EFFECTS OF EMBRYONIC EXPOSURE TO PREDATOR CUES ON PRE- AND POST-HATCHING ANTIPREDATOR BEHAVIOUR IN COMMON CUTTLEFISH (*SEPIA OFFICINALIS*)

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By Lisa Jane Teichroeb

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Head of the Department of Biology
112 Science Place, University of Saskatchewan
Saskatoon, Saskatchewan
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Abstract

Since neonates are often the age-class most susceptible to predation, there should be strong selective pressure on prey for the early development of successful antipredator behaviour. The ability to assess predation risk as early as the embryonic stages may increase an individual's survival, as it would allow young individuals to be better adapted to current predation risk, since present conditions are often a good short-term indicator of future conditions. I exposