used to select shelter in maternal stress groups. More WM (n = 43) chose the shelter in their left visual field (binomial test; p = 0.005, signified by asterisks) while no preference was found in UM-C (n = 40) or SM (n = 43). The proportions were not significantly different between groups (p = 0.08).

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 (Fig. 5A and B) were significantly different 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; Fig. 5C). In addition, WM showed a statistical trend for higher distance travelled than UM-C (exact permutation tests: p = 0.058).
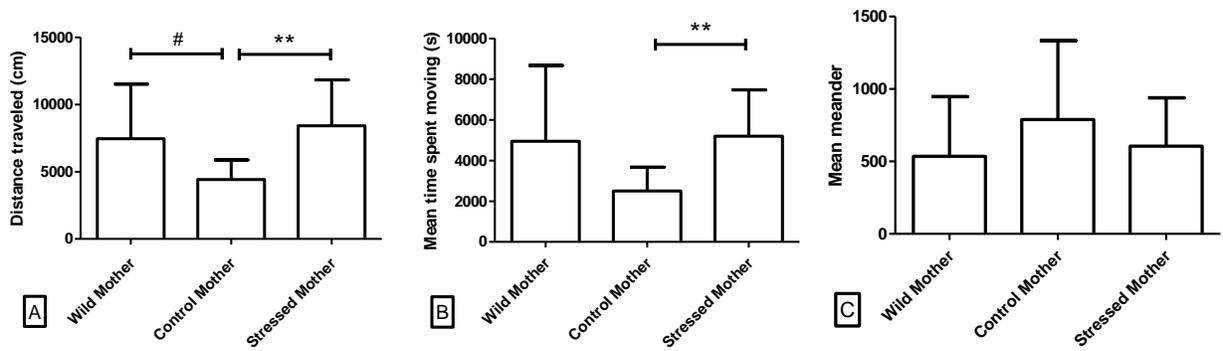
Fig. 24) The total distance traveled (A), time spent moving (B) and mean meander (turn angle/distance traveled; C) ± s.d. of maternal stress groups in the overnight activity analysis test. Significant differences (indicated by connecting brackets with asterisks) exist between UM-C (n = 10) and SM (n = 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. ** indicates p < 0.01; # indicates a statistical tendency (p < 0.08).

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 *versus* after water addition), but not according to treatment groups (*i.e.* WM, SM, UM-C) or cue type (*i.e.* blank water *versus* predator odor), for both distance traveled (p < 0.001; F = 32.666; Fig. 6A) and time moving (p < 0.001; F = 25.284; Fig. 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.
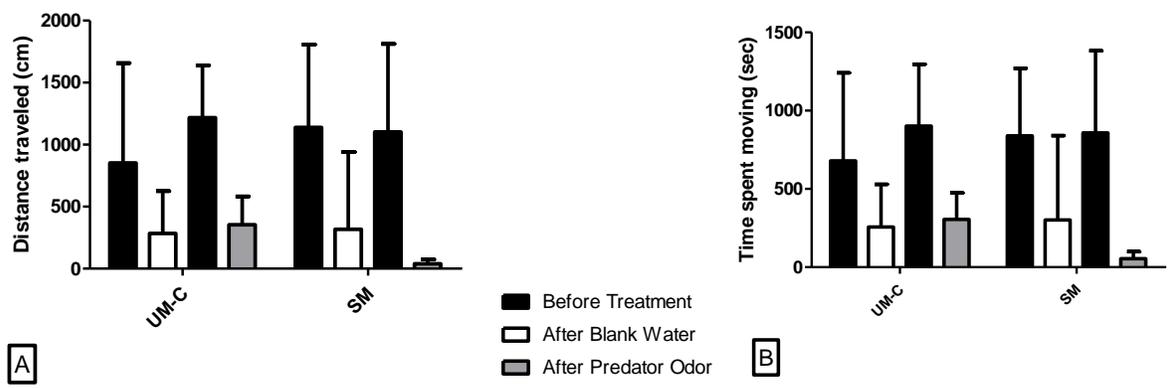
Fig. 25) The total distance traveled (A) and time moving (B) ± s.d. for maternal stress groups in the threat response activity analysis. Differences within groups are indicated by connecting bars; n = 5 for all bars.

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In the embryonic stress groups, the non-parametric analysis for longitudinal data revealed a significant difference within groups according to time (*i.e.* before *versus* after water addition), but not according to treatment groups (*i.e.* UM-C, UM-PE, UM-LE) or cue type (*i.e.* blank water *versus* predator odor), for both distance traveled ($p < 0.001$; F = 37.982; Fig. 7A) and time moving ($p < 0.001$; F = 32.437; Fig. 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.
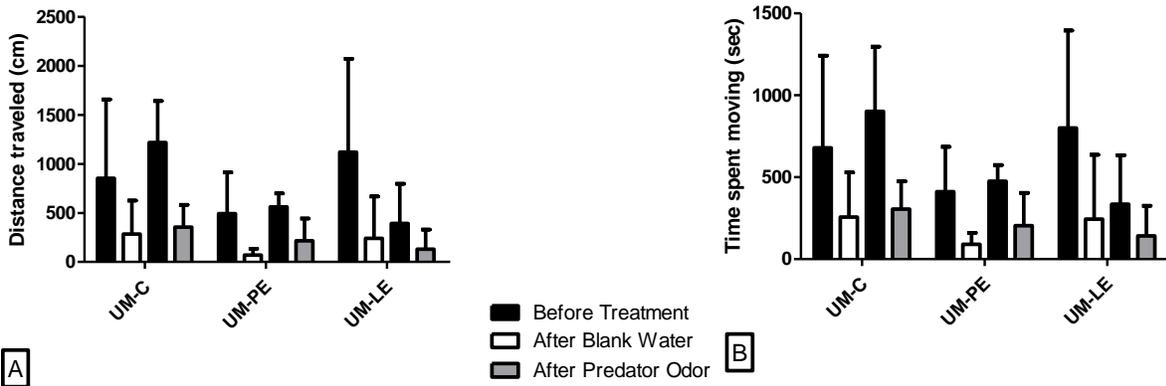
**Fig. 26)** The total distance traveled (A) and time moving (B) ± s.d. for embryonic stress groups in the threat response activity analysis. Differences within groups are indicated by connecting bars; n = 5 for all bars.

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).

Table 7. 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

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, Bowie, et al. 2016) and exposed to certain pharmaceuticals during development (Bidel, Di Poi, et al. 2016; Di Poi et al. 2014a). 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 (Barbosa et al. 2008; Mathger et al. 2007), young cuttlefish usually display a fairly chronic body pattern that often clashes with the background (Hanlon & Messenger, 1988; Poirier, Chichery, & Dickel, 2005). The ability to produce a uniform body pattern emerges during the first few months of life (see O'Brien, Mezrai, Darmaillacq, & Dickel, 2016), 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 towards the left side on Day 5. These results do conflict somewhat with the findings of Jozet-Alves & Hebert (2012); in that study, the

authors showed that prenatal exposure to predator odor induced a left preference three 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 (Clarke et al. 1996; Espmark et al. 2008; Schneider 1992), 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 (Hilakivi, Ota, and Lister 1989; Masterpasqua, Chapman, and Lore 1976; Peters 1986; Sandi, Venero, and Guaza 1996; Wilson, Vazdarjanova, and Terry 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

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offspring of females subjected to a variety of stressors during pregnancy (Fride et al. 1986; Fujioka et al. 2001; Hockman 1961; Patin et al. 2004; Poltyrev et al. 1996; Suchecki and Neto 1991; Vallee et al. 1997). 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. 2006; Dickel, Chichery, and Chichery 2001c), 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.* Dalesman, Rundle, Coleman, & Cotton, 2006; Jacobsen & Stabell, 2004; Snyder & Snyder, 1971), as well as reductions in movement such as cessation of filter feeding or decreases in foraging and migration (*e.g.* Reimer & Tedengren, 1997; Smee & 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 & Hebert, 2012) and changes in prey preference (Mathieu Guibé, Boal, and Dickel 2010b). 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 1999a), 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 versus 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, Laforsch, & Tollrian, 1999; Storm & Lima, 2010). In birds and mammals, such maternal stress effects are likely mediated by the transfer of stress hormones in the egg or placenta (Groothuis et al. 2005; Hayward and Wingfield 2004; 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 versus 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 1999a). 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 & 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 & 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, Mason, Nolte, & Campbell, 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, Slater, & Ford, 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, Boal, and Budelmann 2000c) 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.

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

Acknowledgments

No competing interests declared.

IV. Cognitive effects of prenatal stress.

For several decades, the "prawn in a tube" test, or PIT test, has been used to assess learning and memory in cuttlefish (e.g. Messenger 1977; Dickel, Chichery, and Chichery 1998b; Cartron et al. 2013).

Materials and Methods

Stress Treatments:

On May 10, 2016, 26 adult female cuttlefish (approximately two years old) were captured from the English Channel by trap and transported to the Centre Régional d'Etudes Côtières (CREC, Luc sur Mer, France), which is equipped with a semi-open flow-through seawater system with a mean temperature of 15±1°C and a 16:8h light/dark cycle. Here, the females were randomly divided into an unstressed group and a "stressed" group. The unstressed mothers (UM) were housed in groups of three in 1000L tanks enriched with natural and artificial objects. The stressed mothers (SM) were housed individually in bare 65L tanks and subjected to daily stress treatments. These treatments consisted of removing cuttlefish from the water on a soft mesh platform for 10 sec, three times a day. These treatments occurred between 9h and 17h with the timing randomized each day to avoid habituation. The combination of confined space and daily stress treatments were designed to induce chronic stress.

On arrival, females were mated with males captured concurrently and they began to lay eggs several days later. To ensure that any potential effects of the stress treatment had manifested, and to allow the UM to recover from the stress of capture, only eggs that were laid at least one week after arrival at the CREC were used in the experiments described below. In addition, only eggs from SM that had laid at least 150 eggs were used in order to ensure similar representation between mothers and across stress groups. In total, 11 UM (grouped into four groups since eggs laid in the same tank could not be attributed to individual females) and five SM laid eggs that met these criteria between May 14 and 29. After a sufficient number of eggs had been collected, they were habituated to a temperature of 20±2°C over the course of eight hours and moved to shallow trays floating in 65L aerated tanks for incubation.

The eggs of UM were divided into three treatment groups: Control (UM-C), predator-exposed (UM-PE) and light-exposed (UM-LE). The eggs of UM and SM were allowed to develop without further manipulation. UM-PE eggs were incubated with three sea bass (*Dicentrarchus labrax*) for the remainder of development (three to five weeks). Bright LED light was shined on the UM-LE group for 15m six times a day (90m total) at random intervals. Eggs hatched between July 2 and 24. On the day of hatching,

cuttlefish were placed in an individually-labelled compartment (6.8 x 12.1cm, depth 5.5cm) at a mean temperature of 20±2°C. Starting on day 4, they were fed two shrimp (*Crangon crangon* or *Penaeus serratus*) per day. Individual identity was maintained throughout. Because of higher level of shared parentage between UM-C, UM-PE and UM-LE (the embryonic stressors) versus UM-C and SM (the maternal stressor), the results of the two stressor categories are presented separately.

PIT tests:

Cuttlefish were tested at 3 and 5 weeks of age, with each age-session lasting two weeks and with two to eight individuals tested per day. Cuttlefish were starved for 24hrs prior to testing to ensure feeding motivation. Individuals were moved from their home compartment to the testing arena and allowed to habituate for 10min. The testing arena contained a PIT apparatus: a small glass container containing four shrimp (*C. crangon* or *P. serratus*) with a small pump circulating water to ensure constant movement of the shrimp (Fig. 30).
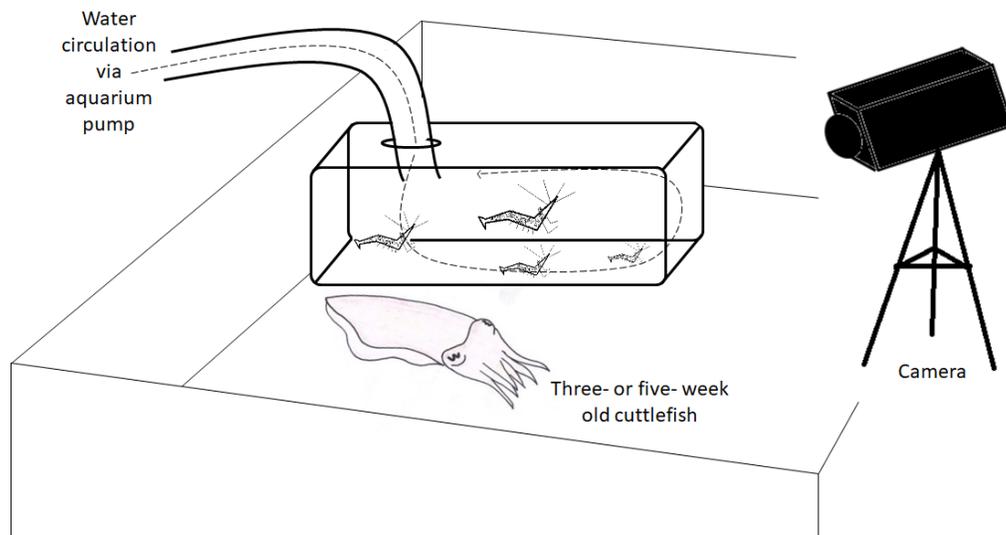
Fig. 27. Prawn-in-a-tube (PIT) apparatus. The tube is composed of glass microscope slides held together by clear plastic. Four live shrimp are trapped between the slides. An aquarium pump keeps water circulating and the shrimp in motion. Cuttlefish drawing by Anne Quiédeville.

Prior to the test, shrimp were hidden from view with an opaque screen. At the start of each trial, the screen was removed and the cuttlefish was allowed visual access to the shrimp for 45min. The screen was then replaced for one hour until the retention trial (Fig. 31). All trials were filmed for later analysis. Videos were analyzed by a single observer to ensure consistency. Strikes (defined as tentacle contact with the side of the PIT apparatus) per minute were counted from the moment of first detection (defined as the moment of orientation towards the apparatus). Each video was analyzed in this way until

an individual achieved the learning criteria (three consecutive minutes without a strike) or until 45 minutes of tube presentation had elapsed, in order to avoid unnecessary stress to the cuttlefish. The mean strikes per minute after detection was plotted for each group until the number of individuals striking fell below n = 5. Six variables were measured: the proportion of each group that reached criterion, the latency to detection (time between removal of the occlusive screen and orientation toward the apparatus), latency to attack (time between detection and the first strike), the initial strike level (number of strikes made in the first 3 min), the mean time to criterion (time between detection and reaching criterion), and the mean strikes to criterion (number of strikes made before three minutes without striking). Differences between groups in these variables could suggest differences in learning abilities, memory capacity, visual abilities, feeding motivation, predation strategy and/ or fatigue.
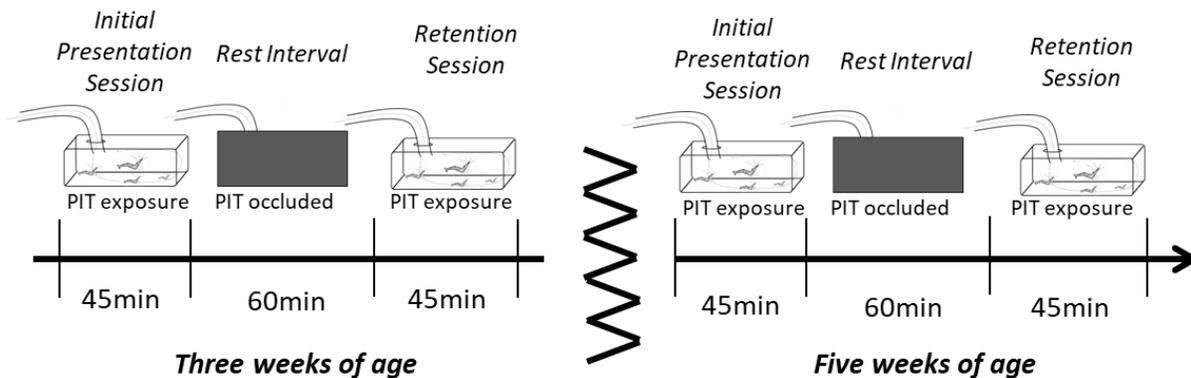
Fig. 28. Timeline of PIT sessions. Juveniles were tested at three and five weeks of age in two 45min sessions separated by an hour-long rest interval.

Group success rates were compared by stress group with a Cochran-Mantel-Haenszel chi-squared test for count data using the mantelhaen.test function in R. The remaining variables were compared by stress group and within groups by performances during the initial session versus the retention session. Latencies and initial strike levels were calculated from all cuttlefish that responded to the PIT apparatus during either or both sessions, while the time and number of strikes to criterion were calculated only from cuttlefish that responded in both the initial and retention sessions in order to ensure a balanced data set (the majority of individuals in all groups responded during both sessions). Latency to detect, latency to attack, strike level in first three minutes, minutes to criterion, strikes to criterion were failed to adhere to parametric assumptions, so a non-parametric repeated measures ANOVA was used to compared between and within groups (ANOVA.test and pair.comparison functions in R package nparLD).

Results

3 Week Maternal Stress

At three weeks of age, the mean strike level of both UM-C and SM fluctuated between 2 and 10 strikes per minute in the initial trial (Fig. 32). In the retention trial, the mean strike level of UM-C fluctuated between 3 and 7 strikes per minute, while SM fluctuated between 4 and 13 strikes per minute (Fig. 33). Non-parametric repeated measures ANOVA showed statistical significance for the factors "session" and "interaction" in latency to attack (p = 0.005 and 0.007 respectively; F = 7.74 and 7.3; UM-C n = 18, SM n = 11), minutes to criterion (p < 0.001 and = 0.047 respectively; F = 16.95 and 3.95; UM-C n = 14, SM n = 7) and strikes to criterion (p < 0.001 and = 0.031 respectively; F = 13.73 and 4.67; UM-C n = 14, SM n = 7; Table 8).

Fig. 29) The mean number of strikes per minute made by the maternal stress groups during their initial exposure to the PIT apparatus at three weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, SM = Stressed Mother.

Fig. 30) The mean number of strikes per minute made by the maternal stress groups during the retention trial exposure to the PIT apparatus at three weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, SM = Stressed Mother.

Table 8) Statistical comparisons of groups and of presentation sessions of the maternal stress groups in six measures of performance in the PIT test at three weeks of age.

	Unstressed Mother-Control, UM-C (n = 18)		Stressed Mother, SM (n = 11)		Statistical Comparison
	Initial Session	Retention Session	Initial Session	Retention Session	
% that reach criterion	77.8%	94.4%	63.6%	81.8%	Cochran-Mantel-Haenszel Chi-Squared Test p = 0.965
Latency to Detection (sec)	245.5 ± 251.9	388.4 ± 605.9	316.4 ± 520.2	600.0 ± 638.3	Stress: p = 0.927 Session: p = 0.21 Interaction: p = 0.551
Latency to Attack (sec)	15.3 ± 17.0	5.6 ± 11.5	9.1 ± 8.8	11.5 ± 14.1	Stress: p = 0.427 Session: p = 0.005 Interaction: p = 0.006
Strikes in the first three minutes	13 ± 7.5	14.1 ± 12.6	20.3 ± 9.0	17.5 ± 19.9	Stress: p = 0.366 Session: p = 0.055 Interaction: p = 0.09
Minutes to reach criterion	17.4 ± 13.0 (n = 14)	11.1 ± 9.3 ("")	24.7 ± 14.2 (n = 7)	9.8 ± 8.7 ("")	Stress: p = 0.958 Session: p < 0.0001 Interaction: p =

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					0.047
Strikes to reach criterion	56.9 ± 50.7 (n = 14)	52.4 ± 103.7 ("")	104.4 ± 79.7 (n = 7)	58.3 ± 86.8 ("")	Stress: p = 0.78 Session: p < 0.001 Interaction: p = 0.031

5 week Maternal Stress:

At five weeks of age, the mean strike level of UM-C fluctuated between 0 and 9 strikes per minute, while SM fluctuated between 0 and 6 (Fig. 34). In the retention trial, the mean strike level of UM-C fluctuated between 1 and 4, while SM fluctuated between 1 and 3 (Fig. 35). Non-parametric repeated measures ANOVA showed statistical significance for the factor "session" in latency to detection ($p < 0.001$; $F = 19.99$; UM-C $n = 15$, SM $n = 12$; Table 9).


Fig. 31) The mean number of strikes per minute made by the maternal stress groups during their initial exposure to the PIT apparatus at five weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below $n = 5$. UM-C = Unstressed Mother-Control, SM = Stressed Mother.


Fig. 32) The mean number of strikes per minute made by the maternal stress groups during the retention trial exposure to the PIT apparatus at five weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the

experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, SM = Stressed Mother.

Table 9) Statistical comparisons of groups and of presentation sessions of the maternal stress groups in six measures of performance in the PIT test at five weeks of age.

	Unstressed Mother-Control, UM-C (n = 15)		Stressed Mother, SM (n = 12)		
	Initial Session	Retention Session	Initial Session	Retention Session	
% that reach criterion	86.7%	93.3%	83.3%	91.7%	Cochran-Mantel-Haenszel Chi-Squared Test p = 0.973
Latency to Detection (sec)	403 ± 352.8	747.1 ± 619.5	157.4 ± 186.8	562.3 ± 588.7	Stress: p = 0.093 Session: p < 0.001 Interaction: p = 0.222
Latency to Attack (sec)	3.5 ± 5.7	4.5 ± 4.9	6.4 ± 10.1	7.7 ± 6.6	Stress: p = 0.058 Session: p = 0.226 Interaction: p = 0.272
Strikes in the first three minutes	8.9 ± 7.0	8.7 ± 6.8	9.9 ± 6.0	7.5 ± 3.9	Stress: p = 0.975 Session: p = 0.272 Interaction: p = 0.279
Minutes to reach criterion	11.7 ± 7.8 (n = 12)	8.6 ± 5.3 ("")	15.7 ± 14.4 (n = 10)	13.5 ± 9.2 ("")	Stress: p = 0.307 Session: p = 0.56 Interaction: p = 0.724
Strikes to reach criterion	22.2 ± 19.0 (n = 12)	14.6 ± 13.0 ("")	44.3 ± 44.0 (n = 10)	17.3 ± 19.2 ("")	Stress: p = 0.385 Session: p = 0.058 Interaction: p = 0.519

3 week Embryonic Stress

At three weeks of age, the mean strike level of UM-C and UM-PE fluctuated between 1 and 10 per minute, and UM-LE fluctuated between 1 and 4 (Fig. 36). In the retention trial, mean strike level of UM-C fluctuated between 3 and 7, while PE fluctuated between 4 and 10, and UM-LE fluctuated between 1 and 7 (Fig. 37). Non-parametric repeated measures ANOVA showed statistical significance for the "stress" factor in strike level during the first 3 min and strikes to reach criterion, and for the "stress" and "session" factors in minutes to criterion (Table 10). Post hoc comparisons showed that UM-LE reached criterion significantly faster than both other groups, and had a significantly lower initial strike level and took significantly fewer strikes to reach criterion than UM-PE.

Fig. 33) The mean number of strikes per minute made by the embryonic stress groups during their initial exposure to the PIT apparatus at three weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, UM-PE = Unstressed Mother-Predator-Exposed, UM-LE = Unstressed Mother-Light-Exposed.


Fig. 34) The mean number of strikes per minute made by the embryonic stress groups during the retention trial exposure to the PIT apparatus at three weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, UM-PE = Unstressed Mother-Predator-Exposed, UM-LE = Unstressed Mother-Light-Exposed.

Table 10) Statistical comparisons of groups and of presentation sessions of the embryonic stress groups in six measures of performance in the PIT test at three weeks of age.

	Unstressed Mother-Control, UM-C (n = 18)		Unstressed Mother-Predator-Exposed, UM-PE (n = 16)		Unstressed Mother-Light-Exposed, UM-LE (n = 8)		Statistical Comparisons	Post hocs
	Initial Session	Retention Session	Initial Session	Retention Session	Initial Session	Retention Session		
% that reach criterion	77.8%	94.4%	62.5%	75%	87.5%	100%	Cochran-Mantel-Haenszel Chi-Squared Test p = 0.908	
Latency to Detection	245.5 ± 251.9	388.4 ± 605.9	219.2 ± 331.9	470.9 ± 621.8	272.5 ± 273.4	243.1 ± 294.4	Stress: p = 0.728 Session: p =	

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(sec)						0.308 Interaction: p = 0.052	
Latency to Attack (sec)	15.3 ± 17.0	5.6 ± 11.5	11.8 ± 16.0	9.1 ± 6.7	5.8 ± 5.6	19.9 ± 21.2	Stress: p = 0.59 Session: p = 0.948 Interaction: p = 0.001	
Strikes in the first three minutes	13 ± 7.5	14.1 ± 12.6	21.3 ± 14.0	22.1 ± 18.2	6.4 ± 5.0	14.3 ± 14.6	Stress: p = 0.016 Session: p = 0.575 Interaction: p = 0.184	UM-PE>UM-LE
Minutes to reach criterion	17.4 ± 13.0 (n = 14)	11.1 ± 9.3 ("")	23.1 ± 16.3 (n = 8)	14.0 ± 10.9 ("")	9.1 ± 8.6 (n =7)	7.9 ± 6.5 ("")	Stress: p = 0.013 Session: p = 0.04 Interaction: p = 0.443	UM-PE>UM-LE UM-C>UM-LE
Strikes to reach criterion	56.9 ± 50.7 (n = 14)	52.4 ± 103.7 ("")	116.5 ± 95.3 (n = 8)	93.6 ± 102.8 ("")	25.1 ± 47.2 (n = 7)	26.6 ± 32.7 ("")	Stress: p = 0.004 Session: p = 0.311 Interaction: p = 0.225	UM-PE>UM-LE

5 week Embryonic Stress

At five weeks of age, the mean strike level of UM-C and UM-LE fluctuated between 1 and 5, while PE fluctuated between 1 and 7 (Fig. 38). In the retention trial, mean strike level of UM-C fluctuated between 1 and 4, while UM-PE fluctuated between 0 and 5, and UM-LE fluctuated between 1 and 3 (Fig. 39). Non-parametric repeated measures ANOVA showed statistical significance for the factors "session" in latency to detect ($p < 0.001$; F = 19.03; UM-C n = 15, UM-PE n = 16,UM- LE n = 12), strikes in the initial 3 min ($p = 0.015$; F = 5.96; UM-C n = 15, PE n = 16, LE n = 12), minutes to criterion ($p < 0.001$; F = 19.53; UM-C n = 13, PE n = 14, UM-LE n = 9) and strikes to criterion ($p = 0.001$; F = 11.08; UM-C n = 13, UM-PE n = 14, UM-LE n = 9; Table 11).

**Fig. 35)** The mean number of strikes per minute made by the embryonic stress groups during the initial exposure to the PIT apparatus at five weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, UM-PE = Unstressed Mother-Predator-Exposed, UM-LE = Unstressed Mother-Light-Exposed.

Fig. 36) The mean number of strikes per minute made by the embryonic stress groups during the retention trial exposure to the PIT apparatus at five weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, UM-PE = Unstressed Mother-Predator-Exposed, UM-LE = Unstressed Mother-Light-Exposed.

Table 11) Statistical comparisons of groups and of presentation sessions of the embryonic stress groups in six measures of performance in the PIT test at five weeks of age.

	Unstressed Mother-Control, UM-C (n = 15)		Unstressed Mother-Predator-Exposed, UM-PE (n = 16)		Unstressed Mother-Light-Exposed, UM-LE (n = 12)		Statistical Comparisons
	Initial Session	Retention Session	Initial Session	Retention Session	Initial Session	Retention Session	
% that reach criterion	86.7%	93.3%	87.5%	100%	75%	100%	Cochran-Mantel-Haenszel Chi-Squared Test p = 0.98
Latency to Detection	403 ± 352.8	747.1 ± 619.5	220.1 ± 275.2	439.3 ± 388.7	218.3 ±	579.3 ± 724.1	Stress: p = 0.089 Session: p <

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(sec)					293.5		0.001 Interaction: p = 0.552
Latency to Attack (sec)	3.5 ± 5.7	4.5 ± 4.9	2.8 ± 2.9	7.1 ± 12.8	5.1 ± 5.7	10.6 ± 15.5	Stress: p = 0.242 Session: p = 0.258 Interaction: p = 0.411
Strikes in the first three minutes	8.9 ± 7.0	8.7 ± 6.8	12.2 ± 7.6	6.1 ± 5.7	11.8 ± 6.9	5.4 ± 4.4	Stress: p = 0.819 Session: p = 0.015 Interaction: p = 0.288
Minutes to reach criterion	11.7 ± 7.8 (n = 12)	8.6 ± 5.3 ("")	19.1 ± 12.7 (n = 14)	12.3 ± 12.1 ("")	18.1 ± 11.3 (n = 9)	8.6 ± 6.5 ("")	Stress: p = 0.323 Session: p = < 0.001 Interaction: p = .089
Strikes to reach criterion	56.9 ± 19.0 (n = 12)	52.4 ± 13.0 ("")	116.5 ± 54.2 (n = 14)	93.6 ± 23.7 ("")	25.1 ± 31.8 (n = 9)	26.6 ± 18.4 ("")	Stress: p = 0.373 Session: p = .001 Interaction: p = 0.387

Discussion

At three and five weeks of age, there were no significant group differences in any measure of PIT test performance between the maternal stress and control groups. Among embryonic stress groups at three weeks of age, light-exposed offspring (UM-LE) took significantly less time to reach criterion than both other groups, suggesting an ability to learn faster. As posited in Article #4, accelerated development due to differences in the embryonic light regime might explain this faster learning in the light-exposed offspring: light influences circadian rhythm and thus might alter embryonic metabolic rate and growth. However, this effect eventually disappeared, as there were no significant differences between groups in any measure of PIT test performance at five weeks of age.

Other than the indications that UM-LE might learn faster at three weeks, there were no other effects of prenatal stress treatments on learning and memory abilities in the PIT task. This suggests that in cuttlefish, prenatal stress does not strongly affect learning and memory, at least not at the ages tested. This contrasts with the numerous behavioral and neurobiological differences (next section) found between stress groups. It also contrasts with the situation in mammals, many of which showed inhibited learning abilities after various forms of prenatal stress, although sometimes they demonstrated stronger abilities in threatening contexts (Braastad 1998). For example, the offspring of

female rats that were shocked during pregnancy showed deficits compared to control animals in four different learning tests (Smith, Wills, and Naylor 1981). Likewise, prenatally-stressed rhesus monkeys showed cognitive impairments in object-permanence tests (Schneider 1992). The lack of stress effect in cuttlefish could indicate that, unlike the mammalian nervous system, the cephalopod cognitive system is largely immune to the effects of prenatal stress. Alternatively, the existence of stress effects in some of the other tests reported in this thesis but not the PIT test might encourage us to formulate new learning and memory tests to use in addition to the PIT.

V. Neurobiological effects of prenatal stress

In order to test for stress-related differences in the cuttlefish brain growth and composition, several neurobiological assays were conducted on representative samples of each stress treatment group from 2015 and 2016. The results and conclusions presented here (section V) and in section IV are preliminary, as we are still working to examine and interpret the potential connections and correlations between these data sets.

Monoamines

The concentrations of various monoamines in neural tissue can be used to gauge the status of various neurotransmitter systems in the brains of animals. We quantified seven monoamines: serotonin (5-HT), its main metabolite 5-hydroxyindolacetic acid (5-HiAA), dopamine and its main metabolite 3,4-dihydroxyphenyl acetic acid (DOPAC), glutamate (Glu), noradrenaline (NA) and octopamine. These monoamines were selected since each has been found to occur in the cephalopod central nervous system (CNS) (Andrews, Messenger, and Tansey 1983; Kime and Messenger 1990; Juorio and Molinoff 1974). Most importantly for this research, serotonin, noradrenaline and dopamine are known to be involved in instigating the stress reactions of vertebrates and mammals—noradrenaline initiates the "fight or flight" hormonal cascade (Cannon 1939), while serotonin and dopamine modulate the response as neurotransmitters (reviewed in (Thierry et al. 1968; Roth et al. 1988; Joca, Ferreira, and Guimarães 2007).

In cephalopods, serotonin is found throughout the CNS, where at least three types of receptors exist. It has been implicated in synaptic transmission and long-term potentiation (LTP), meaning it plays an important role in "higher" cognitive processes (Messenger 1996; Shomrat et al. 2010). The catecholamines—dopamine and noradrenaline—function as neurotransmitters and possibly guide axonal growth in cephalopods (Baratte and Bonnaud 2009). Glutamate also functions as a neurotransmitter in cephalopods, as well as playing a role in LTP (Andrews, Messenger, and Tansey 1983; Hochner et al. 2003). Finally, octopamine, first identified in the cephalopods, is related to noradrenaline and functions as a neurotransmitter and modulator (Kime and Messenger 1990). In addition to raw concentrations, the ratio of serotonin or dopamine to their main metabolites (5-HiAA and DOPAC respectively) also give an indication of turnover rate, with a higher ratio meaning greater turnover of that monoamine (Bidel 2015). Though the neurochemistry of cephalopods is still a nascent field, we hope to correlate behavioral differences between stress groups with monoamine concentrations. These measurements were accomplished in collaboration with Dr. Raphael Delepee at

the Université de Caen's Plate-forme de Recherche et d'Innovation en Spectrométroie de Masse et Métabolomique, SF ICORE 4206 (PRISMM, SF ICORE 4206).

Material and Methods

Representatives from each stress group were selected for monoamine quantification. Samples were collected in 2016 on the day of hatching. Hatchlings were euthanized with an overdose of 2% ethanol following anesthesia with MgCl (17.5g/L) and then stored at -80°C before treatment.

Quantification was achieved by ultra-high-pressure liquid chromatography coupled with tandem-mass spectrometry (UHPLC-MS/MS). Glutamate was also added to the quantification as a precursor of monoamines. Two internal standards were used. The first one was 5-hydroxy-*N*-ω-methyltryptamine (5-HMT) used for the quantification serotonin and 5-hydroxyindolacetic acid. The second one was 3,4-dihydroxybenzylamine (DHBA) used for the quantification of dopamine, noradrenaline, octopamine and 3,4-dihydroxyphenyl acetic acid.

Sample preparation consisted of separating head from the body, weighing this structure (between 3 and 20 mg) and adding 0.5 µL of a solution containing internal standards (5 µg/mL and 50 ng/mL of DHBA and 5-HMT, respectively) per mg of sample. The mixture was stored for 30 min in ice in darkness. Then the volume was augmented with a solution of 2% (w/v) formic acid in water (10 times the volume of internal standards solution). The samples were then crushed for 10 min at 20 Hz in tissuelyser Qiagen (Retsch, Haan, Germany) and centrifuged for 5 min at 10 000 x g at +4°C. A volume of 7 µL of the supernatant was injected in the UHPLC-MS/MS system.

Analyses were carried out on an electrospray triple quadrupole mass spectrometer (LCMS 8030 Plus; Shimadzu, Kyoto, Japan) interfaced to an UHPLC Nexera X2 system consisting of two binary pumps with an auto-sampler and a column oven (Shimadzu). The mobile phase, composed of 0.1% (v/v) aqueous acetic acid (solvent A) and acetonitrile (solvent B), was delivered at a flow rate of 0.6 mL/min under the following gradient conditions: starting from 5% B to 1.5 min and then increasing to 27.5% B in 1.5 min. The column was then equilibrated by holding the initial conditions for 2 min. UHPLC was carried out on a Kinetex XB C18 column (100 mm x 2.1 mm i.d., 1.7 µm particle diameter) from Phenomenex (Shandon, UK). The temperature was fixed at 30°C. The column was equilibrated by maintaining the initial conditions for 2 min.

Mass spectrometry was used for detection of compounds. The UHPLC system was interfaced with an electrospray triple quadrupole mass spectrometer (LCMS 8030 Plus; Shimadzu). The mass spectrometer was used in the Multiple Reaction Monitoring (MRM) acquisition mode after positive ESI except for 3,4-dihydroxy-phenyl acetic acid using negative ESI. LabSolutions 5.86 SP1 software was used to process the data. The measurements were performed at 250°C desolvation temperature, 300°C source temperature, 2.5 L/min cone gas (N2) and 15 L/min desolvation gas (N2). The capillary voltage was +4.5 kV. The mass spectrometer was programmed to allow the $[M+H]^+$ or $[M-H]^-$ ions of monoamines and their metabolites to pass through the first quadrupole (Q1) into the collision cell (Q2). The product ions were monitored through the third quadrupole (Q3) for each compound, two masses were selected, the first one for quantification and the second one for confirmation of the compound according to European Commission Decision 2002/657/EC.

Calibrators were prepared in water at the following concentrations: 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100, 500, 750 and 1000 ng/mL; A weighting of $1/X^2$ was used for all the calibration-curve fittings between 0.1 and 1000 ng/mL for dopamine, serotonin and glutamate, between 0.5 and 1000 ng/mL for all other compounds. The limits of quantification were considered as the lowest concentrations of the calibration curve.

Data were analyzed using R and StatXact (Cytel Studio). Extreme outliers which differed from the first and third interquartiles by 1.5 interquartile ranges or more were excluded from the analysis. Exact permutation tests were used to compare concentrations of UM-C and SM, while exact Kruskal Wallis tests followed by post hoc permutation test were used to compare embryonic stress groups.

Results

For all but two monoamines, no significant differences existed between groups. Thus, the amount of 5-HiAA, dopamine and its main metabolite DOPAC, glutamate and octopamine in the hatchling brain do not appear to be affected by the stress treatments.

All prenatally stressed groups showed lower serotonin (5-HT) concentrations in comparison to controls (Fig. 40A and B). The control group, UM-C had significantly higher serotonin than SM offspring (exact permutation test; p = .0032; test stat = 5.14). Likewise, significant differences existed between embryonic stress groups (Kruskal Wallis; p = 0.0002; test stat = 13.92): UM-C had significantly higher serotonin than UM-PE and UM-LE (post hoc exact permutation tests; p < 0.0001 and = 0.0014). UM-LE had significantly higher serotonin levels than UM-PE (post hoc exact permutation test; p < 0.0001).
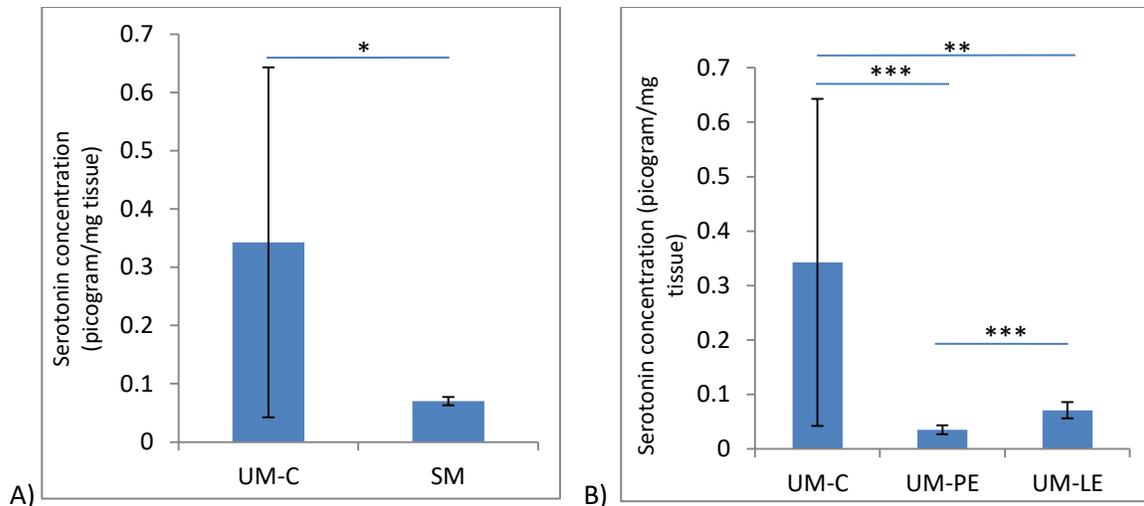
Figure 37. Concentration of serotonin in head tissue of cuttlefish hatchlings from maternal stress groups (A) and embryonic stress groups (B). * indicates < 0.05, ** < 0.01, ** < 0.001. n: UM-C = 15, SM = 13, UM-PE = 15, UM-LE = 14.

The ratio of serotonin to its main metabolite was calculated by dividing 5-HiAA concentration by 5-HT concentration (Figure 41A and B). SM offspring showed a significantly higher ratio than UM-C (exact permutation test; $p < 0.0001$; test stat = 25.68). Significant differences also existed between embryonic stress groups (Kruskal Wallis test; $p = 0.0069$; test stat = 10.03). UM-PE had significantly higher 5-HiAA/5-HT ratios than UM-C and UM-LE (post-hoc permutation test; $p = 0.003$ and 0.003).
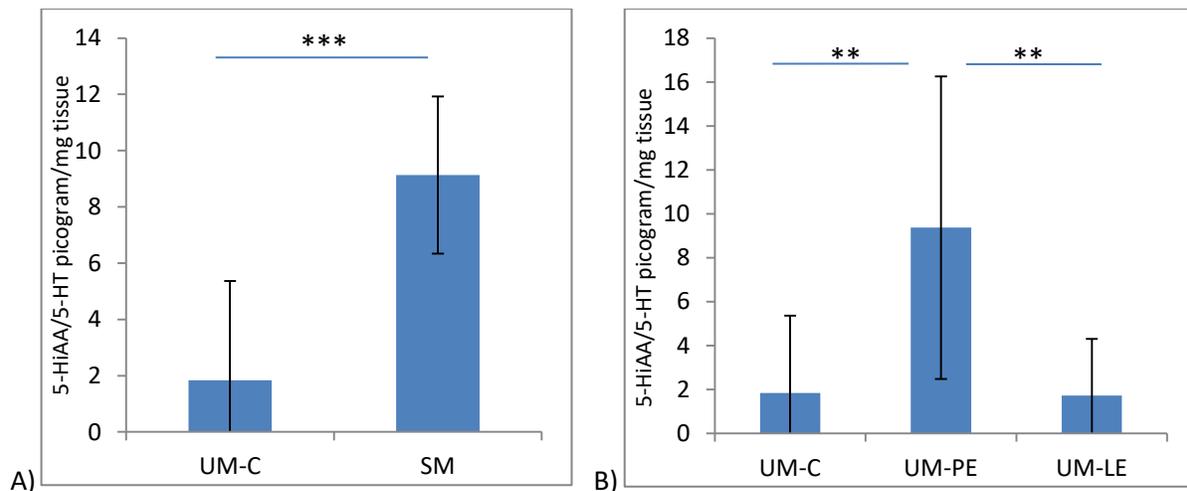
Figure 38. Ratio of serotonin to its main metabolite, 5-HiAA (5-HiAA/5-HT) in head tissue of cuttlefish hatchlings from maternal stress groups (A) and embryonic stress groups (B). * indicates < 0.05, ** < 0.01, ** < 0.001. n: UM-C = 14, SM = 13, UM-PE = 15, UM-LE = 13.

SM offspring showed significantly lower concentrations of noradrenalin (NA) than UM-C (exact permutation test; $p = 0.0492$; test stat = 6.164; Fig. 42A and B). There were also significant differences between embryonic stress groups (Kruskal Wallis; $p < 0.001$; test stat = 20.34). UM-PE had a significantly

lower NA concentration than both UM-C and UM-LE (post hoc permutation test; p = 0.002 and < 0.0001), while UM-LE showed significantly higher levels than UM-C (post hoc permutation test; p = 0.0494).
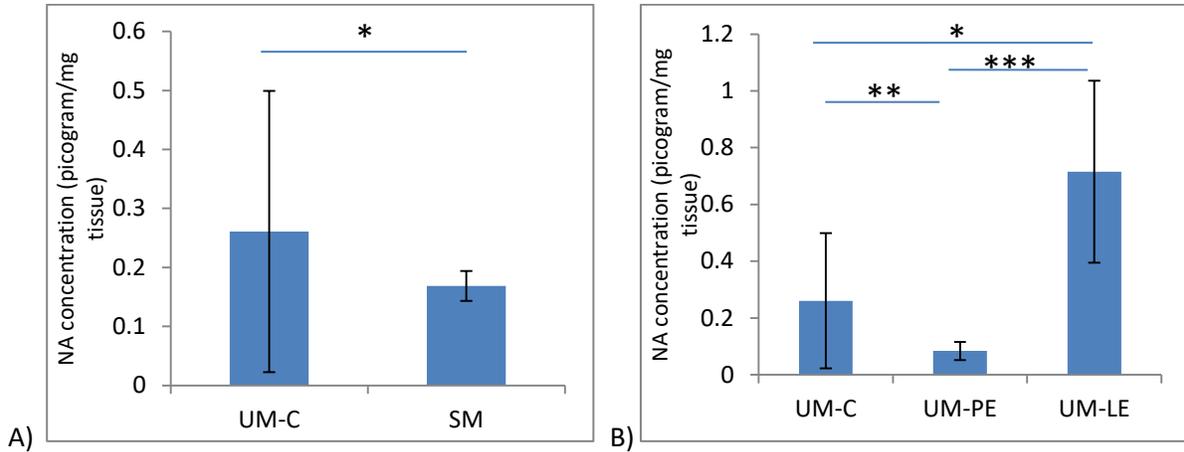
Figure 39. Concentration of noradrenalin (NA) in head tissue of cuttlefish hatchlings from maternal stress groups (A) and embryonic stress groups (B). * indicates < 0.05, ** < 0.01, ** < 0.001. n: UM-C = 15, SM = 13, UM-PE = 15, UM-LE = 15.

Volumetry and Growth

Two thirds of neurons in cuttlefish are contained in eight brachial lobes (Fig 43B), which control movement and innervation of the arms. The remaining third of neurons compose the brain, which is wrapped around the esophagus and located between the eyes (Fig. 43A). The esophagus divides the brain into the supraesophageal mass and the subesophageal mass (Fig. 43B). Each mass is further subdivided into anatomically and functionally distinct brain lobes (Fig. 43B and C). The subesophageal mass contains lobes mainly dedicated to low-level motor control (reviewed in Boycott 1961). The supraesophageal mass contains the higher motor centers along with associative and secondary sensory centers (reviewed in Dickel et al. 2013). Finally, the two large optic lobes project from the sides of the central mass.
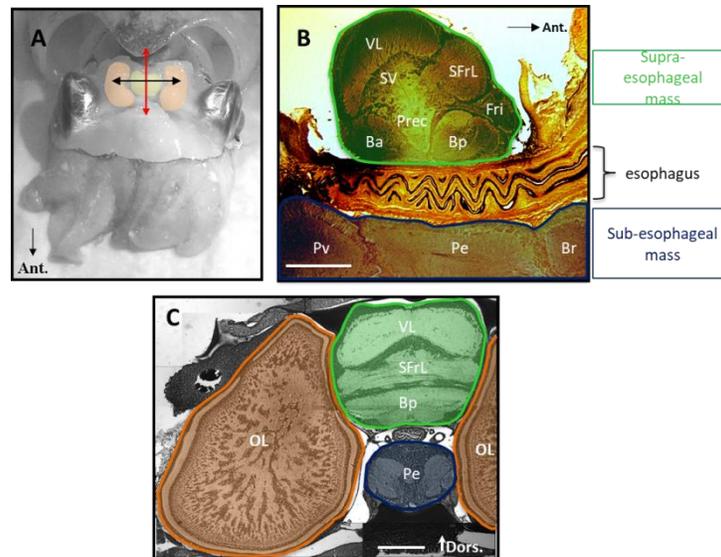
Fig. 40. The cuttlefish brain: Anatomical location (A) and sagittal (B) and frontal (C) histological sections of the cuttlefish brain with the various masses and lobes labeled. VL = vertical lobe, SV = subvertical lobe, SFrL = superior frontal lobe, Fri = frontal inferior lobe, Prec = precommisural lobe, Ba = anterior basal lobe, Bp = posterior basal lobe, Pv = palliovisceral lobe, Pe = peduncle lobe, Br = brachial lobe, OL = optic lobes. Adapted from (Bidel 2015).

The physiology of the cephalopod brain is fairly well-understood thanks to lesioning and electrophysiology experiments and particular tasks have been localized to each (Boycott 1961; Chichery and Chanelet 1976; Chichery and Chichery 1987; Graindorge et al. 2006). For our purposes, the most important lobes consist of the vertical lobe, optic lobes, superior frontal lobe and peduncle lobe. The vertical lobe is involved in learning and the inhibition of predatory behavior (Sanders and Young 1940). In partnership with the vertical lobe, the optic lobes play roles in motor control and visual memory while the frontal superior lobe aids in memory consolidation and storage (Véronique Agin, Chichery, and Chichery 2001). Finally, the peduncle lobe is involved in locomotion and the integration of equilibrium and visual information (Messenger 1967). Because of this anatomical knowledge, we can use the relative size of particular brain lobes as a proxy for the maturity of certain abilities. In particular, the growth of the VL has been correlated with increases in learning and memory as demonstrated by the PIT test (Dickel, Chichery, and Chichery 2001). In the weeks after hatching, this lobe increases in absolute volume and relative size in contrast to the supraesophageal mass as a whole (Fig. 44).
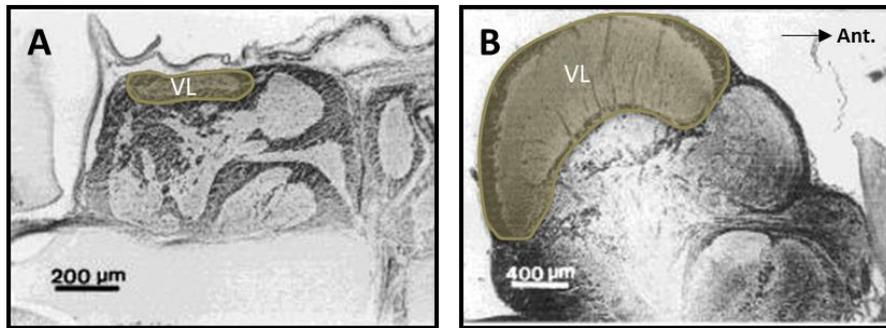
Fig. 41. Sagittal sections demonstrating that the vertical lobe (VL) of cuttlefish increases in both absolute and relative size compared to the rest of the supraesophageal mass from hatching (A) to adulthood (B). Adapted from Dickel et al. 2001 and Bidel, 2015.

In addition to volumetry, we utilized phosphohistone labelling, a method which identifies areas of cell division. Phosphohistone is produced by cells undergoing mitosis. By binding this molecule to a primary antibody, and then adding a dye which labels the primary antibody brown, we can quantify and localize dividing cells under a microscope (Fig. 45). Together, volumetry and phosphohistone labelling allow the comparison of relative neural growth and maturity between stress groups. Preparation of samples was performed by Nawel Mezrai, Celine Thomasse and Nadege Naud (EthoS, Universite de Caen. Measurements and cell counts were conducted by Estelle Paupy (Master's II, Université Jean-Monnet-Saint-Étienne).
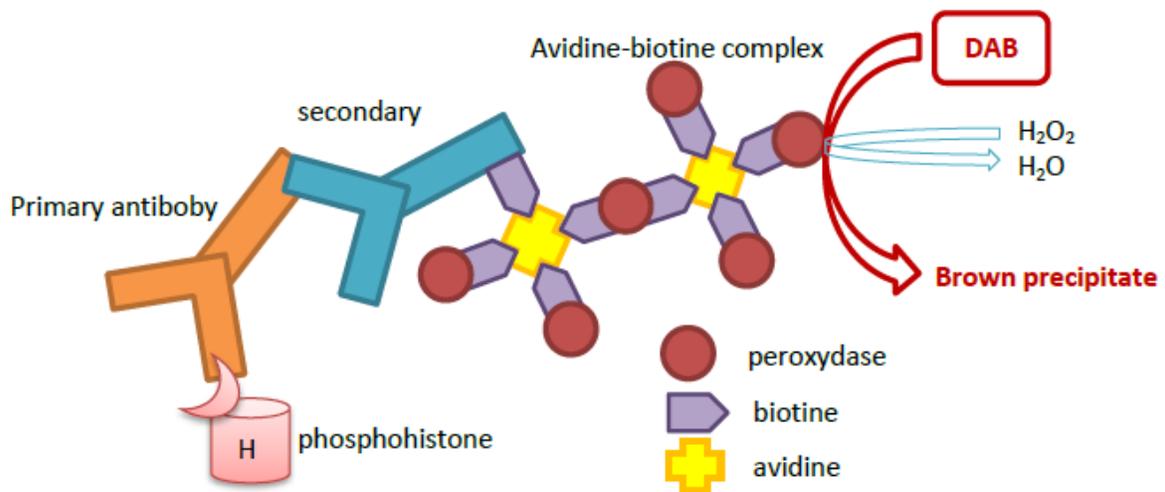
Fig. 42. An illustration of the chemical process of phophohistone H3 labeling. The primary antibodies bind with the phophohistone H3, while the secondary antibody binds with the primary antibody to form a brown precipitate visible under a microscope.

Materials and Methods

In 2015, cuttlefish were euthanized on the day of hatching with an anaesthetizing bath diluted with sea water (ethanol 1% and magnesium chloride 17.5 g.L-1). The cuttlefish were then placed in a 4%

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paraformaldehyde solution for 16 hours at 4°C. After 3 rinses with a phosphate buffer solution (PB0,1M pH 7.4) for 15 min each, samples were placed in the same buffer solution with 20% sucrose for 24hr at 4°C (cryoprotection stage). Specimens were then fixed entirely by inclusion in tissue-TeckTM and then frozen in a isopentane bath and liquid nitrogen. They then finally at -80°C until cross sectioning.

Frontal serial cross sections, 10µm thick, were made using a cryostat at -20°C (Leica® CM3050). Sections were placed on gelatine coated slides and frozen at -80°C until dyeing and labelling. Brains sections were stained with Toluidine blue (1L distilled water with 5g of Toluidine blue and 10g of sodium borate) for 20s, rinsed with water and then dehydrated with a series of ethanol baths (70%, 90%, 100% and 100%) followed by two baths of Roti®-Histol. Slip covers were mounted with a Roti®-Histokitt.

The volume of brain structures were measured using ImageJ ®. One section in four was analyzed for (1) vertical lobe volume/supraesophageal mass volume, (2) superior frontal lobe volume/ supraesophageal mass volume, (3) inferior frontal lobe volume/supraesophageal mass volume, (4) optic lobes volume/supraesophageal mass volume and (5) peduncle lobes volume/supraesophageal mass volume. Limits of the supraesophageal mass were defined anteriorly by the appearance of the inferior frontal lobe and posteriorly by the disappearance of the median basal lobe. The anterior and posterior limits of each lobe were defined by the appearance and the disappearance of the neuropil. The posterior limit of the superior frontal lobe was defined by the sudden fragmentation of the neuropil.

In preparation for phosphohistone analysis, brains sections were rinsed twice with a phosphate buffer solution (PB 0,1M pH 7.4) for 15 min each and then placed in a 3% BSA solution (in PB 0,1M with 0,01% azide). Antibodies are photosensitive, so the following preparation took place in a room with low energy (infrared) light. 300µL of primary antibodies against phosphohistone H3 were placed on each slide (diluted at 1:300 PB 0.1M, 1% BSA and 0.01% azide). The slides were incubated with the antibody solution at 4°C for 16hr in a humidity-saturated environment, then rinsed three times with buffer solution (PB 0.1M with azide), incubated in 0.3% H2O2 for 15min and then rinsed again. 300µL of secondary antibodies against the first antibody were placed on each slide (goat antibodies anti rabbit diluted at 1:300 PB 0.1M, 1% BSA and 0.01% azide) for 2hr. The slides were rinsed again and incubated for 30min with a A/B KIT (avidine-biotine peroxydase complex diluted at 1: 200 in PB 0.1M and azide, prepared 30min before the end of the last incubation). Lastly a diaminobenzidine (DAB) revelation (0.05g DAB for 100mL of TRIS HCl and 25µL of H2O2) was performed for 4 to 10min with regular visual inspection.

The slides were rinsed again and a counterstain with haematoxylin was performed in order to show cytoplasm and nuclei. Lastly the slides were rinsed with water and dehydrated with ethanol baths

(70%, 90%, 100% and 100%) and two baths of Roti®-Histol, and the slip cover was mounted with Roti®-Histokitt. The number of cells in each lobe colored by the chemical reaction was determined using an optic microscope.

Results

Volumetry

The total volume of each brain lobe was expressed as a percentage of total supraesophageal mass volume. Among maternal stress groups, only UM-LE showed a sig diff between left and right peduncle lobes, so only left lobes used for group comparisons of optic and peduncle lobes (Fig. 46). Among maternal stress groups, SM had significantly smaller VL than UM-C (permutation test; $p = 0.0264$). Significant differences also existed among embryonic stress groups (Kruskal Wallis; $p = 0.0075$), with UM-PE and UM-LE having significantly smaller VLs than UM-C (post-hoc permutation tests; $p < 0.05$).
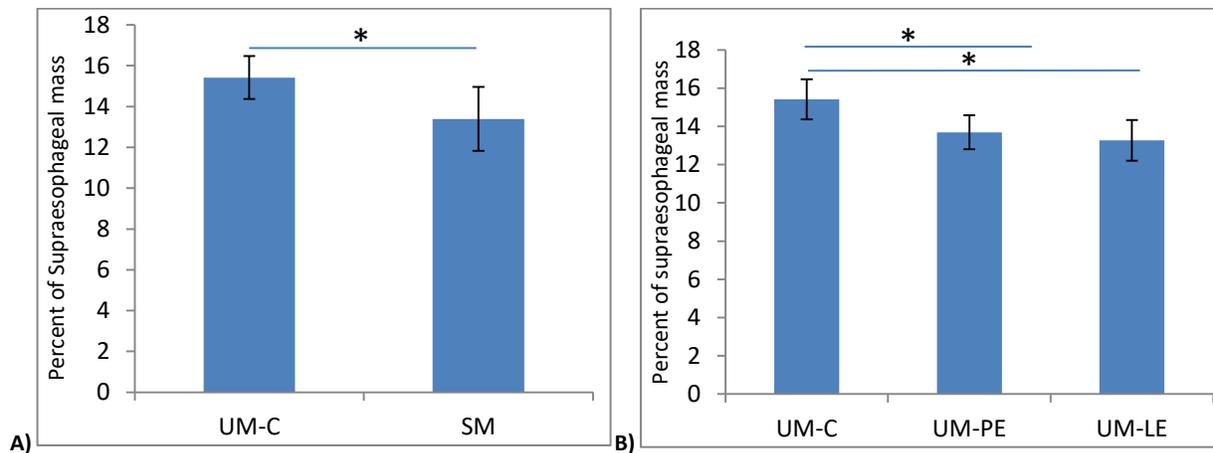
Fig 43. Vertical lobe volumes of maternal stress groups (A) and embryonic stress groups (B) expressed as a percentage of subesophageal mass. n = 6 for all groups.

Phosphohistone

Phosphohistone labeling was expressed as an index of density, with higher values indicating greater, more intense cell division. No group showed significant different between right and left optic and peduncle lobes in terms of density of cell division, so only the results of the left lobes are presented and compared. Among maternal stress groups, SM had more cell division in the LOL and VL than UM-C (permutation tests, $p = 0.0172$ and 0.0246; Fig. 47). No other significant differences existed between groups.
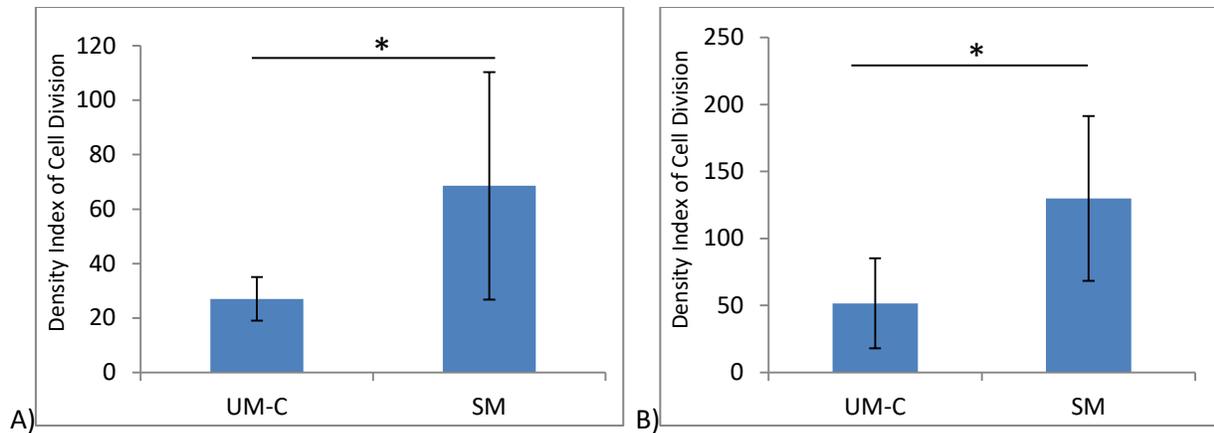
Fig 44. Cell division in VL (A) and LOL (B) of maternal stress groups indicated by phosphohistone labelling. n = 6 for all groups.

Discussion

Monoamines:

All stress groups (SM, UM-PE and UM-LE) showed significantly lower serotonin concentrations than the control group. At the same time, the turnover rate of serotonin (5-HiAA/5-HT) was significantly lower in SM and UM-LE than in controls, but significantly higher in UM-PE. A higher ratio of serotonin to its main metabolite indicates higher turnover (Bidel 2015), meaning a faster rate of serotonin synthesis. In cephalopods, serotonin acts as a neurotransmitter, with motor and chromatic effects, as well as an important role in synaptic transmission and long-term potentiation (LTP) induction (Messenger 1996; Shomrat et al. 2010). Thus, the lower serotonin concentrations measured in all the stress groups, plus the lower turnover rates in SM and UM-LE could be signs of neurological deficits. The higher serotonin turnover rate observed in UM-PE is similar to that observed in rats whose mothers were stressed with crowding and injections (Hayashi et al. 1998). In addition to these stress-induced changes in serotonin, noradrenaline (NA) concentrations were significantly lower in SM and UM-PE and significantly higher in UM-LE than in controls. In cephalopods, NA is associated with faster respiration and higher levels of awareness and cognition (Messenger 1996; Bidel 2015). Thus, the differences between groups might be indicative of differing levels of "alertness" due to prenatal stress, with UM-LE on high alert and the other stress groups at a reduced level of alertness.

Together, the differences in serotonin concentration, the rate of serotonin turnover and noradrenaline concentrations indicate that prenatal stress affects the chemistry of the hatchling cuttlefish brain. Although our assays are good indicators of differences between stress groups, they were whole-brain analyses, and as monoamines have different effects depending on location (e.g. central or peripheral nervous system, particular brain lobes), it is difficult to draw firm conclusions about

how the differences observed here affect the cuttlefish nervous system. However, in the future, more precise techniques, such as HPLC-ECD (Bidel, Corvaisier, et al. 2016) may allow us to localize monoamine levels in specific regions of the brain.

Volumetry and Neurogenesis:

Measurement of the various brain lobes in relation to overall brain size revealed significantly smaller vertical lobes (VL) in all of the stress groups in comparison to the control group. At the same time, phosphohistone staining showed higher neurogenesis (i.e. growth) in the optic lobes (OL) and VL of SM. The VL and the OL are seats of learning and memory in cephalopods, and their relative size and growth is correlated with learning and memory (Dickel, Chichery, and Chichery 2001). The smaller size of VLs in all stress groups suggests that they are cognitively-compromised at hatching in comparison to the control group. In addition, the reduction in neurogenesis in the VL and OL of SM suggests that this group was further neurodevelopmentally-delayed. This would be consistent with numerous studies in rats which find maternal stress associated with reductions in neurogenesis (e.g. Weinstock et al. 1992; Lemaire et al. 2000; Van den Hove et al. 2006). It would also be consistent with the reductions in serotonin and noradrenaline seen in certain stress groups, both of which are associated with mitosis (Bidel 2015). Oddly though, these results contrast with the overall lack of learning differences in the PIT test, where we would expect lower performance in animals with smaller VLs. Indeed, the growth of the VL has been directly correlated with the emergence of predatory pursuit behavior as well as learning and memory performance in the PIT test (Dickel, Chichery, and Chichery 2001a, 1997a). One potential explanation for this is that the brain samples were taken at eclosion, and the differences seen then may have disappeared after several weeks. Whatever the case, the higher neurogenesis in the VL and OL of SM suggests that stress passed from mother to offspring has a stronger effect on VL size and growth than embryonic stress.

VI. Chapter summary

The behavioral experiments, learning tests and neurobiological assays outlined in this chapter demonstrate that both maternal and embryonic stress affect certain aspects of cuttlefish biology and behavior but not others (Fig. 48). The existence of both behavioral and neurobiological differences in maternal and embryonic stress groups demonstrate that stress can be both transferred from mother to offspring and experienced directly by the embryos themselves. The overall lack of learning and memory differences was unexpected given the effects of prenatal stress in other species and the differences in VL size at hatching. Furthermore, the natural stressor was only associated with differences in monoamines and vertical lobe size. This contrasts with the artificial stressor, which ostensibly seemed to benefit the cuttlefish in terms of predatory behavior and learning at three weeks, as well as altering camouflage, VL volume and monoamines. This was also unexpected, given that we had predicted that the natural stressor would have positive effects on fitness, and the artificial stressor mostly negative effects. However, the designation of the effects as "positive" or "negative" is tenuous at best, and deserves further examination.
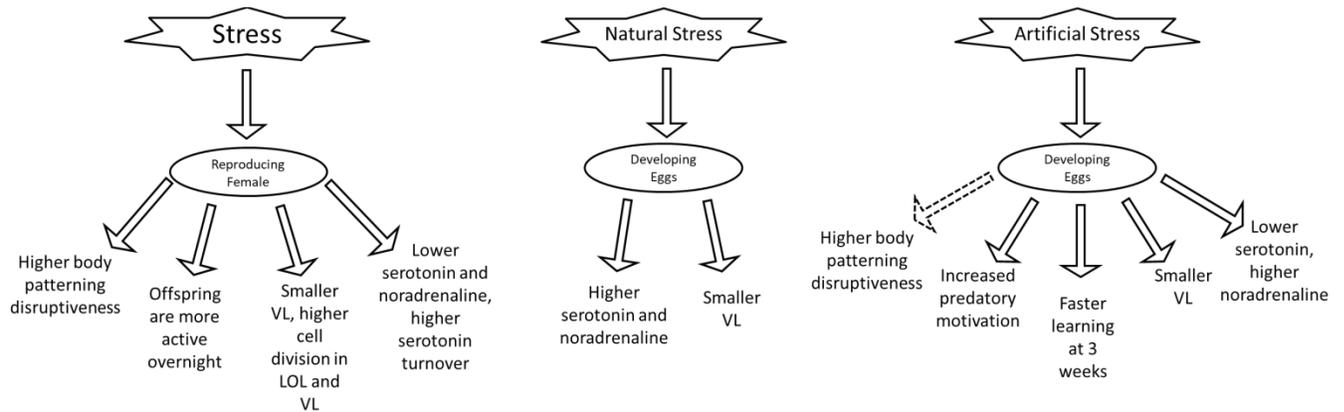
Fig. 45. Schematic summary of Chapter 2 illustrating the effects of the three stressors tested. The dashed arrow indicates a statistical tendency.

Chapter 3: Related Experiments

Chapter 3: Related Experiments

Captivity and/or the lack of environmental enrichment could also be considered as a form of stress. Dickel showed that hatchling rearing environment affects learning and growth (Dickel, Boal, and Budelmann 2000b). Likewise, prenatal rearing environment might have similar effects.

I. Article #5: "The effect of an artificial incubation environment on hatchling size and behavior in the cuttlefish, *Sepia officinalis*"

This manuscript outlines the results of an experiment which investigated whether embryonic development in the lab results in offspring that differ from those that develop in the wild.

THE EFFECT OF AN ARTIFICIAL INCUBATION ENVIRONMENT ON HATCHLING SIZE AND BEHAVIOR IN THE CUTTLEFISH, *SEPIA OFFICINALIS*

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A great deal is known about development in *Sepia officinalis*; however, much of this knowledge comes from animals incubated in laboratory conditions. Since cuttlefish are behaviorally plastic and known as embryos to perceive environmental stimuli from within the egg, we wondered if they are affected by incubation environment and thus whether laboratory-incubated cuttlefish exhibit natural behavior. We investigated the effects of incubation environment on hatchling size, defense and predation behavior in *Sepia officinalis* by comparing artificially-incubated hatchlings to naturally-incubated ones. Contrary to our expectations, no significant differences were apparent in hatchling size, disruptive body patterning and predation. A significant difference did exist between groups in one type of body patterning: Artificially-incubated hatchlings appeared to be better at producing a uniform body pattern than naturally-incubated individuals, possibly due to their prenatal experience with a homogeneous artificial substrate. This difference suggests some caution when interpreting experiments utilizing laboratory-incubated hatchlings, but overall, the limited effect of artificial incubation conditions demonstrated in this experiment bolsters confidence in previous behavioral results. These results are also promising for cuttlefish culture; eggs that would otherwise be lost as bycatch could instead be cultured artificially.

Keywords: Embryonic experience, behavioral plasticity, body patterning, predation, cuttlefish culture

Introduction:

        Sepia officinalis (Linnaeus 1758) rely heavily on both body patterning and predation skills for survival and growth. Cuttlefish skin has both chromatic and textural components that allow it to change appearance and effect crypsis. Cryptic strategies include background matching (general color resemblance or variable color resemblance), disruptive coloration and deceptive resemblance (Cott 1940; Hanlon and Messenger 1998). Though a wide range of body patterns are possible, most work has focused on the two extremes of the spectrum: the uniform pattern, in which homogeneous color and brightness are present over the entire body, and the disruptive pattern, in which the cuttlefish projects many small, irregular, disparate pieces in a way that breaks up the outline of the body (Cott 1940; Boycott 1965). On uniform substrates such as sand or mud, the uniform pattern is generally thought to be employed in background matching, while the disruptive pattern can be used for both background matching (variable color resemblance) on a variegated substrate or as a way to occlude body shape. (However, these patterns may also function in deceptive resemblance, a possibility which will be explored in the discussion.) Cuttlefish are also voracious hunters of shrimp, using vision to target their prey (Wells 1958). They have two methods of attack: a rapid tentacular strike for shrimp, fish and small crabs and a pouncing maneuver for large crabs (Messenger 1968; Duval, Chichery, and Chichery 1984; Chichery and Chichery 1988). While effective prey capture is essential for obtaining food, foraging and prey capture can potentially put a cuttlefish at risk of detection by predators. Thus, both body patterning and predation may be subject to behavioral plasticity.

        Of the 30 stages of development currently recognized in cuttlefish embryos (Lemaire 1970), response to touch and odors is possible by the 23rd (Romagny et al. 2012). 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 (Paulij, Bogaards, and Denucé 1990; Anne-Sophie Darmaillacq, Lesimple, and Dickel 2008c; Romagny et al. 2012). Thus, during the last weeks of embryonic development, cuttlefish embryos are able to perceive the outside world, and may learn and modify their behavior in response. As one example, stage 30 embryos exposed to bright light six times for three minutes at a time over the course of a few hours decreased their behavioral response (measured by mantle contraction) over time, demonstrating habituation to the stimulus (Romagny et al. 2012). Similarly, cuttlefish exposed to a non-preferred prey species (crabs) during the last week of development showed a preference for this prey item after hatching (Darmaillacq, Lesimple, and Dickel 2008; Mathieu Guibé, Boal, and Dickel 2010). Finally, prenatal cues from potential predators influence the strength of brain lateralization in cuttlefish hatchlings (Jozet-Alves and Hebert 2012), a trait

associated with more efficient information-processing in vertebrates (see Jozet-Alves et al. 2012 for discussion). These learning abilities and behavioral plasticity have tremendous potential advantages for young cuttlefish by allowing embryos to adapt to their post-hatching environment before eclosion. For instance, knowledge of the presence of a predator with a particular predation strategy could allow cuttlefish to prioritize the development of suitable defense strategies. Indeed, older cuttlefish discriminate between predators and employ targeted defensive strategies in response to different species (Langridge 2009).

Young cuttlefish are behaviorally plastic. Previous experiments with hatchlings (up to one week old) and juveniles (up to 17 weeks) (Hanlon and Messenger 1988) have demonstrated behavioral plasticity in response to limited enrichment of a laboratory setting with sensory stimuli during the post-embryonic period. For instance, sensory enrichment of a bare tank with a sandy substrate, rocks, shells, artificial algae and the presence of conspecifics is associated with faster growth and memory maturation during the first three months of life (Dickel, Boal, and Budelmann 2000c). Likewise, experience with a sandy substrate soon after hatching results in young that are better at burying in the following days (Poirier, Chichery, and Dickel 2004). It is also worth noting that the brightness of artificial rearing tanks has been shown to affect growth, with a dark (black) background associated with the highest growth (António V. Sykes et al. 2011). Extending the logic of this work to the pre-hatching period, we hypothesized that experiencing an artificial environment during the prenatal period could affect hatchling behavior as well.

With a few exceptions, knowledge about early behavior in cuttlefish comes from experiments on animals hatched in the laboratory (*e.g.* (M. J. Wells 1958; John B. Messenger 1968; Dickel, Boal, and Budelmann 2000c; R. Poirier, Chichery, and Dickel 2004; Chiao, Kelman, and Hanlon 2005; Roseline Poirier, Chichery, and Dickel 2005; C. Jozet-Alves and Hebert 2012), a fact not always indicated by the authors. However, little research attempting to quantify the effects of artificial incubation on behavior in *S. officinalis* exists. This lack of knowledge regarding the effects of artificial incubation is problematic for our ability to generalize conclusions based on the results from artificially-incubated individuals to "natural" behavior. Research with other species and with *Sepia* suggest that a (post-embryonic) laboratory situation can profoundly affect behavioral traits and that behavioral results often diverge between different laboratories due to variance in environmental parameters and experimenters (Dickel, Boal, and Budelmann 2000c; Chesler et al. 2002; R. Poirier, Chichery, and Dickel 2004; Lewejohann et al. 2006; António V. Sykes et al. 2011). This can result directly from differences in experimental protocol or

genetics, but also via an interaction between genotype and laboratory environment (Chesler et al. 2002).

Given the highly-developed brain and behavioral plasticity of cuttlefish (*e.g.* (Dickel, Boal, and Budelmann 2000c; R. Poirier, Chichery, and Dickel 2004; Véronique Agin et al. 2006), coupled with the embryo's ability to perceive, and be influenced by, the world outside the egg (Romagny et al. 2012), it seems likely that artificial conditions during embryonic development may affect the behavior of hatchling *S. officinalis.* If so, it could cause their behavior to differ from animals developed under natural conditions. In that case, experiments on cuttlefish reared in an artificial environment might yield unrealistic behavioral results. The natural environment provides numerous sources of sensory stimulation not present in most laboratory settings. For cuttlefish, these could include natural light, a natural light cycle, water-borne odors from numerous other organisms, water movement (currents, tidal rhythm, etc.) and fluctuations in temperature. In contrast, cuttlefish eggs in most artificial situations are exposed to more stable physical parameters in their environment such as temperature and light, a lack of certain natural stimuli (including currents and organisms such as epibionts and plankton), but a surfeit of unnatural ones (including unnatural color schemes and anthropic handling). No study has directly addressed whether incubation in a standard laboratory setting would have a detectable impact on basic hatchling behavior.

In addition to corroborating or casting doubt on existing experimental results, this question has implications for cuttlefish culture. Female *S. officinalis* lay their eggs on vertical objects on the seabed including seagrasses and algae (Basuyaux and Legrand 2013), as well as basket traps set by commercial fishermen to capture adult cuttlefish. Each year, thousands of cuttlefish eggs are laid on such traps in the English Channel, and when these traps are retrieved, the eggs are discarded, damaged or destroyed (Blanc and Daguzan 1998). Potentially, these eggs could be saved and cultured artificially. Indeed, several authors (*e.g.* Pascual 1978; Forsythe, DeRusha, and Hanlon 1994; Domingues, Sykes, and Andrade 2002) have already managed to culture multiple generations in the laboratory. This raises the possibility of redirecting a normally squandered resource for later release, harvest or experimentation. An assessment of the effects of an artificial incubation environment on some basic behaviors necessary for growth and survival is a critical first step in the pursuit of this possibility.

Our experiments aimed to evaluate the reliability of existing results obtained with cuttlefish incubated *ex situ* and their chances of survival in the first week by quantifying the effect of laboratory incubation on the subsequent hatchling behavior of this species. In order to assess the effect of an artificial incubation environment, we tested hatchling size (one measure), body patterning behavior

(three measures) and predatory behavior (five measures) of hatchlings incubated in the sea and in the lab. These nine measures represent only a small fraction of the behavioral tests possible (other potential assays include prey preference trials, reaction to predator odor and the Prawn-in-a-Tube procedure) but our goal was to assess behavior directly relevant to hatchling survival. These data will help us to assess the validity of prior experimental results obtained from hatchlings incubated in the laboratory and add to our knowledge about cuttlefish culture.

Methods:

Egg Collection and Treatment:

     Sepia officinalis eggs (135) were recovered on May 16, 2014 by a professional diver (OB) from a pre-placed tether at a depth of 3m in the area of Pointe d'Agon (48° 59.547 N - 1° 38.671 W, English Channel) and taken to the Synergie Mer et Littoral (SMEL), a marine research facility in Blainville-sur-Mer, France. These eggs were in the very early stages of embryonic development (<15 days old) and were designated as the artificially-incubated treatment group ("Lab"). At the same time, 150 eggs from the same location were left *in situ* and designated as the naturally-incubated treatment group ("Sea"). "Lab" eggs were placed in a polyethylene basket (29cm x 19cm x 14.5cm) and immersed in an aerated 200L tank matched to local sea surface temperature (14.5°C-18.9°C). Temperature affects the rate of embryonic growth and yolk consumption, as well as the ultimate size of the hatchling (Boletzky 1983, Bouchaud 1991). (Though regional surface sea temperature was monitored throughout the experiment, no local temperature data are available for the eggs left *in-situ*.) Eggs were exposed to artificial illumination from 08h00 to 18h00.

     As the estimated date of hatching approached (based on the technique of accumulated degree days from Basuyaux (2011)), "Sea" eggs were monitored by a professional diver before being collected June 26, 2014 and transported to the laboratory, 7-12 days before the dates of hatching. "Sea" embryos were estimated (Romagny et al. 2012) to be between stages 26 and 30 when collected. After "Sea" egg collection, both "Lab" and "Sea" eggs were placed in separate hatching tanks (90liters, 720 x 470 x 360mm, opaque white) with 100% daily water renewal (18.9°C-19.2°C).

     Hatching occurs mostly at night (Paulij et al. 1991), and hatchlings were collected at 08h00 each morning. (The morning after hatching was designated as "Day 1".) Hatchlings were placed in individually-labeled vials (diameter=4.5cm, height=6.4cm) with numerous perforations for water flow. Cuttlefish remained in these floating tubes in their natal treatment tank until testing. Individual identity was maintained throughout the study. Experiments did not begin on a treatment group until at least

three hatchings had occurred in a single night. All experiments were conducted using water (19.2°C) from hatchlings' natal tank. In total, 47 individuals from the "Sea" group (hatched July 4-7) and 51 from the "Lab" group (hatched July 7-10) were tested.

Mantle Length:

Immediately prior to their first encounter with prey, mantle length (ML) was estimated in order to compare growth between the two incubation conditions. Day 4 was chosen rather than Day 1 as it reflects the consumption of most or all of the embryonic yolk reserve prior to any prey ingestion (Boletzky 1983). To estimate cuttlefish ML, two photographs were taken in which the cuttlefish was lying flat on the bottom and not moving. Using the image analysis software ImageJ, the distance between the tip of the mantle and a point midway between the eyes was measured. Eyes were used instead of the mantle edge since they were easily-identifiable in photographs. The two measurements were averaged, unless they deviated by more than 0.2 cm from each other, in which case a third photograph and measurement were made. The most disparate of the three values was eliminated and the resulting pair of values was averaged.

Behavioral Tests:

Uniform Background Test:

Testing:

On the morning after hatching (Day 1), hatchlings were selected in a randomized order for testing. Between 9h00-10h30, six cuttlefish were placed individually in small (diameter=100cm, height=1cm) uniform medium gray (Mean Gray Value (MGV)=101+/-3.9) arenas and filmed concurrently for 22min. This was repeated until all of the day's hatchlings had been tested. The arenas were lit indirectly with two Zenitech 0221A 500W lights (540lux) mounted on tripods and filmed with a Panasonic HDC-SEM60 camera. After filming, cuttlefish were returned to their individually-labeled tubes in their natal tanks.

Video Analysis:

Using VLC Media Player, two snapshots were captured of each individual at 11 and 21min, allowing the cuttlefish time to habituate to the experimental arena. Snapshots were only taken during moments after cuttlefish had settled and were motionless. Following the method developed by (Di Poi et al. 2014a), we used ImageJ to select the outline of the cuttlefish mantle and measure the

Heterogeneity Index (HI). This value was calculated in ImageJ by an equation using the deviation of the MGV of each individual pixel (x) from the MGV of the whole cuttlefish ($\bar{x}$), and the total number of pixels selected (N): $\mathrm{HI} = \sqrt{\frac{1}{N}\sum(x-\bar{x})^2}$ (Di Poi et al. 2014a). This value measures body pattern uniformity (higher HI=less uniform). The two values were averaged for each individual.

We also used ImageJ to compare the MGV of the cuttlefish to the MGV of the substrate. After selecting the outline of and measuring the MGV of the cuttlefish mantle, we then measured an equivalently-sized portion of the adjacent substrate. By dividing the mantle MGV by the substrate MGV, we were able to calculate a ratio from 0 to 1 expressing the degree of match between the mantle and the substrate (0=no match, 1=perfect match). The two values were averaged for each individual and group medians calculated. A low HI and high MGV match indicate a good match between mantle appearance and the surrounding substrate.

Disruptive Background Test:

Testing:

Using the same set-up and procedure as for the Uniform Background test, cuttlefish were filmed individually on Day 2 for 25min against a "checkered" background (squares=3x3mm) between 10h30 and 12h00. After filming, cuttlefish were returned to their individually-labeled tubes in their natal tanks.

Video Analysis:

Using VLC Media Player, two snapshots were captured of each individual at 15 and 22min. In order to assess disruptiveness, we employed the procedure developed by Barbosa et al. (2007), and graded 11 components of body pattern on a scale of 0-3 based on relative strength of expression. This resulted in a "Disruptive Score" that ranged from 0 to 33 (higher score=more disruptive). The two values were averaged for each individual.

Initial Prey Encounter:

Shrimp Collection:

Shrimp (*Crangon crangon* and *Palaemonetes* sp.) were collected as needed in the vicinity of Blainville-sur-Mer via hand nets in small pools during low tide. They were maintained in aquaria with well-oxygenated water for one to five days prior to testing. Only shrimp between 0.7 cm and 1.4 cm

were selected for predation experiments. One to four hours before testing, shrimp were collected from the larger aquaria and placed in smaller individual containers until the moment of testing.

Testing:

Cuttlefish were tested four days after their date of hatching (between 12h00 and 18h00), from July 7 to 13, 2014 and were not fed prior to testing. Four cuttlefish were tested at a time in individual containers (10.5 x 12 x 5.4cm) filmed by a Canon IXUS camera positioned 40-43cm away and lit indirectly with a Zenitech 0221A max 500 W (500 Lux, light Meter Testo). Testing occurred in 140mL of water from each cuttlefish's respective hatching tank at temperatures equal or slightly above hatching tank temperature (18.3-19.5°C). Cuttlefish were gently removed from their hatching tank in their vials, transported to the testing arena and transferred into their respective container using a spoon. They were allowed to acclimate in the arena for five min before testing. At that point, video recording commenced, and shrimp were poured from small tubes into the testing arena. Video recording was stopped after five min, at which point both shrimp and cuttlefish were removed from the arena and cuttlefish were transferred back into their respective vials and transported back to their hatching tank.

Video Analysis:

Videos were analyzed using VLC Media Player and ImageJ software. Several variables were recorded: Attempted Capture Rate, Capture Rate, Success Rate, Latency to Capture and Distance of Detection. Capture Rate measured the percent of cuttlefish from each group that captured their shrimp. Success Rate was calculated as the percentage of total capture attempts that resulted in a successful capture for each group (failed attempts at capture were defined as tentacle extension without successfully subduing the shrimp). In instances in which the shrimp was successfully captured, we calculated Latency to Capture as the time between shrimp detection [defined as the moment that the cuttlefish oriented towards the shrimp (Messenger 1968)] and successful capture (defined as the moment the cuttlefish's tentacles touched the shrimp and successfully subdued it) for each group. Finally, for Distance of Detection, a snapshot was taken (using VLC Media Player) at the moment of detection and the distance between the shrimp and nearest cuttlefish eye was measured with ImageJ software. Together, these five variables are indirect measures of feeding motivation, visual acuity, cautiousness and overall predation ability.

Statistical Analysis:

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R and StatXact®7 (Cytel Studio$^{©}$) were used to conduct all statistical analyses. Data analyzed were ML, HI, Disruptive Score, Percentage of Cuttlefish that Captured Shrimp, Success Rate (total number successful captures/total number of attempted captures), Latency to Capture and Distance of Detection. The ML met parametric assumptions (Shapiro Wilk, α=0.05, p>0.05), so data are reported as means +/- SEM and a T-test was used to compare the two groups. All other data failed to meet parametric assumptions (Shapiro Wilk, α=0.05, p<.0001), so data are reported as medians +/- SEM and non-parametric Fisher Exact tests and permutation tests were employed.

Ethical Considerations:

All procedures were carried out in accordance with the Directive of the European Parliament and of the Council of the European Union (2010/63/UE) regarding the care and use of animals for experimental procedures, and approved by the regional ethical committee (Comité d'Ethique NOrmandie en Matière d'EXpérimentation Animale, CENOMEXA; agreement number 54) (cuttlefish: project authorization on September 25, 2014). Experiments were supervised by several individuals (ASD, CJA, LD and OB) certified to work with cephalopods.

Results:

Mean Mantle Length on Day 4:

Mean ML on Day 4 of "Sea" cuttlefish (n=47) was 1.04cm (+/- 0.01cm SEM) and 1.03cm (+/- 0.01cm SEM) for "Lab" cuttlefish (n=51) with no significant difference between groups (T-test, α=0.05, p=0.28).

Uniform Background Test:

Heterogeneity Index:

Median HI was 7.12 (+/- 0.22 SEM) for "Sea" (n=51) and 8.21 (+/- 0.30 SEM) for "Lab" (n=47) (Fig. 49a). A higher HI indicated a more disruptive body pattern. A permutation test showed that "Sea"-incubated cuttlefish had significantly lower* HI (a more uniform* body pattern) than "Lab"-incubated cuttlefish (permutation test, α=0.05, p=0.02).
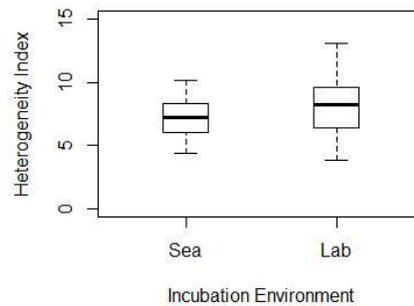
Fig. 46a Heterogeneity Index (HI) against a uniform background on Day 1 reflects the ability to produce a uniform body pattern. Data are displayed as median (bars), inter-quartiles (boxes) and minimum/maximum values (whiskers). Lab-incubated cuttlefish show more disruptiveness (higher HI) than Sea-incubated* (permutation test, n=47, 51, alpha=0.05, p=0.02).

*modified from error in original article.

Mean Gray Value match:

Median MGV match was 0.79 (+/- 0.06 SEM) for "Sea" (n=51) and 0.78 (+/- 0.06 SEM) for "Lab" (n=47) (Fig. 49b). A higher MGV match indicated better correspondence to substrate color. A permutation test showed no significant difference between groups (permutation test, α=0.05, p=0.95).

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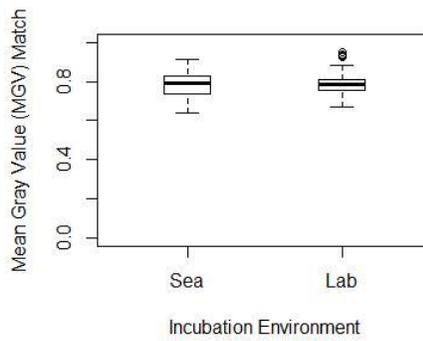
Fig. 46b Mean Gray Value (MGV) match to a uniform background on Day 1 reflects the ability to produce general color resemblance. Data are displayed as median (bars), inter-quartiles (boxes), minimum/maximum values (whiskers) and outliers (circles). There is no significant difference between groups (permutation test, n=47, 51, alpha=0.05, p=0.95).

Disruptive Background Test:

Disruptive Score:

Median Disruptive Score (out of 33) was 9.50 (+/- 0.90 SEM) for "Sea" (n=51) and 8.37 (+/- 0.74 SEM) for "Lab" (n=47) (Fig. 50). A higher Disruptive Score indicated a more disruptive body pattern. A permutation test showed no significant difference between groups (permutation test, α=0.05, p=0.77).
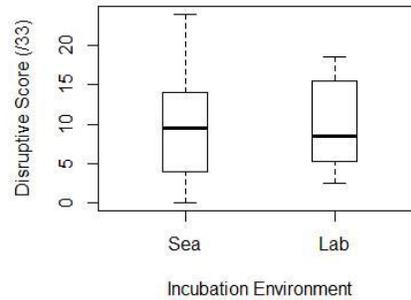
Fig. 47 Disruptive Score (/33) against a checkered background over time on Day 2 reflects disruptive body-patterning ability. Data are displayed as median (bars), inter-quartiles (boxes) and minimum/maximum values (whiskers). There is no significant difference between groups (permutation test, n=47, 51, alpha=0.05, p=0.77).

Initial Prey Encounter:

Attempted Capture Rate:

Thirty-one of 47 (66%) of "Sea"-incubated hatchlings and 37 of 51 (73%) "Lab"-incubated hatchlings attempted shrimp capture with no significant difference between groups (Fisher Exact test, α=0.05, p=0.52) (Table 12).

Table 12. Attempted Capture Rate, Capture Rate and Success Rate during the initial encounter with prey on Day 4. No significant difference exists between treatment groups in these tests (Fisher Exact test, n=47, 51, alpha=0.05).

	Definition	Sea	Lab	p
Attempted Capture Rate	percentage of cuttlefish that attempted captured	66.00%	73.00%	0.52
Capture Rate	percentage of cuttlefish that captured shrimp	61.70%	70.60%	0.40
Success Rate	the percentage of successful captures	87.90%	90.00%	0.99

Capture Rate:

Twenty-nine of 47 (61.7%) of "Sea"-incubated hatchlings and 36 of 51 (70.6%) "Lab"-incubated hatchlings captured their shrimp during the first five minutes of their initial prey encounter with no significant difference between groups (Fisher Exact test, α=0.05, p=0.40) (Table 1).

Success Rate:

"Sea"-incubated cuttlefish (n=29) made a total of 33 attempts to capture shrimp, of which 29 (87.9%) were successful. "Lab"-incubated cuttlefish (n=36) made a total of 41 attempts to capture shrimp, of which 36 (90%) were successful, with no significant difference between treatment groups (Fisher Exact test, α=0.05, p>0.99) (Table 1).

Latency to Capture:

An extreme outlier (>120sec) was removed from each group prior to analysis. The median latency between detection and attack was 5.5sec (+/- 2.41sec SEM) for "Sea" (n=28) and 5sec (+/- 1.36sec SEM) for "Lab" (n=35) with no significant difference between groups (permutation test, α=0.05, p=0.08) (Fig. 51).
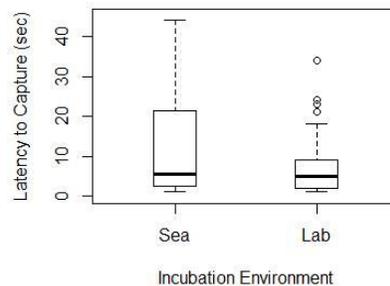
Fig. 48a Latency to Capture (sec) is the time between detection and capture on Day 4. One extreme outlier was eliminated from each group before analysis. Data are displayed as median (bars), inter-quartiles (boxes), minimum/maximum values (whiskers) and outliers (circles). No significant difference exists between treatment groups (permutation test, n=28, 35, alpha=0.05, p=0.08).

Distance of Detection:

The median latency between detection and attack was 4.31cm (+/-0.50cm SEM) for "Sea" (n=29) and 3.24cm (+/- 0.49cm SEM) for "Lab" (n=36) with no significant difference between groups (permutation test, α=0.05, p=0.65) (Fig. 51b).
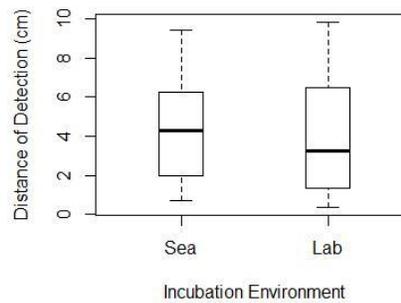
Fig. 48b Distance of detection (cm) on Day 4. Data are displayed as median (bars), inter-quartiles (boxes) and minimum/maximum values (whiskers). No significant difference exists between treatment groups (permutation test, n=29, 36, alpha=0.05, p=0.65).

Discussion:

We investigated the effects that an artificial incubation environment has on subsequent hatchling size and behavior. Contrary to our expectations, we saw only one difference between "Sea" and "Lab" groups in nine measures of size, body patterning and predation, suggesting that artificial incubation conditions had little effect on rates of development and subsequent hatchling behavior.

Mantle length estimated on Day 4 represents embryonic and hatchling growth determined exclusively by yolk reserves. Water temperature can have dramatic effects on the pace and duration of embryonic growth, which in turn affects the rate of yolk consumption, feeding motivation and predatory behavior. Higher temperatures accelerate growth and yolk absorption, resulting in shorter development time (which can vary as much as 40 to 90 days) but also in smaller hatchlings (Boletzky 1983; Olivier Bouchaud 1991b). Temperature is also associated with differences in the emergence of hatchling pursuit behavior (Dickel, Chichery, and Chichery 1997). While the "Lab" cuttlefish in our experiment (and in typical artificial environments) experienced steady water temperatures, it is likely that the thermal regimes experienced by the "Sea" group included frequent fluctuations due to currents and tides. Because of this more complex temperature regime, we had expected that "Sea" hatchlings would differ in size from "Lab" cuttlefish maintained at the same mean temperature. In contrast, ML was similar between the two treatment groups, suggesting that the overall thermal mean is more relevant to embryonic growth than any thermal fluctuations experienced. This possibility deserves further exploration, as no *in situ* temperature measurements were made during this experiment, and prior investigations of temperature and embryo growth have involved steady thermal regimes (*e.g.* (S. V. Boletzky 1983; Olivier Bouchaud 1991b)).

In addition to size, we measured two body patterns. These tests have been used extensively in the literature (*e.g.*Chiao, Kelman, and Hanlon 2005; Barbosa et al. 2007; Barbosa et al. 2008; Di Poi et al. 2014a) to measure defensive ability, since body patterning is the primary means of defense in cuttlefish (Hanlon and Messenger 1998). Heterogeneity Index (HI) measures the overall uniformity of pattern and a hatchling with low HI is considered to be well-camouflaged on a uniform background. Hatchlings are notoriously poor at producing uniform body patterns (Hanlon and Messenger 1988; Dickel et al. 2006), and we hypothesized that animals enriched by the natural environment would be better at this task. Results show some evidence that this is indeed the case. Heterogeneity Index against a uniform background ranged between 3.87 and 13.06 and "Lab" hatchlings showed a significantly higher HI (more disruptive) than "Sea" hatchlings. The fact that "Sea" incubated cuttlefish are slightly better at

producing uniform body patterns is strange given that they likely experienced a heterogeous background during development, while "Lab" cuttlefish experienced an artificially homogeneous substrate (PVC plastic) during development. This disparity between our expectations and experimental results deserves further inquiry. Moreover, the potential for incubation environment to influence body patterning in cuttlefish must be considered when interpreting past and future behavioral results.*

 In contrast, while "Lab" cuttlefish showed stronger uniform pattern-matching in this test, the same was not true of their ability to match the color of the background. Mean Gray Value match assessed the correspondence between the overall color of the mantle to that of the immediately adjacent substrate, and values closer to 1 indicate a high degree of correspondence. Mean Gray Value match ranged between 0.64 and 0.95, and there was no significant difference between the MGV match of "Sea" and "Lab", suggesting that unlike HI, the color-matching abilities are not affected by incubation environment.

Body patterning against a checkered background was also tested and the overall "disruptiveness" evaluated. Disruptive Score allowed us to obtain an overall sense of difference in the disruptive pattern between incubation groups. Disruptive Scores ranged from 8 to 10 (out of 33) and did not significantly differ between groups. We had expected "Sea" cuttlefish to be more efficient in their cryptic abilities due to their prenatal exposure to a variegated natural background. In contrast, the lack of difference between the "Sea"- and "Lab"-incubated hatchlings in Disruptive Score suggests that this feature of body patterning behavior is not affected by incubation environment.

While we have followed traditional body pattern interpretations and concluded that a less disruptive (lower HI) and better color match (higher MGV match) on a uniform substrate and that a higher Disruptive Score on a checkered substrate are most adaptive, it is not entirely clear that this is the case. Cuttlefish have multiple strategies for creating crypsis (background matching, disruptive coloration and deceptive resemblance). Any particular body pattern may function in multiple cryptic strategies and two different patterns may be equally effective in certain circumstances (Hanlon and Messenger 1988). For instance, a cuttlefish displaying a disruptive body pattern on a uniform background may not be attempting background matching, but rather deceptive resemblance of stone or shell fragments. This possibility is supported by the fact that hatchling cuttlefish often display seemingly "inappropriate" body patterns (*i.e.* a disruptive body pattern on a uniform substrate) for the first two to three days (Hanlon and Messenger 1988). Perhaps we have misinterpreted the "ideal" cryptic strategy. It seems instead that the proper strategy is determined by size and thus changes throughout the lifetime

of an individual (Hanlon and Messenger 1988). Further investigation is necessary to resolve this question, and the interpretation of our camouflage results may change as we learn more about strategies for different size classes on a variety of substrates.

Finally, we measured five aspects of predatory behavior during hatchlings' first encounter with their preferred prey, shrimp (Wells 1958): Attempted Capture Rate, Capture Rate, Success Rate, Latency to Capture and Distance of Detection. Successful predation is critical at this time, since hatchlings' yolk reserves are nearly depleted and they are at their most vulnerable (Wells 1958). While many factors influence these variables, making it impossible to impute a single cause to each measure, together they give us an overall sense of motivation and predation efficiency in hatchling cuttlefish and allow us to identify differences due to incubation environment. We hypothesized that the enrichment present in the natural incubation environment would stimulate development and result in more adept hatchlings with higher Capture Rate and visual skills. We also reasoned that hatchlings incubated in the natural environment would show more cautiousness due to their experience with visual and odor cues from other organisms and differences in feeding motivation due to their exposure to a more variable temperature regime. In contrast, none of these variables differed significantly between groups and Capture Rate was very high (between 85% and 90% in accordance with Messenger 1968). This suggests that lab-raised hatchlings would be equally capable of feeding themselves during this critical period of growth.

The literature suggests a high degree of behavioral plasticity in hatchling and juvenile cuttlefish. In terms of growth, enrichment and environmental factors such as dark tank color promote growth (Dickel, Boal, and Budelmann 2000c; António V. Sykes et al. 2011), while experience with external stimuli such as a natural substrate or conspecifics alter behavior (Poirier, Chichery, and Dickel 2004; Poirier, Chichery, and Dickel 2005). Given the cuttlefish's high plasticity in other areas, the strong evolutionary pressure on the tested behaviors and the embryo's ability to perceive beyond the egg, it seemed logical that prenatal environment would have dramatic effects on size and behavior. This was not supported by our data. One potential reason for this unexpected outcome is that our experiment focused on hatchlings fewer than five days old, and it is possible that differences due to incubation environment would manifest later in development or in some unmeasured aspect of post-predatory growth or behavior. Behavioral plasticity probably requires a fair amount of brain development, especially of such crucial structures in the supraesophageal mass as the vertical lobe. These structures are still developing during the first few months (Dickel, Chichery, and Chichery 1997, 1998; Dickel et al.

2013), so perhaps behavioral plasticity only develops later. Alternatively, it may simply be that these particular aspects of behavior are highly pre-programmed and not subject to plasticity.

There were some significant limitations to the data we were able to collect during this experiment. Logistical constraints necessitated that "Sea" eggs be removed from the wild 7 to 12 days prior to the majority of eclosions (stages 26-30) when embryos were most sensitive to external stimulation. It is also possible that the laying site from which the eggs were collected was atypically barren of stimulation and enrichment, which would limit the effect of natural incubation. This could be addressed in the future by collecting eggs from multiple laying sites and via censuses of the marine life that typically occurs at these sites. Most importantly, we were only able to investigate a limited range of behaviors in our experiment. It is possible that plasticity is not manifest in hatchling growth, body patterning or predation, but is present in other aspects of behavior, such as learning, memory or activity.

Despite the limitations in experimental design which constrain our conclusions from this experiment, the lack of strong differences between groups is manifest. The overall lack of difference between incubation groups bolsters confidence in existing behavioral findings and has practical implications for cuttlefish conservation. These results, added to the success of several authors (*e.g.* Pascual 1978; Forsythe, DeRusha, and Hanlon 1994; Domingues, Sykes, and Andrade 2002) in culturing multiple generations in the laboratory, suggest that artificial incubation does not alter natural hatchling behavior. This implies, but in no way guarantees, that the survival, at least of hatchlings, will not be compromised, although survival was not tested directly in our experiment. In the future, cuttlefish eggs laid on basket traps normally lost during the harvest of adults could instead be incubated and repurposed for conservation, research or as an additional source of product. Indeed, larger-scale trials examining the feasibility of egg and hatchling incubation in artificial tanks are already in progress.

The next obvious step in the characterization of the effects of artificial incubation is to repeat these experiments with cuttlefish collected from the wild at hatching and compare them to eggs spawned by captive females. This may allow us to gauge more fully the entire course of embryonic development as well as the contribution that a laboratory setting can have on the embryo indirectly through any maternal transmission of hormones (as seen in birds—Groothuis and Schwabl 2008). In addition, direct observation of eggs and hatchlings in the field would give a clearer picture of the stimuli experienced by embryos in the wild and the natural behavior of hatchlings. Despite the difficulties, field observations (via SCUBA, remote sensing or photography) are certainly possible with today's technology and would do much to elucidate the effect of prenatal stimulation on subsequent behavior. For

example, measurements of physiological parameters such as turbidity would allow us to estimate the visual field of cuttlefish embryos and censuses of marine fauna at laying sites would provide an idea of what odor cues might be sensed. Finally, behavioral experiments conducted in the natural environment would be the ultimate assessment of the effect of incubation environment.

Conclusion:

This experiment provides evidence that incubation in a laboratory environment does not strongly affect three fundamental aspects of behavior and survival in cuttlefish hatchlings: embryonic growth, body patterning and predation ability. The one difference identified in uniform body patterning urges further investigation as well as caution when interpreting results from body pattern experiments with artificially-incubated hatchlings. (We may also need to reevaluate "conventional" interpretations of what constitutes the best cryptic strategy in a particular situation.) The overall lack of differences between naturally- and artificially-incubated hatchlings bolsters confidence in existing experimental data. Our results are also encouraging from a conservation perspective; artificially-incubated cuttlefish could augment fishery stocks or replace wild-caught cuttlefish in certain situations. Future work on this question should strive to test cuttlefish spawned from captive females and collected directly from the wild at the moment of hatching and to test a broader range of behaviors for the entire juvenile period.

II. Chapter Summary

Up until the final two weeks of development, egg incubation environment only affected one aspect of body patterning behavior and not predation. This was similar to the effect of spawning environment, except in the direction of body patterning disruptiveness (Fig. 52). This demonstrates that both the environment in which females lay their eggs and the environment in which those eggs develop are potential sources of future behavioral variation. Like stress, these effects may be transmitted both indirectly through the mother or directly experienced by offspring.
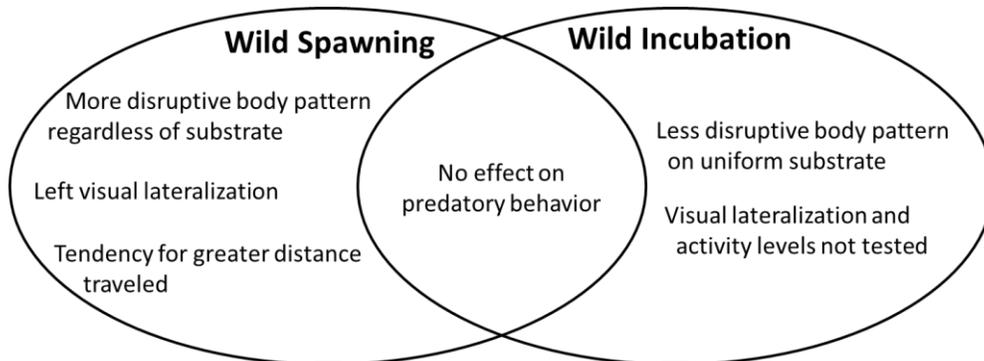
Wild Spawning

More disruptive body pattern regardless of substrate

Left visual lateralization

Tendency for greater distance traveled

No effect on predatory behavior

Wild Incubation

Less disruptive body pattern on uniform substrate

Visual lateralization and activity levels not tested

Fig. 49. Comparison of the effects of spawning in the natural environment to spawning + incubation in the natural environment.

General Discussion

General Discussion

I. Prenatal Stress and Cuttlefish

In this thesis, two major categories and two subcategories of stress were investigated in order to a) determine if prenatal stress affects cuttlefish, and, if so, to b) discern the pathways by which stress transmits its effects. The results of all behavioral, learning and neurobiological experiments conducted during the course of this thesis are summarized in Table 13. Comparing maternal stress in isolation demonstrated that stress affects reproducing females (decreasing their reproductive output) and that they pass this onto their offspring (altering their behavior and neurobiology). Testing separate embryo-only stressors indicated that embryos also respond on their own with changed behavior, learning and neurobiology. Thus, it appears that when prenatal stress occurs, all three of the potential avenues of stress effect transmission—through the mother, from mother to offspring and reaction of the offspring themselves (see Fig. 1)—are possible in cuttlefish.

Table 13) Summary of prenatal stress experiments. Results refer to comparisons with the Unstressed Mother Control (UM-C) group. 5-HT = serotonin, NA = noradrenaline, VL = vertical lobe, LOL = left optic lobe.

	Egg Production	Egg Survival	Hatching Size	Body Patterning	Turning Bias	Predatory Behavior	Activity Patterns	Threat Response	Learning and Memory	Brain Development
	2015/2016 data	2015/ 2016 data	2015/2016 data	2015 data	2015 data	2015 data	2015 data	2016 data	2016 data	2015/2016 data
Maternal stress exposure	*Reduced number of eggs laid and hatched, possible production of transparent eggs*	*Reduced the number of eggs that hatched*	Results varied by year, but no effect overall	*Higher disruptiveness*	No effect	No effect	*Distance traveled, velocity and time spent moving were higher*	No effect	No effect at either age tested	*Lower 5-HT and NA concentrations, higher 5-HT turnover, smaller VL, higher cell division in LOL and VL*
Embryonic predatory exposure (natural stressor)	NA	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect at either age tested	*Lower 5-HT and NA concentrations, smaller VL*
Embryonic light exposure (artificial stressor)	NA	No effect	No effect	*Tendency for higher disruptiveness*	No effect	*Higher attempted capture rate*	No effect	No effect	*Faster learning at 3 weeks*	*Lower 5-HT concentration, higher NA, smaller VL*

Notably, and in contrast to our predictions, cuttlefish responded more strongly to the artificial embryonic stressor than the naturally-occurring one. Both seabass and cuttlefish are well-established species in the English Channel, and seabass are known to predate on hatchling cuttlefish (Blanc and Daguzan 1999b). From earlier experiments, we knew that prenatal sea bass odor is associated with changes in visual lateralization (Jozet-Alves and Hebert 2012). These facts, paired with the impressive behavioral flexibility of cuttlefish led us to predict that natural selection would have produced a

behavioral reaction to this predator. Instead, predator-exposed cuttlefish showed no differences from the control group in any of our behavioral tests, although they did show changes in brain chemistry and morphology. The presence of neurobiological changes in offspring from predator-exposed eggs demonstrates that the cuttlefish embryos are physically affected by prenatal stress, while the lack of any behavioral reaction suggests habituation to the prenatal predator cues as discussed in Article #4. Indeed, other species can tailor their innate anti-predator reactions according to the level of threat. Many gastropods, a sister group to cephalopods, show reactions to predator odors, and these reactions are stronger when they are paired with alarm cues from conspecifics (*e.g.* McCarthy and Fisher 2000; Jacobsen and Stabell 2004; Dalesman et al. 2006). At the same time, we know that cuttlefish habituate to certain prenatal stimuli (Romagny et al. 2012), so it seems plausible that the predator-odor, paired with the lack of alarm cue from the numerous other cuttlefish embryos present, did not result in behavioral changes because the cuttlefish learned to perceive it as non-threatening.

In contrast to the natural stressor, the artificial stressor showed both behavioral and learning differences, as well as differences in brain chemistry and morphology. Since a mismatch between a stress response and the stressor itself often leads to negative side effects, we had predicted that this the artificial stressor (which was not something the species could have ever experienced historically in order to evolve a reaction via natural selection) would have a "negative" effect on offspring. Arguably though, the light-exposed group showed "positive" effects in the behaviors tested—better learning abilities and greater feeding motivation leading to a higher predation rate. One hypothesis to explain this is that prenatal exposure to an unprecedented stressor actually induced a generalized alarm response that resulted in acceleration in development. Alternatively, prenatal light exposure is known to affect circadian rhythm in a range of animals, including cuttlefish, which can hatch earlier or later depending on the light regime (Paulij et al. 1991). In this case, the light may have accelerated development.

Finally, we were also able to make some indirect comparisons between artificial incubation and artificial spawning and found similar impacts on the behaviors tested (no effect on predatory behavior, minor effects on body patterning). This shows that the environment, as well as specific stressors, can potentially impact the post-natal behavior of offspring, a fact which must be taken into account when designing and interpreting experiments with young cuttlefish.

II. An Ethological Model of Prenatal Stress

The results presented here allow us to begin formulating a rough model of prenatal stress in cuttlefish from Tinbergen's four perspectives. In terms of the mechanisms of prenatal stress transmission (Tinbergen's first perspective), we hypothesized that female cuttlefish might transfer corticosterone to offspring in the egg yolk. This has been the subject of some interest among vertebrates, particularly birds, which have been found to impregnate their egg yolk with stress hormones that affect the hypothalamic–pituitary–adrenal (HPA) axis of their offspring (e.g. Charil et al. 2010). We failed to find definitive evidence for or against this hypothesis in cuttlefish, but our work does suggest that other possible avenues of stress transmission from mother to offspring are possible, since offspring of stressed mother differed from other stress groups nonetheless. Hopefully, as molecular and neurological analytic techniques become more refined and widely-available, we will be able to pin down the exact mechanisms that lead to behavioral changes after prenatal stress.

Our experiments had a great deal of insight to shed on Tinbergen's second and third perspectives, the ontogenic and the adaptive. From the differences in behavior of the artificial stress group, we see that, ontogenically, the prenatal period cannot be ignored as a source of behavioral influence, since stressors experienced by the embryo appear to alter future behavior. Previous generations of researchers had largely overlooked this time, since it was presumed that embryos could not perceive the outside world, although this view has been overthrown in more recent decades. Our results provide further evidence (along with Darmaillacq, Lesimple, and Dickel 2008; Romagny et al. 2012; Jozet-Alves and Hebert 2012) that cuttlefish can perceive the world outside the egg membrane and alter their future behavior in response. From an adaptive perspective, we found indications that maternal stress may be a better indicator of future prospects for embryonic cuttlefish than direct embryonic experience: many more behavioral differences were apparent in association with maternal stress than with embryonic. On the other hand, one could argue that the responses to the embryonic stressor were more clearly "adaptive" (better learning and predation), suggesting that direct embryonic experience is more informative to cuttlefish after all. Clearly, the responses of cuttlefish are complex, and further experiments are required to understand the interplay of selective forces driving stress-induced post-natal behavior.

Finally, we were able to address prenatal stress from Tinbergen's fourth perspective, the phylogenic, by comparing our results in cuttlefish to the other PReSTO'Cog models. All four models (chickens, quail, zebrafish and trout) showed effects of maternal stress as well as artificial and natural embryonic stressors. These results imply that existence of stress responses is highly conserved across

phylogenetic groups. Also, it seems that like cuttlefish, the effects of prenatal stress in animals manifest on a multitude of levels, and are complicated by numerous interactions.

Conclusion

I. Thesis Summary

In this thesis, the questions of "if" and "how" prenatal stress affects cuttlefish was investigated from Tinbergen's four ethological perspectives through a suite of physiological, behavioral, cognitive and neurobiological tests. Stressors were applied to both reproducing females (maternal stress) and developing embryos (embryonic stress) in order to compare their relative impact and to uncover the routes by which these effects are transmitted. These results demonstrate unequivocally that prenatal stress affects cuttlefish, and strongly suggests that all three of the three potential avenues of stress (on the mother, from the mother to offspring and on the developing offspring directly) seem to be operating in this species. Figure 53 illustrates the ranges of prenatal influences that were found to alter post-natal behavior. By comparing stress groups, we made several tentative deductions about prenatal stress in cuttlefish from Tinbergen's first three perspectives. From a mechanistic perspective, we found no evidence of corticosterone transfer, but some possibility that prenatal stress effects are mediated by changes in neurobiology. When examining stress from the ontogenic perspective, we found further confirmation that embryonic experience alters future behavior in this species. From an adaptive perspective, we had mixed indications about whether maternal stress or an artificial embryonic stressor had a stronger and more positive effect on offspring behavior. Finally, by comparing our cuttlefish results to other those of the other Presto'Cog animal models and using Tinbergen's fourth perspective, we see that prenatal stress effects seem to something that is shared by a broad range of animal groups.
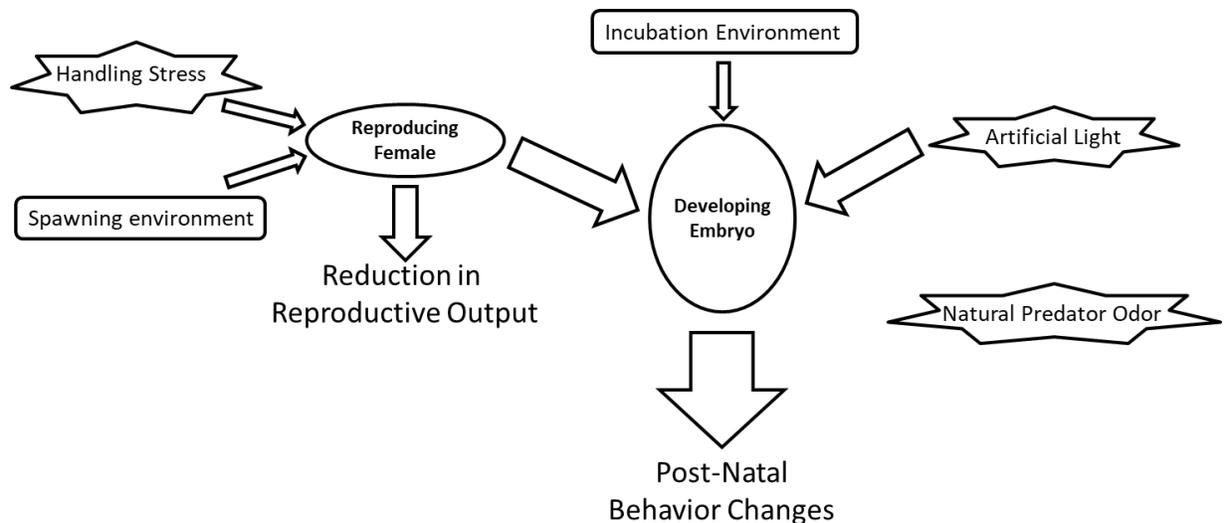
Fig. 50. A schematic representation of the range of variables that were found to influence post-natal behavior. Note that embryonic predator-exposure did not affect post-natal behavior.

Further work is necessary to confirm and extend these conclusions. Replicating these experiments in cuttlefish with other types of prenatal stressors will give further insight into the factors that are relevant indicators of future conditions to cuttlefish embryos. One particularly intriguing possibility would be to compare the effects of subjecting reproducing females to the same randomized artificial light regime as the embryos were exposed to, so that we may directly compare the effects of maternal transmission versus direct experience. Our comparisons of the effects of spawning environment to incubation environment (Chapter 3) give some preliminary clues to this question by showing that both have a similar effect on offspring predatory behavior, but opposing effects on body patterning disruptiveness. Unfortunately, we did not test the full range of behaviors in the incubation experiment. Nevertheless, it is apparent that maternal or embryonic environment can exert and influence on post-natal behavior.

In order to better-understand the mechanisms of stress effect, our lab is currently testing the responses of embryos directly, while they are still in the egg, by observing changes in mantle contractions or food preferences (Mezrai, in preparation). This is made possible by certain experimentally-conducive characteristics of cuttlefish eggs, including the lack of parental care and a semi-transparent membrane. Identifying and testing other species with similar traits would reveal whether the trends seen here widespread throughout the animal kingdom or another example of cephalopod distinctiveness. In particular, it would be especially enlightening to see if maternal stress transmission and embryonic stress exist in "social" invertebrates like ants and bees, which have a completely different social structure than most other animal groups.

By studying prenatal stress in animals, ethologists can gain insight that can be used to improve animal welfare as well as human health and society. A better understanding of prenatal stress across a broad range of species might lead to improvements in the way we treat pregnant female animals in agriculture and laboratories. For instance, the existence of prenatal stress effects from handled mother to offspring in this species and a wide range of others (*e.g.* salmon, Sigholt et al. 1997; farmed blue foxes, Braastad 1998; cattle, Grandin, Oldfield, and Boyd 1998) adds further incentive for the agricultural industry to avoid things like gestation crates for pigs, which may not only decrease animal welfare but the amount and quality of meat product produced. Likewise, a better understanding of prenatal stress may lead to ways to mitigate its negative impacts on children and adults, and perhaps to prevent it from even occurring in the first place. For instance, the children of women who have been known to have experienced a stressor during pregnancy could be targeted for extra maternal attention or early-childhood therapy, since parental care has been shown to mitigate some of the negative effects

of prenatal stress in rodents (reviewed in Francis and Meaney 1999). This research would also help us predict what sorts of things may actually be stressful to a fetus. Here, we saw possible habituation in response to a predator odor that we expected to be very stressful. This suggests that we should be aware of the possibility that human embryos could habituate to stressors as well. Indeed, human fetuses are known to habituate to repeated vibriotactile stimuli perceived from within the womb (Leader et al. 1982).

II. Article #6: "The Future of Cephalopod Research; Perspectives of Three Early-Career Researchers."

Given the insight a cephalopod mollusc has provided here, it seems logical that cephalopods should continue to be used as a model with which to study animal behavior and search for broad commonalities across phylogenetic distance. The following manuscript outlines my views on the future of research of this group.

The future of cephalopod science: Perspectives from three early-career researchers

Behavior, Cognition and Neuroscience
By Caitlin E. O'Brien;
<u>Excerpt</u> from a collaboration with Drs. Katina Roumbedakis and Inger Winkelmann

Introduction:

The charismatic behavior, sophisticated cognition and advanced neurophysiology of cephalopods is intriguing to scientists and the general public alike. This is reflected in both their popularity as aquarium specimens (despite the challenges associated with keeping them) and their prevalence on social media. They, or creatures strongly resembling them, are also often depicted in works of fiction (*e.g. Finding Dory* (2016), *The Arrival* (2016), *Life* (2017)). Some of their most unique characteristics have also served as inspiration for many recent developments in technology, such as adaptive camouflage based on cephalopod skin (Wang et al. 2014; Yu et al. 2014), suction cups for wound repair (Choi et al. 2016), propulsion and buoyancy systems for Autonomous Underwater Vehicles (Song et al. 2016) and as models for soft robotics (Renda et al. 2012; Laschi et al. 2012). A number of other cephalopod features, such as sophisticated "camera" eyes and advanced cognition, have experienced convergent evolution with vertebrates, allowing cephalopods to serve as a phylogenetically-distant reference point from which to examine our own evolution. Unfortunately, despite their great popularity and relevance, only about 8% (60 species) of the 800 or so species known have been thoroughly described. Here, potential developments in the study of cephalopod behavior, cognition and neurobiology are discussed, and some recent examples of progress are highlighted.

Behavior:

Our understanding of cephalopod behavior is limited mainly to laboratory observations. Without the ecological context of the natural environment, we often cannot perceive the true "purpose" of particular behaviors, leading to misinterpretations. In order to improve our understanding of cephalopod behavior in particular, more field observations and field experiments are necessary. Like many animals, cephalopods are influenced by and adapt to their surroundings (e.g. (Dickel, Boal, and Budelmann 2000c; Anderson and Wood 2001), constraining or altering the behavioral phenotype. While there are obvious difficulties to field work, the insight gained will be well-worth the effort. A recent study by Schnell et al. (2015) is a good illustration of this: via controlled laboratory experiments, the authors found that the white lateral stripe displayed by female *Sepia apama* signals non-receptivity for mating (they are less likely to mate when showing it). However, observations of natural behavior in the field showed that males largely ignored this and mated anyway. This combination of laboratory tests with natural observations allowed observers to deduce the intended meaning of an intraspecific signal but also provide contextual evidence about its relevance and efficacy in actual mating situations. Fieldwork can not only validate and contextualize laboratory experiments, but will improve overall animal welfare in experiments by minimizing disturbance and manipulations of animals.

The effort to increase our canon of field data will likely be aided by the general trends of technological development and decreasing costs of data acquisition tools. Various types of tagging have been utilized successfully in recent years to answer questions about geographic range, migration and diving habits (*e.g.* (Bazzino et al. 2010; Liu et al. 2016; António V. Sykes et al. 2017). Remote monitoring through videography and photography is another increasingly accessible option thanks to the profusion of low-cost cameras that have come on the market in recent years. Remotely-operated underwater vehicles (ROVs), AUVs and submersibles have also become more affordable, and they have greatly expanded our knowledge of deep-sea cephalopod behavior (*e.g.* Trueblood et al. 2015; Thomas, Robison, and Johnsen 2017), even capturing footage of the elusive giant squid (Robey, 2012). In addition to embracing the benefits of evolving technology, the community of cephalopod researchers should consider creating a shared, open access data repository of video footage and data sets. Such a repository would allow students and researchers lacking funds, facilities or animals to conduct their own experiments and contribute to the body of knowledge. Other authors (Xavier et al. 2015) have urged a community-wide shift in focus from data acquisition to data analysis, and open access to shared data would accelerate this process. Finally, the reuse of raw data and footage would improve welfare by reducing the total number of animals manipulated (Fiorito et al. 2014).

When we do conduct laboratory work, the cephalopod research community would be wise to make greater efforts at standardization across experiments. Due to the sensitivity and advanced perceptive abilities of cephalopods, even minor methodological differences can skew results and lead to inaccurate conclusions. For example, the standard method of measuring learning and memory in cuttlefish is the "Prawn-in-the-Tube" (PIT) procedure (see Messenger 1973 for methodological description) which has been used for decades by a number of research groups. While this standardized method theoretically allows direct comparisons to be made between experiments conducted at different times, the more recent discovery that cuttlefish and other cephalopods are able to perceive differences in the polarization of light has led to the realization that the seemingly irrelevant choice of tube material (e.g. glass versus plastic, each of which alters the properties of light in different ways) could potentially affect results (Cartron, Darmaillacq, and Dickel 2013). One technique to increase standardization across experiments and research groups is video playback of standardized behavioral stimuli (e.g. approach of a predator, prey item or conspecific). Such a method has already been used by Pronk, Wilson, and Harcourt (2010) to study the reactions of octopus over time and between individuals, and other labs have developed cephalopod-specific video playback systems (G. Fiorito pers. comm.). If such video clips were shared to a common video library, experiments could be replicated by different labs in a standardized fashion using identical audiovisual playback equipment.

In addition to standardizing and replicating within the same species, the cephalopod research community should also strive to duplicate the same observations and experiments across multiple species. Having corresponding data on closely related animals allows us to make comparisons and draw conclusions about the entire lineage by giving us a sense of what behaviors are evolutionary conserved from earlier shared ancestors and which represent novel adaptations to the particular environment of that species. In the family Hominidae for example, social differences between such congeners as apes, chimps and bonobos allow us to assess the factors driving behavioral evolution (*e.g.* Stanford 1998; Malone, Fuentes, and White 2012). Similar comparisons between such commonly-studied cephalopod species as *Octopus vulgaris*, *Loligo vulgaris* and *S. officinalis* would be a good place to start, although the eventual goal should be to assess behavior across a wide variety of species, including the non-coleoid cephalopod *Nautilus* spp., which can serve as an ancestral reference point (R. Crook and Basil 2008).

We would also benefit greatly from the formal investigation of inter-individual differences and behavioral plasticity in this group. Anecdotal observations by aquarists and researchers give the distinct impression that individual animals have distinct "personalities." Indeed, Carere et al. (2015) found that in *S. officinalis*, certain behaviors were expressed predictably and consistently over time, although the

expression of other behaviors differed between testing situations. Further research into this subject may indicate that we need to apply different tactics and interpretations at the population level, such as distinguishing between "personality types" when calculating group means. Ultimately, we may find that this plasticity explains many of their idiosyncrasies, their evolutionary persistence through three mass extinctions and their current ecological success despite the effects of global climate change (Doubleday et al. 2016). In theory, individual plasticity can fortify animals against the rapid changes in conditions the world is currently experiencing (e.g. bleached coral reefs, invasive species, changing temperature regimes). Perhaps we can even learn ecological "lessons" from cephalopods that will aid in conservation of other species. At the very least, increased understanding of their behavioral ecology can be used to guide future environmental regulations.

An increasingly relevant subject will be whether or not cephalopod behavior is affected by environmental pollutants. As neurologically-complex organisms often residing in nearshore environments polluted by pharmaceutical residues, pesticides, and other chemicals, the cephalopod nervous system is potentially affected, perhaps in subtle ways that are not immediately apparent. Indeed, the SSRI Fluoxetine has been shown to affect young *S. officinalis* in different ways depending on age and dose (Di Poi et al. 2013; Bidel, Di Poi, et al. 2016). Moreover, in one case, differences could not be identified with standard behavioral tests but only neurobiological assays (Bidel, Di Poi, et al. 2016). Considering the rapid pace of anthropologically-induced environmental change, it is important that we get a behavioral "baseline" of vulnerable species as quickly as possible.

Evolution of Sophisticated Cognition:

Cephalopods demonstrate unexpectedly advanced cognitive abilities (summarized in (Hochner, Shomrat, and Fiorito 2006; Godfrey-Smith 2013; J. A. Mather and Dickel 2017) and should play a larger role in scientific discussions about cognitive evolution. Because of their higher learning abilities, large and centralized brains, and sensory apparatuses (e.g. eyes, statocysts) that have undergone convergent evolution with vertebrates, cephalopods have an enormous potential to reveal general evolutionary principals driving cognition. Scientists studying other phyla should be made aware of the powerful comparative role cephalopods can play in understanding of the evolution of animal cognition. It is my hope that in the future, more direct comparisons between cephalopods and "cognitively advanced" vertebrates, such as mammals and birds, will be made.

The existence of advanced cognition in cephalopods raises an important philosophical question: What drove it to evolve to such a high degree in an otherwise cognitively-unsophisticated group (*i.e.* a

group most closely related to bivalves and gastropods)? Complex nervous systems and cognition come at a high metabolic cost for organisms (Godfrey-Smith 2013), and in cephalopods, the size of the brain limits the amount of food that can be ingested per swallow and puts animals at risk of brain injury (Huffard 2013). There must be strong pressure driving its development in the face of these disadvantages. Comparisons with birds and mammals suggest that a variable environment is an indispensable driver of advanced cognition, since that is a factor common to all three groups (Vitti 2013), but more investigation is necessary before any concrete conclusions can be drawn.

One step that can be taken in the effort to answer this question is to deduce how and when the physiological coleoids increased their neural tissue and became so encephalized (factors suspected to be the physiological basis for their cognition). Was it driven by the rise of and competition with bony fishes as suggested by Packard (1972)? More recent authors have countered that cognitive development actually occurred before bony fishes due to competition with the first jawed fishes and other cephalopods (Grasso and Basil 2009). Other authors suggest physiological innovations such as the loss of the hard external shell (Mather 2011)and the advent of sophisticated vision (Vitti 2013) as key. The cognitive abilities and behavioral plasticity of cephalopods may also be related to recently-discovered abilities to edit their own RNA (Liscovitch-Brauer et al. 2017).

In order to understand the evolutionary development of cognition in this group, we need a more comprehensive survey of the learning abilities of nautilus, the putative "ancestral" condition from which modern coleoids evolved (Shigeno et al. 2008; Shigeno, Takenori, and Boletzky 2010; Sasaki, Shigeno, and Tanabe 2010; Basil et al. 2011). Comparisons of the coleoids (150 myo) with their smaller-brained, less-encephalized living fossil (400 myo) nautilus relatives would allow us to deduce the role of various senses and neural structures in the cognitive abilities of cephalopods. The nautilus has only 13 brain lobes compared to the 40 of octopus, and importantly, lacks a vertical lobe (VL)—the structure thought to be the seat of higher cognitive processes in coleoids. Recent experiments with nautilus have demonstrated that they possess more advanced cognitive abilities than once thought, including rapid learning, biphasic memory and advanced olfactory spatial navigation skills (Crook and Basil 2008; Crook, Hanlon, and Basil 2009; Basil et al. 2011). This contradicts traditional interpretations, and suggests that either a prototype VL is present in the nautilus (perhaps the plexiform layer and subesophogeal nerve cords), or that they have evolved their advanced cognition utilizing different brain lobes (Basil et al. 2011).

Inquiries into the cognitive evolution of cephalopods would also be greatly facilitated by increasing the amount of genomic and paleontological data available. Yoshida et al. (2015) compared

gene expression in the eyes of nautilus, squid, other molluscs and humans, and were able to identify at least three types of genetic innovations that occurred during evolution of the cephalopod eye, including the duplication and subsequent re-purposing of some genes. Recently, the entire genome of *O. bimaculoides* was sequenced, and already, examination of these data has shown that unlike other molluscs, this species has experienced expansion of some of the same gene families involved in vertebrate neuronal development (Albertin et al. 2015). Another study used data from the same 180 genes across 26 species to test hypotheses about divergence times (Tanner et al. 2017). The complete sequencing of other species along with open access to these genomes would certainly lead to further revelations.

While the recent boom in genetic data has led to some neglect of more traditional paleontological and morphological methods (Xavier et al. 2015), new techniques are being used to extract more information from existing fossil specimens. For example, Klug et al. (2016) used UV light to reveal structures not normally visible in a fossilized belemnite (*Acanthoteuthis speciosus*), including cranial cartilage, vague imprints of the statocysts and the first-ever evidence of a belemnitid radula. Though we lack a fossil record for most soft-bodied cephalopods, a few specimens do exist. Recently, Kruta et al. (2016)were able to reconstruct soft body parts in three dimensions (including the eyes and some suckers) from a fossilized octopus using synchrotron microtomography. The presence of suckers in this specimen forces researchers to re-evaluate the advent of this structure, which was thought to be a more recent development. Other possible tools include isotope analysis of fossil material and X-ray tomography, a method which allows the internal investigation of fossils and which can reveal preserved soft tissues. Synthesis of paleontology, traditional phylogenetic methods and "modern" biology promises to be very fruitful. One recent example is a phylogenetic analysis conducted with a new morphological dataset gleaned from both extinct and extant forms, which was able to confirm many of the putative relationships between coleoid groups, but found a few to be para- or polyphyletic (Sutton, Perales-Raya, and Gilbert 2016).

Study of cephalopods may ultimately demonstrate that cognition is an inevitable emergent property past a certain level of neural size or centralization. Or it may demonstrate that certain kinds of structures and organizational features are essential before advanced cognition can evolve. Either way, a better understanding of cephalopod cognition would give us insight into the evolution of cognition in animals. Integrating all the techniques at our disposal—genetic, paleontological, phylogenetic—will greatly facilitate progress.

Neuroscience:

Neuroscience is an area of fast-paced change, with many promising new techniques that have the potential to address the questions about cephalopod behavior and cognition discussed above. It is impossible to survey them all here, but a few that have recently added to our knowledge on these fronts will be highlighted.

Using anatomical and histological comparisons between the hatchlings of six different species of coleiods, Wild et al. (2015) showed that the sizes and shapes of the visual and nervous systems demonstrate plasticity according to each species' respective ecological niche. This information could be useful in situations where the origin of a specimen is unknown—measurement of the relative size of various neural structures might yield clues about its ecological niche, much the same way as tooth shape suggests diet in vertebrates. Another group Wollesen et al. (2014) compared the expression of four genes encoding transcription factors important for nervous system development in squid to that of other bilaterians. They found that the roles of these genes have been largely conserved across these widely divergent groups, and thus represent a shared legacy. Bidel, Corvaisier, et al. (2016) recently validated a method to quantify dopamine, serotonin, norepinephrine and their metabolites simultaneously in brains of cuttlefish using high performance liquid chromatography (HPLC) electro-chemical detection. Array tomography is another imaging method which might soon be possible with cephalopods. With this technique, tissues are stabilized by a glass substrate that allows samples to be stained with multiple markers so that both brain structure and 20 or more neurotransmitters can be viewed simultaneously and visualized in three dimensions (F. Bidel, pers. comm.). Another method on the cusp of accessibility is primary neuronal cell culture, which may one day be used to reconstruct cephalopod brain networks in vitro (F. Bidel, pers. comm.).

As we continue to make neurobiological progress, we should make every effort to avoid unnecessary pain, suffering, death and or lasting harm (PSDLH). The first step will be to determine whether or not cephalopods experience pain and suffering, and to validate our standards of anesthesia for this group (Andrews et al. 2013). Such work is especially important given recent legislative changes (Fiorito et al. 2015) and our growing knowledge of their sensory and intellectual sophistication. Luckily, technological advances and cost-reductions have made some non-invasive methods available. Portable ultrasound machines have recently been used to study brain size in octopus (Grimaldi, Agnisola, and Fiorito 2007), while non-destructive X-ray microtomography has been used to map the brain of bobtail squid (Kerbl et al. 2013). As we utilize these methods to glean new data, this and existing information should be digitized and shared (Xavier et al. 2015). In particular, the development of a shared digital

brain atlas like those that exist for rodents (*e.g.* Allen Brain Atlas) based on the existing data from early

lesion and electrical stimulation data is currently within reach. This has been done already for two

species of squid (Shigeno et al. 2001; Yamamoto, Shimazaki, and Shigeno 2003), and should be repeated

with other commonly-studied cephalopod models, like *Octopus vulgaris*, *Loligo vulgaris* and *S. officinalis*.

Some other important research topics to be pursued in the near future include investigation of

the interplay between PNS and CNS, especially regarding how the 8 arms are controlled. For example,

are all arms represented equally, or is there a hierarchy among them, with a certain arm or arms

dominating like in human beings? This question could be pursued through both behavioral and

neurobiological inquiry; frequency of arm use could be compared to the relative size of the brachial

lobes. Another topic that deserves more inquiry is sleep; cephalopods appear to undergo periods of

behavioral and physiological quiescence that strongly resembles sleep in vertebrates (*e.g.* Mather 2008;

Meisel et al. 2011; Frank et al. 2012). A better understanding of this phenomenon in cephalopods would

give insight into the phylogenetic origins and biological reasons for sleep. Observations of neuroactivity

might soon be made non-invasively and could yield insight into more general question of why sleep is

physiologically necessary in animals. Finally, greater exploration of the nautilus nervous system would

provide a better idea of the presumably more basic nervous system from which the modern coleoid

brain evolved.

Final Thoughts:

People are naturally drawn to "charismatic" animals, and often feel more empathy towards

creatures that display human-like characteristics such as intelligence and apparent personality. These

traits probably account for a great deal of the growing popularity of cephalopods with the non-scientific

public. This popularity might be leveraged to promote conservation and to encourage research and sea

exploration. For instance, the cephalopods could serve as compelling representatives that draw

attention to the consequences of anthropological damage to vulnerable habitats, while exciting video

footage taken by submersibles could be used to highlight the benefits of deep sea exploration.

Researchers should also be on the lookout for new creative ways to disseminate knowledge and

augment public awareness and interest. Some recent novel outreach ideas include an interactive

museum exhibit that encourages visitors to participate in their own neuroscientific data analysis

("Surprising Minds" at the Brighton Sea Life Centre, UK), a graphic novella illustrating the results of a

scientific study ("Cuttlefish Brawl" by Shanna Baker and illustrated by Mark Garrison) and a virtual

reality game allowing visitors to see through the eyes of a cuttlefish ("Eye Sea" by Darmaillacq et al.

2016). However, even while we encourage and harness their popularity for good causes, we must also be aware of the pitfalls that come with fame and work to ensure that their popularity does not lead to over-exploitation by fisheries or the aquarium trade.

Public interest in cephalopods might also be channeled into non-traditional uses, such as citizen science via crowd-sourced data collection and analysis. Dozens to hundreds of photographs and videos of cephalopods are shared to social media every year. There is no reason why such media cannot be put to scientific use by posting them to an open access online repository as suggested by Xavier et al. (2015). Aquarists, divers and fishermen should be encouraged to share observations, photographs, videos and data with the cephalopod research community. We could also harness public aid in analyzing large data sets. The National Aeronautics and Space Administration (NASA, USA) encourages public participation in hunting for exoplanets ("Backyard Worlds: Planet 9"); we could use similar initiatives to crowd-source analysis of things like the manual assessment of cuttlefish body pattern components per the method first described Mathger et al. (2007) or for measuring the size of brain structures from digitized thin sections.

The development of a shared, open-access platform (CephsInAction website) dedicated to cephalopod research would facilitate progress. With a rapidly-changing climate and growing food demands, it is ever more important that we continue to generate and disseminate data that can guide fisheries and environmental practices in order to mitigate human impact. In the near future, we may face some difficulty in funding basic research. Hopefully, rather than let this constrain progress, we can use this as an opportunity to synthesize existing research and data. There are many researchers who may not have access to animals or proper equipment to conduct their own experiments, but could make use of shared data or media. Shared open-access tools and data should also help us pursue our research in a way which minimizes pain, suffering and lasting harm, by reducing the total number of animals that need to be manipulated and by promoting best-practices. In addition, researchers working with other invertebrates that are not currently regulated but will likely be in the future (e.g. bees, decapods), could refer to this platform in developing their own welfare practices. Finally, researchers could use this platform to share information with each other regarding the health and maintenance of animals in their care, and publicize their own research findings.

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Figures and Tables

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Fig. 1. The three potential avenues of prenatal stress transmission.

Fig. 2. A) The giant Pacific octopus, *Enteroctopus dolfleini* (photo by C.E. O'Brien)*;* B) the giant squid (Architeuthis dux (photo by Brian J. Skerry, National Geographic); C) the common cuttlefish, *Sepia officinalis* (photo by Hans Hillewaert); D) the chambered nautilus, *Nautilus pompilius* (photo by William Cho).

Fig. 3. Schematic representation of the categories of stress investigated.

Fig. 4. 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).

Fig. 5. Fecal corticosterone concentration (mean ng corticosterone mg^{-1} dry feces ± s.d.) over time, measured from fecal samples collected in 2016. No significant difference exists between groups (GLMM: $X^2 \geq 0.07$, n per data point = 4 (means calculated from the pooled feces of one to four individuals over two days), p = 0.79). Data are displayed as group means (dots) ± standard deviation (whiskers).

Fig. 6. A normal (ink-stained) *S. officinalis* egg (left) and a translucent 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.

Fig. 7. Large ponds once used for oyster aquaculture at the CABaNor Aquaculture Cooperative (Blainville sur Mer, Normandy). a) Overhead view, note the channels connecting the ponds to the sea at high tide (photo by O. Basuyaux). b) Close-up view of an experimental pond (photo by C. E. O'Brien).

Fig. 8. Schematic of the egg-laying site choice test: two modified cuttlefish traps were provided. One contained a potential hatchling predator, and both had six ropes, of a type and diameter known to "attract" cuttlefish eggs in the wild, affixed to them.

Fig. 9. Schematic summary of Chapter 1. Stress to the mother during egg-laying reduces her reproductive output and likely the quality of her eggs as well. At the same time, a naturally-occurring and an artificial stressor applied directly to developing embryos had no effect on hatching rate or size. The dashed arrow indicates a statistical tendency.

Fig. 10. Stage 30 *Sepia officinalis* embryo (approx. mantle length 6mm) seen through the transparent egg membranes*.* Photo by Nawel Mezrai.

Fig. 11. Cuttlefish eggs (a) *in situ*, 26 June, 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 2.5-3.0cm in length (Boletzky 1983). Photo by Olivier Basuyaux, of the Synergie Mer et Littoral (SMEL).

Fig. 12. Hatchling cuttlefish (approx. mantle length 10mm) displaying a disruptive body pattern on a uniform substrate. Photo by Anne-Sophie Darmaillacq.

Fig. 13. Three-month old juvenile cuttlefish (approx. mantle length 65mm). Photo by Caitlin E. O'Brien.

Fig. 14. Eyes of the cuttlefish *Sepia elongata* caught off the coast of Eilat (Gulf of Aqaba, Israel; photo AS Darmaillacq).

Fig. 15. 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 = oesophagus. Modified from Jozet-Alves et al. 2012a.

Fig. 16. 7-day-old cuttlefish's prey choice depending on whether they have been exposed to white crabs during embryonic development ('exposed') or not ('control'). *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 (Mathieu Guibé et al. 2012).

Fig. 17. Proportion of the cuttlefish (N=10 per group) that showed an optomotor response (OMR) to BWG (luminance only; black) or Pol (polarization; grey) 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. 2013).

Fig. 18. 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.

Fig. 19. Stage 30 embryo (less than 1 cm) showing a mottle-disruptive coloration inside the egg. It has also squirted ink; note the cloud ink in the perivitellin fluid. Note that the embryo is seen from under through a peeled *S. officinalis* egg (photo C.E. O'Brien).

Fig. 20. Schematic representation of the experimental design.

Fig. 21. Timeline of stress treatments and behavioral tests. All tests except for the threat response activity analysis occurred in 2015.

Fig. 22. 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. * indicates p < 0.05; *** indicates p < 0.001.

Fig. 23. 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).

Fig. 24. 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. ** indicates p < 0.01; # indicates a statistical tendency (p < 0.08).

Fig. 25. 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.

Fig. 26. 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.

Fig. 27. Prawn-in-a-tube (PIT) apparatus. The tube is composed of glass microscope slides held together by clear plastic. Four live shrimp are trapped between the slides. An aquarium pump keeps water circulating and the shrimp in motion. Cuttlefish drawing by Anne Quiédeville.

Fig. 28. Timeline of PIT sessions. Juveniles were tested at three and five weeks of age in two 45min sessions separated by an hour-long rest interval.

Fig. 29. The mean number of strikes per minute made by the maternal stress groups during their initial exposure to the PIT apparatus at three weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, SM = Stressed Mother.

Fig. 30. The mean number of strikes per minute made by the maternal stress groups during the retention trial exposure to the PIT apparatus at three weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, SM = Stressed Mother.

Fig. 31. The mean number of strikes per minute made by the maternal stress groups during their initial exposure to the PIT apparatus at five weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, SM = Stressed Mother.

Fig. 32. The mean number of strikes per minute made by the maternal stress groups during the retention trial exposure to the PIT apparatus at five weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, SM = Stressed Mother.

Fig. 33. The mean number of strikes per minute made by the embryonic stress groups during their initial exposure to the PIT apparatus at three weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, UM-PE = Unstressed Mother-Predator-Exposed, UM-LE = Unstressed Mother-Light-Exposed.

Fig. 34. The mean number of strikes per minute made by the embryonic stress groups during the retention trial exposure to the PIT apparatus at three weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, UM-PE = Unstressed Mother-Predator-Exposed, UM-LE = Unstressed Mother-Light-Exposed.

Fig. 35. The mean number of strikes per minute made by the embryonic stress groups during the initial exposure to the PIT apparatus at five weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, UM-PE = Unstressed Mother-Predator-Exposed, UM-LE = Unstressed Mother-Light-Exposed.

Fig. 36. The mean number of strikes per minute made by the embryonic stress groups during the retention trial exposure to the PIT apparatus at five weeks of age. For each individual cuttlefish, timing commenced at the moment of first detection and orientation towards the apparatus, and ends either when no strikes were made for three consecutive minutes, or when the experimental trial time (45min) had elapsed. Each group is plotted until its sample size falls below n = 5. UM-C = Unstressed Mother-Control, UM-PE = Unstressed Mother-Predator-Exposed, UM-LE = Unstressed Mother-Light-Exposed.

Fig. 37. Concentration of serotonin in head tissue of cuttlefish hatchlings from maternal stress groups (A) and embryonic stress groups (B). * indicates < 0.05, ** < 0.01, ** < 0.001. n: UM-C = 15, SM = 13, UM-PE = 15, UM-LE = 14.

Fig. 38. Ratio of serotonin to its main metabolite, 5-HiAA (5-HiAA/5-HT) in head tissue of cuttlefish hatchlings from maternal stress groups (A) and embryonic stress groups (B). * indicates < 0.05, ** < 0.01, ** < 0.001. n: UM-C = 14, SM = 13, UM-PE = 15, UM-LE = 13.

Fig. 39. Concentration of noradrenalin (NA) in head tissue of cuttlefish hatchlings from maternal stress groups (A) and embryonic stress groups (B). * indicates < 0.05, ** < 0.01, ** < 0.001. n: UM-C = 15, SM = 13, UM-PE = 15, UM-LE = 15.

Fig. 40. The cuttlefish brain: Anatomical location (A) and sagittal (B) and frontal (C) histological sections of the cuttlefish brain with the various masses and lobes labeled. VL = vertical lobe, SV = subvertical lobe, SFrL = superior frontal lobe, Fri = frontal inferior lobe, Prec = precommisural lobe, Ba = anterior basal lobe, Bp = posterior basal lobe, Pv = palliovisceral lobe, Pe = peduncle lobe, Br = brachial lobe, OL = optic lobes. Adapted from (Bidel 2015).

Fig. 41. Sagittal sections demonstrating that the vertical lobe (VL) of cuttlefish increases in both absolute and relative size compared to the rest of the supraesophageal mass from hatching (A) to adulthood (B). Adapted from Dickel et al. 2001 and Bidel, 2015.

Fig. 42. An illustration of the chemical process of phophohistone H3 labeling. The primary antibodies bind with the phophohistone H3, while the secondary antibody binds with the primary antibody to form a brown precipitate visible under a microscope.

Fig 43. Vertical lobe volumes of maternal stress groups (A) and embryonic stress groups (B) expressed as a percentage of subesophageal mass. n = 6 for all groups.

Fig 44. Cell division in VL (A) and LOL (B) of maternal stress groups indicated by phosphohistone labelling. n = 6 for all groups.

Fig. 45. Schematic summary of Chapter 2 illustrating the effects of the three stressors tested. The dashed arrow indicates a statistical tendency.

Fig. 46a. Heterogeneity Index (HI) against a uniform background on Day 1 reflects the ability to produce a uniform body pattern. Data are displayed as median (bars), inter-quartiles (boxes) and minimum/maximum values (whiskers). Lab-incubated cuttlefish show more disruptiveness (higher HI) than Sea-incubated* (permutation test, n=47, 51, alpha=0.05, p=0.02).

Fig. 46b. Mean Gray Value (MGV) match to a uniform background on Day 1 reflects the ability to produce general color resemblance. Data are displayed as median (bars), inter-quartiles (boxes), minimum/maximum values (whiskers) and outliers (circles). There is no significant difference between groups (permutation test, n=47, 51, alpha=0.05, p=0.95).

Fig. 47. Disruptive Score (/33) against a checkered background over time on Day 2 reflects disruptive body-patterning ability. Data are displayed as median (bars), inter-quartiles (boxes) and minimum/maximum values (whiskers). There is no significant difference between groups (permutation test, n=47, 51, alpha=0.05, p=0.77).

Fig. 48a. Latency to Capture (sec) is the time between detection and capture on Day 4. One extreme outlier was eliminated from each group before analysis. Data are displayed as median (bars), inter-quartiles (boxes), minimum/maximum values (whiskers) and outliers (circles). No significant difference exists between treatment groups (permutation test, n=28, 35, alpha=0.05, p=0.08).

Fig. 48b. Distance of detection (cm) on Day 4. Data are displayed as median (bars), inter-quartiles (boxes) and minimum/maximum values (whiskers). No significant difference exists between treatment groups (permutation test, n=29, 36, alpha=0.05, p=0.65).

Fig. 49. Comparison of the effects of spawning in the natural environment to spawning + incubation in the natural environment.

Fig. 50. A schematic representation of the range of variables that were found to influence post-natal behavior. Note that embryonic predator-exposure did not affect post-natal behavior.

Table 1. Composition of female stress groups by year. All data from 2015 and 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.

Table 2. Proportion of egg layers, size (DML and weight), lifespan after capture (days), the correlation between lifespan after capture and number of eggs laid and remaining oocytes (mean ± s.d.) of female cuttlefish. 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 Fisher exact test, all others with exact permutation tests (these calculations include both egg-layers and non-layers).

Table 3. Hatching rate and mean hatching size (dorsal mantle length (mm), mean ± s.d.) of eggs and offspring from the maternal stress experiment. Hatching rates were compared with a chi-squared test, while hatching sizes were compared with a two-tailed independent T-test.

Table 4. Hatching rate and mean hatching size (DML, mean ± s.d.) of eggs and offspring from the embryonic stress experiment. Hatching rates were compared with a Cochran-Mantel-Haenszel chi-squared test, while hatching sizes were compared with a two-way ANOVA.

Table 5. A summary of development in *Sepia officinalis*. Modified from Dickel et al. 2006. Sources are cited throughout the text.

Table 6. 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. Both group comparisons and post hocs are chi squared exact tests (sequential Bonferroni correction).

Table 7. Summary of behavioral test results in comparison to the unstressed control mothers.

Table 8. Statistical comparisons of groups and of presentation sessions of the maternal stress groups in six measures of performance in the PIT test at three weeks of age.

Table 9. Statistical comparisons of groups and of presentation sessions of the maternal stress groups in six measures of performance in the PIT test at five weeks of age.

Table 10. Statistical comparisons of groups and of presentation sessions of the embryonic stress groups in six measures of performance in the PIT test at three weeks of age.

Table 11. Statistical comparisons of groups and of presentation sessions of the embryonic stress groups in six measures of performance in the PIT test at five weeks of age.

Table 12. Attempted Capture Rate, Capture Rate and Success Rate during the initial encounter with prey on Day 4. No significant difference exists between treatment groups in these tests (Fisher Exact test, n=47, 51, alpha=0.05).

Table 13. Summary of prenatal stress experiments. Results refer to comparisons with the Unstressed Mother Control (UM-C) group. 5-HT = serotonin, NA = noradrenaline, VL = vertical lobe, LOL = left optic lobe.

Synthèse de « Les effets du stress prénatal sur la seiche *Sepia officinalis* »
par Caitlin E. O'Brien
Relecture par Nawel Mezrai

Introduction

En 1963, Niko Tinbergen a décrit une méthode d'investigation pour l'analyse comportementale, identifiant quatre perspectives analytiques directrices: mécaniques (c'est-à-dire les processus physiologiques et moléculaires qui causent un comportement), ontogénétiques (c'est-à-dire les événements au cours du développement qui affectent le comportement), adaptatifs (c.-à-d. dans lequel un comportement augmente la survie ou la reproduction) et phylogénétique (c'est-à-dire le degré auquel le comportement est façonné par l'ascendance). Ces quatre perspectives forment le fondement de l'éthologie, l'étude du comportement animal (Tinbergen 1963). À l'origine, les éthologues s'intéressaient principalement à la recherche fondamentale documentant le comportement animal. Cependant, depuis les années 1970, avec la création de la Société internationale pour l'éthologie appliquée (ISAE), les éthologues sont de plus en plus intéressés par les processus généraux qui peuvent expliquer les tendances générales du comportement animal et humain. Dans le même temps, l'un des principaux objectifs de l'ISAE et de la communauté éthologique est d'améliorer le bien-être des espèces captives dans les zoos, les aquariums, les laboratoires et les installations agricoles en augmentant notre capacité à équilibrer les besoins humains avec les besoins des animaux, qu'ils soient physiologiques ou comportementaux. Pour cette raison, une grande attention s'est récemment concentrée sur l'étude du stress. Bien que la définition exacte du stress soit parfois controversée, elle se réfère à une suite de changements physiologiques, morphologiques et comportementaux qui se posent face à des défis externes dans une tentative de rétablir l'homéostasie ou pour atténuer l'impact du facteur stressant.

Le stress peut avoir des effets «positifs» et «négatifs» sur les organismes. Lorsque le facteur de stress est à court terme et qui a été rencontré pendant l'histoire de l'évolution de l'espèce, la réponse au stress devrait être en mesure d'atténuer ses effets négatifs et d'augmenter la forme physique globale. Cependant, lorsque le facteur de stress est chronique ou nouveau, la réponse de stress de l'organisme peut réellement avoir un impact néfaste sur la santé et la condition physique que le stress lui-même. Ceci est bien illustré dans notre propre espèce: en cas de danger immédiat, comme un prédateur ou une automobile à venir, l'axe hypothalamique-hypophysaire-surrénal (HPA) va initier une série de changements physiologiques automatiques ("lutte ou fuite") qui permettent d'échapper à la situation aussi rapidement que possible (Cannon 1932). Cependant, à plus long terme, l'activation

continue du même axe HPA peut endommager divers systèmes corporels (par exemple, le système immunitaire), dégrader la santé (par exemple, troubles du sommeil) et réduire la qualité de vie (par exemple, l'anxiété). De même, de nombreux problèmes de santé et de société résultent d'une inadéquation entre nos réponses au stress évoluées et les défis modernes. Ces problèmes de santé ont de vastes implications sociétales, ce qui entraîne d'énormes dépenses dans les soins sanitaires et les services sociaux, ainsi que la perte de productivité et la baisse du rendement en milieu de travail (Greenberg et al. 1999). La prise de conscience croissante de ces effets négatifs du stress a engendré un grand nombre de travaux visant à mieux comprendre ces effets en nous-mêmes et sur l'évolution des espèces (Selye 1976).

Dans l'étude du stress, la période de reproduction, de frai et de développement embryonnaire est particulièrement intéressante en raison de son importance dans l'établissement de modèles de physiologie, de morphologie et de comportement futurs (Gottlieb et Wagner 1991; Bremner, Lewkowicz, et Spence 2012; Houdelier et al. 2013). En effet, le stress pendant ce temps (appelé «stress prénatal») peut avoir des effets profonds non observés lorsque le même stimulus se produit ailleurs dans le cycle de vie. Alors que le stress prénatal permet souvent aux organismes de prédire et de s'adapter aux défis présents dans l'environnement postnatal (Gluckman et Hanson 2004), cela peut aussi entraîner des problèmes de vie. Le stress prénatal chez l'homme a été lié à des troubles du comportement, de la cognition et de l'émotion, tels que le trouble déficitaire de l'attention avec hyperactivité (TDAH), le syndrome de stress post-traumatique (SSPT), la dépression, l'anxiété et la schizophrénie (Charil et al. 2010).

Il existe trois pistes possibles par lesquelles le stress prénatal peut exercer ses effets: 1) sur la mère elle-même (en affectant la fécondité, le comportement d'accouplement ou la ponte), 2) par la mère à la progéniture (p. Ex. Par transmission hormonale ou peut-être par spermatozoïdes) ou 3) perception directe et réaction au stress par l'embryon (Fig. 1). La compréhension de la contribution relative de ces trois voies potentielles de stress et de leurs interactions est nécessaire pour comprendre comment le stress peut avoir une incidence sur la santé, la société, le comportement et l'évolution des organismes. Par exemple, les facteurs de stress appliqués aux femelles au cours de la période de reproduction («stress maternel») ont montré une incidence sur la survie, le comportement, l'apprentissage et l'anxiété des descendants dans divers groupes tels que les primates, les rongeurs, les oiseaux et les poissons (Braastad 1998 pour revue; Schreck, Contreras-Sanchez, et Fitzpatrick 2001; Henriksen, Rettenbacher, et Groothuis 2011). Ces effets peuvent résulter d'une réaction directe du

stress par la mère elle-même (affectant le nombre ou la composition génétique de ses embryons) ou le transfert de la mère à la progéniture par l'approvisionnement ou des hormones dans le placenta ou le jaune d'œuf (Groothuis et al. 2005; Hayward et Wingfield 2004; Lemaire et al. 2000; Weinstock 2008). Les embryons eux-mêmes peuvent également percevoir et réagir aux facteurs de stress («stress embryonnaire») appliqués aux femelles au cours de la ponte ou de la naissance. Afin de distinguer les effets médiés par la mère et la perception directe, les embryons doivent être isolés de la femelle et ont les facteurs de stress leur sont directement appliqués. Cependant, il est actuellement difficile ou impossible de séparer une mère de sa progéniture en développement dans des espèces ayant un développement interne (mais voir Roberts 2017) ou des soins maternels de la progéniture après la naissance. Cependant, les espèces ovipares - celles dans lesquelles les embryons se développent à l'extérieur de la femelle - permettent de séparer la mère de la progéniture dès que les œufs sont pondus.
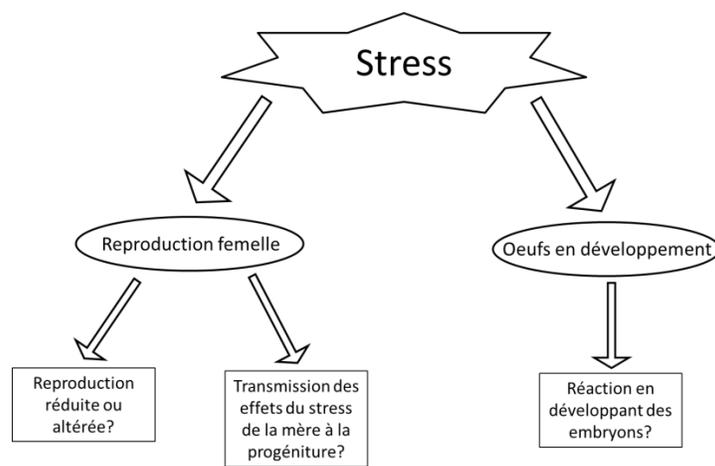
Figure 1. Les trois pistes possibles de transmission du stress prénatal.

Il y a aussi la question de la pertinence écologique des facteurs de stress particuliers pour les embryons. Les facteurs de stress naturels, tels que l'odeur d'un prédateur, devraient, théoriquement, susciter une réponse évolutive et adaptative. En effet, lorsque les indices de prédateurs sont appliqués aux animaux juvéniles et adultes, cela induit souvent une modification du phénotype ou du comportement adapté face à ce prédateur. Un exemple bien connu de ceci se produit chez les puces d'eau du genre *Daphnia*; lorsqu'un prédateur est détecté, ils développent des épines et des pointes qui réduisent la capacité du prédateur à les consommer facilement (Walls et Ketola 1989). En revanche, un stimulus artificiel qui ne se rencontre pas naturellement, comme la lumière vive ou les bruits forts, appliqué directement aux embryons en développement («stress artificiel») devrait confondre la capacité

de l'espèce à répondre, entraînant des réponses inadaptées. Par exemple, l'exposition à des niveaux élevés de bruit anthropique a été liée à la fois aux réponses au stress et aux changements de comportement chez les cétacés, ce qui peut avoir des conséquences aussi graves que l'échouage et la mort (Weilgart 2007).

Cette thèse s'inscrit dans une étude éthologique plus large intitulée "Effets de stress prénatals sur le développement précoce des comportements et des capacités cognitives", ou "PReSTO'Cog" pour abréger. PReSTO'Cog est une collaboration entre cinq laboratoires à travers la France. Chaque laboratoire étudie un modèle animal différent: la poule domestique, la caille japonaise, la truite, le poisson zèbre et la seiche européenne. Ces espèces représentent une gamme variée de groupes d'animaux: les invertébrés (seiches) et les vertébrés (poissons, oiseaux) ainsi que les espèces sauvages (seiches) et les espèces domestiquées (poule) et les poïkilotherms (seiche, poisson) et les homéothermies (oiseaux). Tous sont ovipares et nidifuges, ce qui permet à la progéniture d'être isolée expérimentalement de la femelle pendant le développement embryonnaire et sans l'influence post-natale de l'interaction maternelle. Enfin, ils sont également relativement autonomes à la naissance, ce qui permet un test de comportement immédiat de la progéniture. En comparant ces espèces phylogénétiquement éloignées, nous abordons le sujet du stress prénatal à partir des troisièmes et quatrième perspectives de Tinbergen - les indices adaptatifs et phylogénétiques - découvrant les pressions évolutives et les antécédents familiaux qui ont conduit au comportement que nous observons aujourd'hui dans ces espèces. En fin de compte, de telles idées pourraient s'appliquer à d'autres groupes d'animaux, y compris les mammifères, ce qui entraînerait des améliorations du bien-être humain et animal.

Le thème unifiant de ce projet est de déterminer si le stress prénatal induit des changements dans la progéniture et si le type de facteur de stress prénatal a eu une incidence sur la manière dont la progéniture réagit. Les effets des facteurs de stress maternel sur les descendants diffèrent-ils de ceux des facteurs de stress appliqués directement aux embryons eux-mêmes ? Est-ce que la réponse à un facteur de stress artificiel diffère de ce qui se produit en réponse à un phénomène naturel ? Les effets du stress prénatal sont évalués à travers une gamme de tests physiologiques, comportementaux et d'apprentissage sur les jeunes descendants. Nous recherchons également des indices sur les mécanismes de ces effets, en particulier les preuves endocrinologiques pour le transfert des hormones du stress de la mère à la progéniture et des changements dans la croissance et la morphologie du cerveau. Ces questions sondent le comportement des premières perspectives de Tinbergen - l'analyse

mécanique et ontogénétique - des processus biologiques innés et des influences externes qui interagissent pour produire un répertoire comportemental particulier.

Modèle d'étude

Dans cette thèse, le stress prénatal est étudié à partir des quatre perspectives éthologiques décrites par Tinbergen (1963) à travers un large spectre de tests physiologiques, comportementaux, cognitifs et neurobiologiques pour déterminer si et comment le stress prénatal affecte la seiche. Deux types principaux de stress ont été étudiés: les facteurs de stress ont été appliqués à la fois aux femelles reproductrices (stress maternel) et aux embryons en développement (stress embryonnaire). Deux types de facteurs de stress embryonnaires ont été appliqués: l'un naturel et l'autre artificiel (figure 2).

Beaucoup d'invertébrés sont à la fois ovipares et nidifuges, ce qui en fait des candidats potentiels pour étudier les façons dont le stress peut affecter une espèce comme discuté ci-dessus. En outre, les invertébrés représentent 97% des espèces sur terre, vivent dans presque toutes les parties de la planète et démontrent une diversité impressionnante dans les modes de vie et de comportement. À côté des arthropodes, les mollusques invertébrés sont le deuxième phylum le plus peuplé et, comme les arthropodes, ont colonisé avec succès les habitats maritimes, d'eau douce et terrestre. Certains mollusques ont également évolué de plusieurs façons convergentes avec les vertébrés (par exemple, le «poumon» des gastéropodes terrestres), ce qui en fait de bons modèles comparatifs. Le céphalopode est un groupe de mollusques composé d'environ 700 espèces existantes (Roger T. Hanlon and Messenger 1998), divisé en quatre groupes principaux: poulpes, calmars, seiches et nautilus. Ils sont exceptionnellement sophistiqués, démontrant des capacités perceptuelles avancées, l'apprentissage, la mémoire, la résolution de problèmes et la plasticité (Hochner, Shomrat, et Fiorito 2006). Les céphalopodes ont des systèmes nerveux fortement centralisés (Budelmann 1995) et peu importants, avec des rapports cerveau / poids corporel supérieurs à ceux des poissons et des reptiles et approchant ceux des mammifères et des oiseaux. Le système nerveux a été bien étudié au cours du siècle dernier, avec des tâches cognitives spécifiques localisées dans des lobes spécifiques du cerveau (Dickel et al. 2013 pour revue). Les céphalopodes affichent des adaptations convergentes (par exemple, l'effet céphalopode et les vertébrés) et divergentes (par exemple, propulsion par pont céphalopodique par rapport à la propulsion musculaire des poissons) à des défis évolutifs comme autres groupes d'animaux (Packard 1972; Hochner, Shomrat, et Fiorito 2006). Leur sophistication et leur position d'invertébrés signifient que les céphalopodes ont beaucoup d'intuition pour offrir des contrepoints aux modèles de vertébrés plus traditionnels, et leurs comportements uniques et extraordinaires remettent souvent en

question nos notions sur les principes généraux qui sous-tendent le comportement animal. Il a même été posé que la concurrence entre les poissons et les céphalopodes a façonné l'évolution de nombreux traits dans les deux groupes pendant le mésozoïque (Packard 1972).

Comme les autres modèles PReSTO'Cog, la seiche commune *Sepia officinalis* (Linnaeus 1758) est un excellent modèle pour étudier les effets du stress prénatal parce qu'il est ovipare et nidifuge. *S. officinalis* est également une espèce importante sur le plan commercial et scientifique: des pêches existent à la fois dans l'Atlantique et la Méditerranée (Dunn 1999) et elle est cultivée dans plusieurs laboratoires et aquacoles (Pascual 1978; Forsythe, DeRusha, et Hanlon 1994; Domingues, Sykes, and Andrade 2002). En effet, avec *Octopus vulgaris*, c'est l'une des espèces de céphalopodes les plus étudiées. Plus important encore, les seiches et autres céphalopodes ont l'avantage d'être phylogénétiquement éloignés des modèles animaux plus typiques comme les rats et les singes: ils sont un groupe d'invertébré séparé des vertébrés par des centaines de millions d'années d'évolution distinctes, ce qui leur permet de servir de référence indiquez si les effets de stress que nous observons dans différentes espèces sont des produits d'ascendance partagée ou des développements évolutifs distincts (troisième et quatrième questions de Tinbergen). En fin de compte, une meilleure compréhension des effets du stress prénatal chez *S. officinalis* donnera un aperçu général des processus et des stratégies par lesquels les organismes survivent.

En outre, cette recherche aidera à combler les lacunes dans la connaissance des besoins biologiques spécifiques de la seiche, en particulier ceux concernant le logement, la reproduction et les marqueurs comportementaux du bien-être. L'aperçu de ce travail améliorera la capacité des aquaculteurs et des chercheurs à établir des normes de soins et des pratiques courantes. Cela est particulièrement nécessaire en raison de l'inclusion récente des seiches et d'autres céphalopodes dans la législation européenne en matière de protection des animaux (directive 2010/63/UE) régissant l'utilisation des animaux dans les procédures expérimentales. Cela pourrait aussi contribuer aux futurs efforts d'élevage et de relance captifs, ce qui risque de devenir de plus en plus nécessaire avec la demande alimentaire croissante et le changement climatique. Par exemple, *Sepia apama*, la seiche australienne géante, a récemment été désignée comme «presque menacée» en raison de la pêche intensive d'agrégats de reproduction dans des endroits spécifiques et fait face à une diminution prévue de 20% des niveaux de population si les taux de capture actuels se poursuivent (UICN 2017). Et de façon alarmante, les journaux britanniques locaux signalent que les pêcheurs profitent de la réglementation lâche et récoltent des seiches dans le sud-ouest du Royaume-Uni à des taux sans précédent en réponse

à la demande en Asie (Jones, 2017; Payne, 2017). Si cette tendance se poursuit, nous aurons bientôt de meilleures incitations écologiques et économiques pour augmenter artificiellement les stocks naturels. Une stratégie particulièrement économique consisterait à récupérer et à cultiver les œufs posés sur les pièges à la seiche et normalement perdus lorsque l'engin est nettoyé à la fin de la saison des récoltes (Blanc and Daguzan 1998). Déjà, les efforts pilotes visant à cultiver des œufs de seiche et des éleveurs dans de vastes étangs extérieurs ont été entrepris avec un succès modéré (Roussel et Basuyaux, 2016). Le travail présenté ici permettra d'en informer ces efforts.

Aperçu

Dans cette thèse, le stress prénatal est étudié à partir des quatre perspectives éthologiques décrites par Tinbergen (1963) à travers un large spectre de tests physiologiques, comportementaux, cognitifs et neurobiologiques pour déterminer si et comment le stress prénatal affecte la seiche. Deux types principaux de stress ont été étudiés: les facteurs de stress ont été appliqués à la fois aux femelles reproductrices (stress maternel) et aux embryons en développement (stress embryonnaire). Deux types de facteurs de stress embryonnaires ont été appliqués: l'un naturel et l'autre artificiel (figure 2).

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                    │  Stress prénatal │
                    └─────────────────┘
                             │
            ┌────────────────┴────────────────┐
   ┌─────────────────┐              ┌──────────────────────┐
   │ Stress maternel │              │  Stress embryonnaire │
   └─────────────────┘              └──────────────────────┘
                                              │
                                  ┌───────────┴───────────┐
                        ┌──────────────────┐   ┌────────────────────┐
                        │ Stresseur naturel│   │ Stresseur artificiel│
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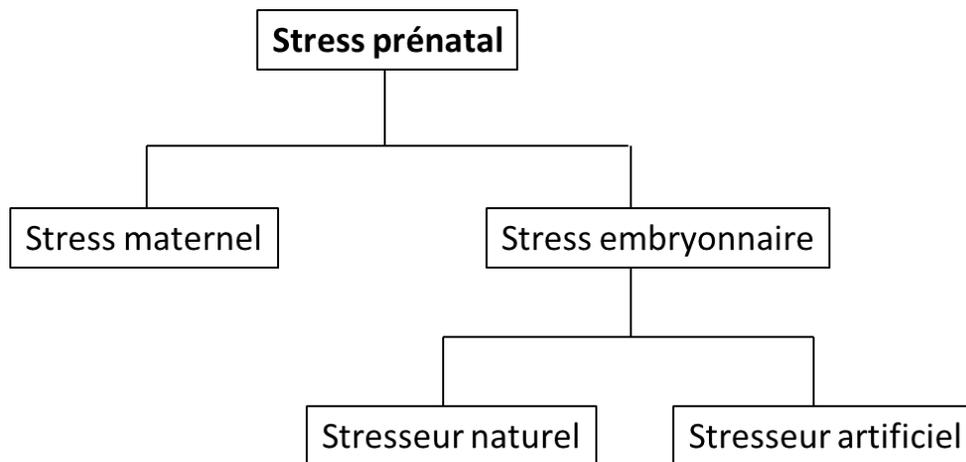
Figure 2. Représentation schématique des catégories de stress étudiées.

Nous avons conçu une expérience à long terme pour tester les effets du stress maternel et embryonnaire sur le rendement reproducteur, la croissance embryonnaire, le comportement, l'apprentissage et la neurobiologie chez *S. officinalis*. Puisque l'espace confiné des réservoirs et le retrait occasionnel de l'eau sont souvent des aspects inévitables de la capture de seiches et de l'aquaculture, nous avons utilisé ces facteurs comme facteurs de stress aigu chronique et répété pour créer un groupe

de traitement «mère stressée». Nous avons comparé ce groupe aux «mères témoins non stressées» (UM-C). Dans certains tests, nous avons également comparé ces deux groupes à un groupe «mère sauvage» (WM). Dans une deuxième expérience, nous avons examiné les effets du stress embryonnaire. Nous avons subdivisé les œufs UM-C en trois groupes; un groupe témoin (UM-C), un groupe exposé à un facteur de stress d'origine naturelle et un groupe exposé à un facteur de stress artificiel tout au long du développement embryonnaire. En tant que facteur de stress naturel, nous avons sélectionné le bar *Dicentrarchus labrax*, un prédateur commun dans la Manche qui se nourrit de petites seiches (UM-PE). En tant que facteur de stress artificiel, nous avons sélectionné des épisodes de lumière artificielle brillante (LED) chronométrés au hasard tout au long de la journée au cours du développement embryonnaire. Nous avons ensuite appliqué une batterie de tests à la progéniture de ces groupes de stress après l'éclosion afin d'évaluer ses effets.

Chapitre 1

Au chapitre 1, nous présentons des données démontrant que le stress maternel réduit nettement la ponte des seiches. Cette différence n'a pas pu être expliquée par la taille des femelles ou le temps de survie: Il n'y avait pas de différence de poids moyen ou de LMD entre les groupes de stress et les femelles des deux groupes ont survécu un peu plus de deux semaines après leur capture avant de mourir naturellement. En plus de réduire le nombre d'œufs pondus par SM, moins d'œufs SM ont éclos. Le taux d'éclosion de l'UM-C (57,14%) est tombé à mi-portée des taux d'éclosion déclarés dans la littérature sur l'aquaculture, alors que le taux de SM était très faible (22,27%). Une telle différence dans le taux d'éclosion n'a pas été observée dans les groupes de stress embryonnaires, suggérant que les facteurs de stress appliqués aux mères peuvent avoir un impact plus important sur l'éclosion que les facteurs de stress appliqués directement à la progéniture. Ni le stress maternel ni embryonnaire n'a été associé à des différences significatives dans la taille des éclosions.

Fait intéressant, 25% de SM et aucun des UM-C qui ont produit des œufs viables pondent partiellement ou entièrement translucides. Dans la plupart des cas, la membrane de l'œuf de *S. officinalis* est imprégnée d'encre noire de la mère, bien que des œufs translucides soient parfois observés en aquaculture et dans la nature. Dans notre expérience, le ratio de SM affichant ce trait ne diffère pas significativement de l'UM-C, mais il constitue une tendance statistique et nous croyons qu'il peut être lié au traitement de stress. La présence d'œufs translucides dans une couvée pourrait servir de marqueur de la présence de facteurs de stress pendant la ponte, donnant des indications indirectes sur les conditions de ponte à l'état sauvage ou la pertinence d'un environnement d'élevage en captivité.

En plus de tester les effets du stress sur la reproduction, nous avons également cherché un indicateur simple de stress chez les femelles pondeuses. Chez de nombreux autres animaux (par exemple, les oiseaux, les mammifères, les poissons), le cortisol et / ou les glucocorticoïdes sont sécrétés en réponse au stress et interviennent dans la plupart de ses effets (Moberg 1991). L'échantillonnage fécal est relativement simple et non intrusif, nous avons donc prélevé des échantillons quotidiens et de la corticostérone quantifiée. Malheureusement, les niveaux de corticostérone fécale ne se sont pas avérés être un indicateur particulièrement bon du stress chez cette espèce (au moins pendant la reproduction). Nous avons également tenté de relier le matériel de reproduction inutilisé des femelles décédées à des niveaux de stress. Les œufs prêts à la fécondation, à l'encapsulation et à la ponte sont stockés dans la cavité palléale qui, à la mort, peut contenir jusqu'à 500 ovocytes (Boletzky 1988; Laptikhovsky et al. 2003). Puisque les ovocytes inutilisés dans la cavité palléale des femelles décédées indiquent un potentiel de reproduction inutilisé, nous avons examiné le nombre restant au décès, en supposant qu'il y aurait plus de résidus dans la SM au décès. Cependant, il n'y avait pas de différence entre les deux groupes, et donc ce trait ne peut pas être utilisé comme un mesure de les niveaux de stress. Bien que ces deux facteurs ne puissent constituer de simples marqueurs de stress chez les seiches, les aquaculteurs et les biologistes des pêches pourraient un jour utiliser un nombre réduit d'œufs et l'absence d'encre dans les œufs pour indiquer la présence de stress maternel chez les seiches.

Les données présentées au chapitre 1 montrent que le stress peut influencer la seiche par au moins une des trois voies potentielles en réduisant la production reproductive totale des femelles et la qualité des œufs produits. Cela fait allusion à l'existence de compromis entre la réponse du stress maternel et sa production reproductive. Plus important encore, il montre que le stress peut avoir un impact sur la seiche avant même que ses œufs ne soient pondus, un facteur que nous devons prendre en compte lors de la formulation des explications mécaniques et adaptatives (première et troisième perspective de Tinbergen) du comportement de la seiche. Il suggère également une ligne directrice très spécifique pour l'aquaculture de la seiche: minimiser la manipulation des femelles reproductrices afin de maximiser le rendement reproducteur et la qualité des œufs.

Chapitre 2

Après avoir examiné en profondeur les jeunes seiches au moyen de deux articles de synthèse, le chapitre 2 présente les résultats de nombreuses expériences comportementales, de tests d'apprentissage et de tests neurobiologiques.

Modelage du corps

Nous avons mesuré la «perturbation» de la structure du corps de la seiche en utilisant le protocole «d'hétérogénéité» (HI) décrit dans (Di Poi et al. 2014) sur un fond uniforme en damier gris et un damier noir et blanc. Dans tous les groupes, le HI moyen sur le fond perturbateur était constamment plus élevé que celui sur l'uniforme, suggérant que toutes les seiches ont ajusté leurs modèles de corps à l'arrière-plan. Des différences significatives ont également été observées entre les groupes: Dans notre expérience, le stress maternel et artificiel (lumière LED) a augmenté la perturbation moyenne du schéma corporel affiché, bien que les différences entre les groupes de stress maternels étaient plus importantes et plus significatives qu'entre les groupes de stress embryonnaires. Nos résultats suggèrent également que la captivité femelle pendant la ponte peut induire un biais de groupe pour une plus grande perturbation chez sa progéniture, puisque la progéniture des mères sauvages avait l'HI le plus élevé globalement.

Prédation

Les motivations de l'alimentation et les capacités prédatrices ont été testées 4 jours après l'éclosion en plaçant la seiche dans une petite arène avec des crevettes pendant 15 minutes. Près de deux fois plus de descendants UM-LE ont tenté de capturer que UM-C ou UM-PE. Les UM-LE étaient également capables de détecter les proies à une distance significativement plus grande que les UM-PE, bien qu'ils soient statistiquement identiques au groupe témoin. Il convient également de noter que même si une plus grande proportion de crevettes capturées par UM-LE, elles n'étaient pas de meilleurs prédateurs que les autres groupes, puisque la capture et le succès ne différaient pas significativement entre les groupes (près de 100%).

Latéralité visuelle

Suite à des travaux démontrant l'existence d'un biais de rotation à gauche chez de jeunes seiches, nous avons testé la latéralisation visuelle de la seiche de cinq jours dans un labyrinthe en forme de T. Aucun biais au niveau du groupe n'a été trouvé dans le groupe témoin. Parmi tous les autres groupes, seul le groupe WM a affiché une préférence au niveau du groupe vers le côté gauche le jour 5. Le fait que la préférence oculaire existe dans le groupe MW suggère que lorsque la ponte et le développement précoce se produisent dans la nature, la maturation de la préférence d'utilisation de l'œil gauche est plus rapide.

Activité nocturne

Nous avons mesuré les niveaux d'activité nocturne de base chez les nouveau-nés en utilisant un logiciel de suivi comportemental. Nous n'avons trouvé aucune différence entre les groupes de stress embryonnaires, tandis que la progéniture mère stressée était associée à une plus grande activité que la progéniture mère témoin: la distance totale parcourue et le temps passé à bouger étaient plus élevés en moyenne que la progéniture UM-C. Nous avons également observé une tendance statistique pour les nouveau-nés MW à parcourir une plus grande distance que les UM-C. Ces résultats indiquent que la progéniture des mères stressées était plus active, passant plus de temps en mouvement et par conséquent parcourant une plus grande distance que la progéniture de l'UM-C.

Réponse aux menaces

Nous avons également suivi l'activité en réponse à l'introduction d'odeurs de prédateurs ou d'eau vierge dans leur environnement immédiat. Une réduction de l'activité a été observée dans tous les groupes après l'ajout de l'un ou l'autre stimulus dans le prolongement d'une tendance à la diminution progressive de l'activité au fil du temps mais aucun groupe n'a présenté de réponse à l'odeur du prédateur différente de celle de l'eau vierge.

Tests d'apprentissage PIT

Nous avons appliqué un test d'apprentissage standard aux mineurs à trois et cinq semaines d'âge. Il n'y avait pas de différences significatives entre les groupes dans la mesure de la performance du test PIT entre le stress maternel et les groupes témoins. Parmi les groupes de stress embryonnaires à l'âge de trois semaines, la progéniture exposée à la lumière (UM-LE) a pris beaucoup moins de temps pour atteindre le critère que les deux autres groupes, suggérant une capacité à apprendre plus rapidement. Outre les indications selon lesquelles UM-LE pourrait apprendre plus rapidement à trois semaines, il n'y avait pas d'autres effets des traitements du stress prénatal sur les capacités d'apprentissage et de mémoire dans la tâche PIT.

Monoamines

Nous avons quantifié la concentration de sept monoamines et de leurs principaux métabolites dans le tissu neural des nouveau-nés. Tous les groupes de stress (SM, UM-PE et UM-LE) présentaient des concentrations de sérotonine significativement plus faibles que le groupe témoin. Dans le même temps, le taux de renouvellement de la sérotonine (5-HiAA / 5-HT) était significativement plus faible chez les

sujets SM et UM-LE que chez les témoins, mais significativement plus élevé chez les sujets UM-PE. Un rapport plus élevé de la sérotonine à son métabolite principal indique un taux de renouvellement plus élevé (Bidel 2015), ce qui signifie un taux plus rapide de synthèse de la sérotonine. Dans les céphalopodes, la sérotonine agit comme un neurotransmetteur, avec des effets moteurs et chromatiques, ainsi qu'un rôle important dans la transmission synaptique et l'induction de la potentialisation à long terme (Messenger 1996; Shomrat et al. 2010). Ainsi, les concentrations plus faibles de sérotonine mesurées dans tous les groupes de stress, plus les taux de renouvellement plus faibles de SM et d'UM-LE pourraient être des signes de déficits neurologiques. Les concentrations de noradrénaline (NA) étaient significativement plus faibles dans les SM et les UM-PE et significativement plus élevées dans les UM-LE que chez les témoins. Dans les céphalopodes, la NA est associée à une respiration plus rapide et à des niveaux plus élevés de conscience et de cognition (Messenger 1996; Bidel 2015). Ainsi, les différences entre les groupes pourraient indiquer des niveaux différents de «vigilance» en raison du stress prénatal, avec UM-LE en état d'alerte élevé et les autres groupes de stress à un niveau réduit de vigilance. Ensemble, les différences dans la concentration de sérotonine, le taux de renouvellement de la sérotonine et les concentrations de noradrénaline indiquent que le stress prénatal affecte la chimie du cerveau de la seiche qui naît.

Volumétrie et neurogenèse

En utilisant la volumétrie et le marquage à la phosphohistone, nous avons quantifié la croissance et la division cellulaire dans le cerveau. La mesure des différents lobes cérébraux par rapport à la taille globale du cerveau a révélé des lobes verticaux (VL) significativement plus petits dans tous les groupes de stress par rapport au groupe témoin. Dans le même temps, la coloration à la phosphohistone a montré une neurogenèse plus élevée (c'est-à-dire une croissance) dans les lobes optiques (OL) et VL de SM. Le VL et l'OL sont des sièges d'apprentissage et de mémoire chez les céphalopodes, et leur taille relative et leur croissance sont corrélées avec l'apprentissage et la mémoire (Dickel, Chichery, et Chichery 2001). La plus petite taille des LV dans tous les groupes de stress suggère qu'ils sont cognitivement compromis à l'éclosion par rapport au groupe témoin. En outre, la réduction de la neurogenèse dans la VL et l'OL de SM suggère que ce groupe a été retardé dans le neurodéveloppement. La neurogenèse supérieure dans le VL et l'OL de SM suggère que le stress transmis de la mère à la progéniture a un effet plus fort sur la taille de la VL et la croissance que le stress embryonnaire.

L'existence de différences comportementales et neurobiologiques dans les groupes de stress maternels et embryonnaires démontre que le stress peut être transféré de la mère à la progéniture ainsi que vécu directement par les embryons eux-mêmes. Le manque général d'apprentissage et les différences de mémoire étaient inattendus étant donné les effets du stress prénatal chez les autres espèces et les différences de taille de la LV à l'éclosion.

Chapitre 3

Dans une expérience distincte mais liée, nous avons analysé les effets de l'environnement d'incubation en comparant un groupe d'œufs recueillis en laboratoire («Lab») à un groupe laissé in situ jusqu'à la quasi-éclosion («Mer»). Nous avons ensuite mesuré la taille, les modèles corporels et les capacités de prédation de la progéniture. ML était similaire entre les deux groupes de traitement, et la structuration du corps contre un fond quadrillé ne différait pas significativement entre les groupes. Nous avons mesuré cinq aspects du comportement prédateur lors de la première rencontre des nouveau-nés avec leurs proies préférées, la crevette (Wells 1958): Tentative de capture, taux de capture, taux de réussite, temps de capture et distance de détection. Aucune de ces variables ne différait significativement entre les groupes et le taux de capture était très élevé (entre 85% et 90% selon Messenger 1968). Ceci suggère que les nouveau-nés élevés en laboratoire seraient également capables de se nourrir pendant cette période critique de croissance. Une différence existait entre les groupes: les nouveau-nés incubés en laboratoire présentaient un nombre significativement plus élevé d'individus HI (plus perturbateurs) que les nouveau-nés «Mer». Cela suggère que l'environnement d'incubation peut affecter la structure du corps dans certains contextes.

Le chapitre 3 démontre que l'incubation dans un environnement de laboratoire n'affecte pas fortement trois aspects fondamentaux du comportement et de la survie chez les nouveau-nés de seiche: la croissance embryonnaire, la structure du corps et la capacité de prédation. Le manque général de différences entre les nouveau-nés naturellement et artificiellement incubés renforce la confiance dans les données expérimentales existantes. Nos résultats sont également encourageants du point de vue de la conservation; la seiche artificiellement incubée pourrait augmenter les stocks de poissons ou remplacer la seiche sauvage dans certaines situations.

Les effets de l'environnement d'incubation étaient semblables à l'effet de l'environnement de reproduction, sauf dans le sens de la perturbation du modelage du corps. Cela suggère que l'environnement dans lequel les femelles pondent leurs œufs et l'environnement dans lequel ces œufs

se développent sont des sources potentielles de variation comportementale future. Comme le stress, ces effets peuvent être transmis indirectement par la mère ou directement par la progéniture.

Discussion

Les résultats de toutes les expériences comportementales, d'apprentissage et neurobiologiques menées au cours de cette thèse sont résumés dans le tableau 1. La comparaison du stress maternel isolément a démontré que le stress affecte la reproduction des femelles (diminution de leur production reproductive) et qu'elles la transmettent à leur progéniture (modification de leur comportement et de leur neurobiologie). L'étude de facteurs de stress distincts uniquement pour l'embryon indiquait que les embryons réagissaient eux-mêmes avec un comportement, un apprentissage et une neurobiologie modifiés. Ainsi, il semble que lorsque le stress prénatal survient, les trois voies possibles de la transmission de l'effet de stress - à travers la mère, de la mère à la progéniture et la réaction de la progéniture elle-même (voir Fig. 1) - sont possibles chez les seiches.

Tableau 1) Résumé des expériences de stress prénatal. Les résultats se rapportent aux comparaisons avec le groupe Unstressed Mother Control (UM-C). 5-HT = sérotonine, NA = noradrénaline, VL = lobe vertical, LOL = lobe optique gauche.

	Production d'oeufs	Survie des oeufs	Taille d'éclosion	Modelage du corps	Biais de rotation	Comportement prédateur	Modèles d'activité	Réponse aux menaces	Apprentissage et mémoire	Le développement du cerveau
	Données 2015/2016	Données 2015/2016	Données 2015/2016	Données 2015	Données 2015	Données 2015	Données 2015	Données 2016	Données 2016	Données 2015/2016
Exposition au stress maternel	*Nombre réduit d'œufs pondus et éclos, production possible d'œufs transparents*	*Réduction du nombre d'œufs éclos*	Les résultats variaient selon l'année, mais aucun effet global	*Perturbation plus élevée*	Aucun effet	Aucun effet	*La distance parcourue, la vitesse et le temps passé à bouger étaient plus élevés*	Aucun effet	Aucun effet à l'un ou l'autre âge testé	*Des concentrations plus basses de 5-HT et de NA, un taux de renouvellement de 5-HT plus élevé, une VL plus faible, une division cellulaire plus élevée dans LOL et VL*
L'exposition prédatrice embryonnaire (stresseur naturel)	N'est pas applicable	Aucun effet	Aucun effet	Aucun effet	Aucun effet	Aucun effet	Aucun effet	Aucun effet	Aucun effet à l'un ou l'autre âge testé	*Des concentrations plus basses de 5-HT et de NA, plus petit VL*
L'exposition à la lumière embryonnaire (facteur de stress artificiel)	N'est pas applicable	Aucun effet	Aucun effet	*Tendance à une plus grande perturbation*	Aucun effet	*Taux de tentatives de capture plus élevé*	Aucun effet	Aucun effet	*Apprentissage plus rapide à 3 semaines*	*Concentration de 5-HT inférieure, NA supérieur, VL plus faible*

Notamment, et contrairement à nos prédictions, les seiches ont répondu plus fortement au facteur de stress embryonnaire artificiel que celui qui est naturel. Le bar et la seiche sont des espèces bien établies dans la Manche, et on sait que le bar est antérieur à la seiche (Blanc et Daguzan 1999). À partir d'expériences antérieures, nous savions que la présentation prénatale de l'odeur du prédateur est associée à des changements de latéralisation visuelle (Jozet-Alves et Hebert 2012). Ces faits, associés à la flexibilité comportementale impressionnante des seiches, nous ont permis de prédire que la sélection

naturelle aurait produit une réaction comportementale à ce prédateur. Au lieu de cela, les seiches exposées aux prédateurs n'ont montré aucune différence par rapport au groupe témoin dans aucun de nos tests de comportement, bien qu'ils aient montré des changements dans la chimie et la morphologie du cerveau. La présence de changements neurobiologiques dans les descendants des œufs exposés aux prédateurs démontre que les embryons de seiche sont physiquement affectés par le stress prénatal, alors que l'absence de réaction comportementale suggère une habituation aux indices de prédateurs prénataux, comme indiqué à l'article 4. En effet, d'autres espèces peuvent adapter leurs réactions innées anti-prédateurs en fonction du niveau de menace. De nombreux gastéropodes, un groupe proche des céphalopodes, montrent des réactions aux odeurs de prédateur, et ces réactions sont plus fortes lorsqu'elles sont associées à des signaux d'alarme de conspécifiques (p. Ex. McCarthy et Fisher 2000; Jacobsen et Stabell 2004; Dalesman et al. 2006). Dans le même temps, nous savons que la seiche s'habitue à certains stimuli prénataux (Romagny et al. 2012), il semble donc plausible que l'odeur prédateur, associée à l'absence de signal d'alarme provenant des nombreux autres embryons de seiche présents, n'a pas entraîne des changements de comportement parce que la seiche a appris à le considérer comme non menaçant.

Contrairement au facteur de stress naturel, le facteur de stress artificiel a montré des différences comportementales et d'apprentissage, ainsi que des différences dans la chimie et la morphologie du cerveau. Étant donné qu'un décalage entre une réponse au stress et le facteur de stress lui-même entraîne souvent des effets secondaires négatifs, nous avons prédit que le facteur de stress artificiel (ce qui n'était pas quelque chose que les espèces auraient pu expérimenter historiquement pour faire évoluer une réaction par sélection naturelle) aurait un effet "négatif" sur la progéniture. Cependant, le groupe exposé à la lumière a montré des effets «positifs» dans les comportements testés: de meilleures capacités d'apprentissage et une moindre motivation alimentaire conduisant à un taux de prédation plus élevé. Une hypothèse d'expliquer cela est que l'exposition prénatale à un facteur de stress sans précédent a induit une réponse d'alarme généralisée qui a entraîné une accélération du développement. Alternativement, on sait que l'exposition prénatale à la lumière affecte le rythme circadien dans une gamme d'animaux, y compris les seiches, qui peuvent éclore plus tôt ou plus tard en fonction du régime léger (Paulij et al. 1991). Dans ce cas, la lumière peut avoir un développement accéléré.

Enfin, nous avons également pu faire des comparaisons indirectes entre l'incubation artificielle et la reproduction artificielle et avons constaté des impacts similaires sur les comportements testés (pas

d'effet sur le comportement prédateur, les effets mineurs sur le modelage du corps). Cela montre que l'environnement, ainsi que les facteurs de stress spécifiques, peuvent avoir un impact sur le comportement post-natal de la progéniture, ce qui doit être pris en compte lors de la conception et l'interprétation d'expériences avec de jeunes seiches.

Les résultats présentés nous permettent de commencer à formuler un modèle approximatif de stress prénatal dans les seiches des quatre perspectives de Tinbergen. En ce qui concerne les mécanismes de transmission du stress prénatal (première perspective de Tinbergen), nous émettons l'hypothèse que chez la seiche, les femelles pourraient transférer de la corticostérone à la progéniture dans l'œuf. Cela a fait l'objet d'un intérêt particulier chez les vertébrés, en particulier les oiseaux, qui ont été trouvés pour imprégner leur jaune d'œuf avec des hormones de stress qui affectent l'axe hypothalamique-hypophysaire et surrénal (HPA) de leur progéniture (p. Ex. Charil et al. 2010). Nous n'avons pas trouvé de preuve définitive à l'encontre de cette hypothèse ou contre cette hypothèse, mais notre travail suggère que d'autres possibilités possibles de transmission du stress de la mère à la progéniture sont possibles, puisque la progéniture de la mère stressée différait d'autres groupes de stress néanmoins. Espérons que, au fur et à mesure que les techniques analytiques moléculaires et neurologiques deviennent plus raffinées et largement disponibles, nous pourrons déterminer les mécanismes exacts qui conduisent à des changements de comportement après le stress prénatal.

Nos expériences ont eu beaucoup de perspicacité pour se lancer sur les perspectives deuxième et troisième de Tinbergen, l'ontogénique et l'adaptatif. D'après les différences de comportement du groupe du stress artificiel, on voit que, de façon ontogénique, la période prénatale ne peut pas être ignorée comme source d'influence comportementale, puisque les facteurs stressants subis par l'embryon semblent modifier les comportements futurs. Les générations antérieures de chercheurs ont largement négligé cette fois, car on présumait que les embryons ne pouvaient pas percevoir le monde extérieur, bien que ce point de vue ait été renversé au cours des dernières décennies. Nos résultats fournissent des preuves supplémentaires (avec Darmaillacq, Lesimple, et Dickel 2008; Romagny et al. 2012; Jozet-Alves et Hebert 2012), que la seiche peut percevoir le monde en dehors de la membrane de l'œuf et modifier son comportement futur en réponse. D'un point de vue adaptatif, nous avons constaté que le stress maternel pourrait être un meilleur indicateur des perspectives d'avenir pour les seiches embryonnaires que l'expérience embryonnaire directe: beaucoup plus de différences comportementales étaient apparentes en association avec le stress maternel qu'avec l'embryonnaire. D'autre part, on pourrait soutenir que les réponses au facteur de stress embryonnaire étaient plus clairement

«adaptables» (meilleure apprentissage et prédation), ce qui suggère que l'expérience embryonnaire directe est plus instructive pour la seiche après tout. De toute évidence, les réponses des seiches sont complexes, et d'autres expériences sont nécessaires pour comprendre l'interaction des forces sélectives conduisant le comportement post-natal induit par le stress.

Enfin, nous avons pu aborder le stress prénatal à partir de la quatrième perspective de Tinbergen, le phylogénique, en comparant nos résultats de la seiche aux autres modèles PReSTO'Cog. Les quatre modèles (poulets, caille, poisson zèbre et truite) ont montré des effets sur le stress maternel ainsi que sur les facteurs de stress embryonnaires artificiels et naturels. Ces résultats impliquent que l'existence de réponses au stress est fortement conservée dans les groupes phylogénétiques. En outre, il semble que, comme les seiches, les effets du stress prénatal chez les animaux se manifestent sur une multitude de niveaux et sont compliqués par de nombreuses interactions.

Résumé

Les résultats de cette thèse démontrent sans équivoque que le stress prénatal affecte les seiches et suggère fortement que trois des trois avenues potentielles de stress (chez la mère, de la mère à la progéniture et de la progéniture en développement directement) semblent fonctionner chez cette espèce. La figure 3 illustre la gamme des influences prénatales qui ont été trouvées pour modifier le comportement post-natal. En comparant les groupes de stress, nous avons fait plusieurs déductions provisoires sur le stress prénatal chez les seiches des trois premières perspectives de Tinbergen. Du point de vue mécaniste, nous n'avons trouvé aucune preuve de transfert de corticostérone, mais certaines possibilités que les effets du stress prénatal sont médiées par des changements dans la neurobiologie. Lors de l'examen du stress de la perspective ontogénique, nous avons constaté une confirmation supplémentaire que l'expérience embryonnaire modifie les comportements futurs dans cette espèce. D'un point de vue adaptatif, nous avions des indications mitigées sur le fait que le stress maternel ou un facteur de stress embryonnaire artificiel avait un effet plus fort et plus positif sur le comportement de la progéniture. Enfin, en comparant nos résultats de seiches à ceux des autres modèles animaux de Presto'Cog et en utilisant la quatrième perspective de Tinbergen, nous voyons que les effets de stress prénatal semblent être partagés par une large gamme de groupes d'animaux.
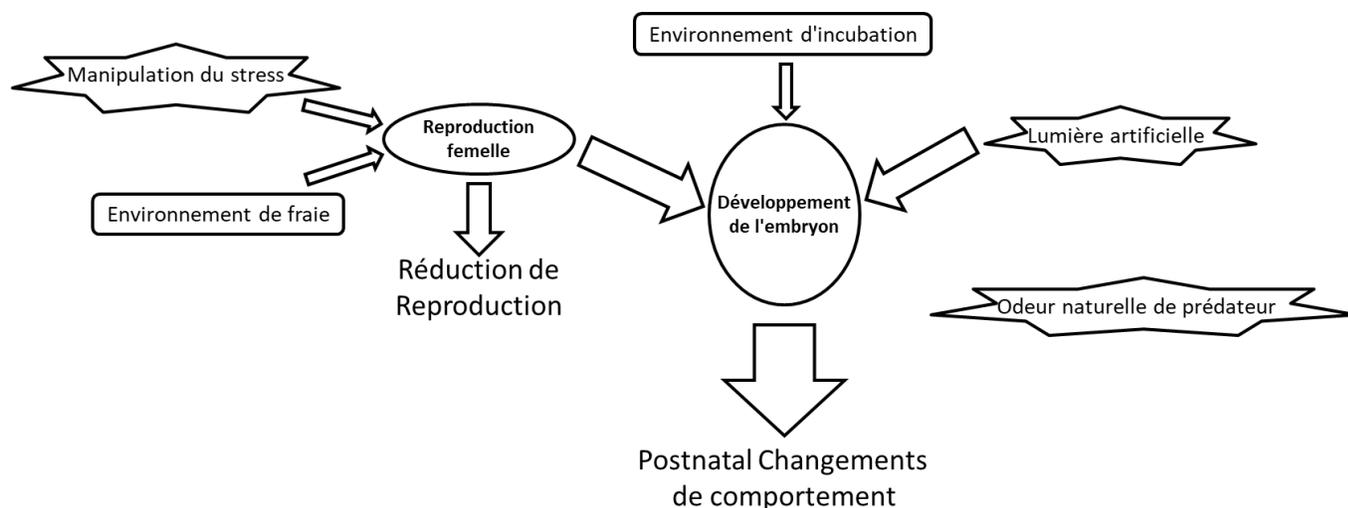
Figure 3. Une représentation schématique de la gamme de variables qui ont été trouvées pour influencer le comportement post-natal. Notez que l'exposition au prédateur embryonnaire n'a pas d'incidence sur le comportement post-natal.

Travail futur

Afin de mieux comprendre les mécanismes de l'effet du stress, notre laboratoire teste actuellement les réponses des embryons directement, alors qu'ils sont encore dans l'œuf, en observant les changements dans la respiration ou les préférences alimentaires (Mezrai, en préparation). Ceci est possible grâce à certaines caractéristiques expérimentales des œufs de seiche, y compris le manque de soins parentaux et une membrane semi-transparente. L'identification et le test d'autres espèces ayant des traits similaires révèlent si les tendances observées ici sont répandues dans tout le règne animal ou un autre exemple de caractère distinctif des céphalopodes. En particulier, il serait particulièrement intéressant de voir si la transmission du stress maternel et le stress embryonnaire existent dans les invertébrés «sociaux» comme les fourmis et les abeilles, qui ont une structure sociale complètement différente de celle de la plupart des autres groupes d'animaux.

En étudiant le stress prénatal chez les animaux, les éthologues peuvent comprendre ce qui peut être utilisé pour améliorer le bien-être des animaux ainsi que la santé humaine et la société. Une meilleure compréhension du stress prénatal dans un large éventail d'espèces pourrait entraîner des améliorations dans la façon dont nous traitons les femelles gestantes dans l'agriculture et les laboratoires. Par exemple, l'existence d'effets de stress prénatal de la mère manipulée à la progéniture dans cette espèce et dans un large éventail d'autres (p. Ex. Le saumon, Sigholt et al. 1997; les renards bleus cultivés, Braastad 1998; bovins, Grandin, Oldfield, et Boyd 1998) ajoute une nouvelle incitation à l'industrie agricole pour éviter des choses comme les caisses de gestation pour les porcs, ce qui peut non

seulement diminuer le bien-être des animaux, mais aussi la quantité et la qualité du produit à base de viande. De même, une meilleure compréhension du stress prénatal peut conduire à des moyens d'atténuer ses effets négatifs sur les enfants et les adultes, et peut-être même pour empêcher qu'il ne se produise même en premier lieu. Par exemple, les enfants humains de femmes qui ont été connus pour avoir subi un stress pendant la grossesse pourraient être ciblés pour une attention maternelle supplémentaire ou une thérapie prématurée, car les soins parentaux ont atténué certains des effets négatifs du stress prénatal chez les rongeurs (examiné dans Francis et Meaney 1999). Cette recherche nous aiderait également à prédire quelles sortes de choses peuvent être stressantes pour un fœtus. Ici, nous avons vu une habituation possible en réponse à une odeur de prédateur que nous nous attendions à être très stressante. Cela suggère que nous devrions être conscients de la possibilité que les embryons humains puissent également s'habituer aux facteurs de stress. En effet, les fœtus humains sont habitués à des stimuli vibriotactile répétés perçus dans l'utérus (Leader et al. 1982).

Resumé

Le stress prénatal est un sujet d'intérêt éthologique croissant en raison de ses effets sur la santé humaine et le bien-être des animaux. Cette thése de doctorat s'intéresse à la seiche Sepia officinalis, un modèle pratique dans lequel la progéniture en développement peut être séparée de leurs mères pour examiner diverses sources potentielles de stress en isolement expérimental. Plusieurs catégories de facteurs de stress ont été appliquées aux embryons et aux juvéniles et la progéniture résultante a été testée dans une série d'épreuves physiologiques et comportementales. L'objectif était de déterminer si différents types de stress prénatal affectent la seiche et, dans l'affirmative, comment ces effets se transmettent. Les données présentées démontrent que les stresseurs appliqués aux femelles reproductrices (stress maternel), ainsi que les stresseurs appliqués directement aux embryons (stress embryonnaire), affectent le comportement postnatal (y compris la structuration corporelle, la latéralisation cérébrale, la prédation et les schémas d'activité) la mémoire et / ou la neurobiologie (y compris les concentrations et le renouvellement de la monoamine, la taille des différents lobes cérébraux et la division cellulaire). Les résultats mettent en évidence la présence de trois voies par lesquelles le stress peut exercer des effets: sur le nombre de descendants produits par la femelle, la transmission de la femelle à sa progéniture et directement sur la progéniture elle-même. Les expériences ont également démontré qu'un facteur de stress complètement artificiel (lumière forte) affectait un éventail plus large de comportements chez la progéniture qu'un stress naturel (odeur de prédateur). Enfin, les données ont montré que l'environnement d'incubation et d'élevage peuvent également affecter la progéniture et méritent donc une attention particulière dans la formulation et l'interprétation des expériences avec cette espèce. Ces découvertes informent à la fois les pratiques de bien-être des seiches et d'autres céphalopodes (par exemple, réduisent la manipulation pour maximiser la reproduction) et élucident et renforcent les principes éthologiques qui s'appliquent au stress animal en général (par exemple la transmission des effets de stress de la mère à la progéniture). Compte tenu des informations fournies ici et dans de nombreuses autres études, la seiche et d'autres céphalopodes devraient continuer à servir de modèles comportementaux en éthologie et en biologie en général.

Mots clés: odeur de prédateur, stress maternel, facteur de stress artificiel, structuration du corps, latéralisation cérébrale, activité, réponse à une menace, comportement prédateur, apprentissage, test PIT, monoamine, volumétrie, marquage phosphohistone

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Abstract

Prenatal stress is a subject of growing ethological interest due to its effects on human health and animal welfare. This Ph.D. thesis utilizes the cuttlefish *Sepia officinalis*, a convenient model in which developing offspring can be separated from their mothers to examine various potential sources of stress in experimental isolation. Several categories of stressors were applied to cuttlefish and cuttlefish eggs and the resulting offspring were tested in a range of physiological and behavioral tests. The goal was to determine if various types of prenatal stress affect cuttlefish, and if so, how these effects are transmitted. The data presented demonstrate that both stressors applied to reproducing females (maternal stress), as well as stressors applied directly to embryos (embryonic stress), affected post-natal behavior (including body patterning, brain lateralization, predation and activity patterns), learning, memory and/or neurobiology (including monoamine concentrations and turnover, the size of various brain lobes and cell division). The results highlight the presence of three pathways by which stress can exert effects: on the number of offspring produced by the female, transmission from the female to her offspring and directly on the offspring themselves. The experiments also demonstrated that a completely artificial stressor (bright light) affected a wider range of behaviors in offspring than a natural-occurring one (predator odor). Finally, the data showed that incubation and spawning environment can also affect offspring, and thus deserve attention in the formulation and interpretation of experiments with this species. These findings inform both welfare practices for cuttlefish and other cephalopods (e.g. reduce handling to maximize reproduction) as well as elucidating and reinforcing ethological principles that apply to animal stress in general (e.g. the transmission of stress effects from mother to offspring). Given the insight provided here and in numerous other studies, cuttlefish and other cephalopods should continue to serve as behavioral models in ethology and biology in general.

Keywords: Predator odor, maternal stress, artificial stressor, body patterning, brain lateralization, activity, threat response, predatory behavior, learning, PIT test, monoamine, volumetry, phosphohistone labeling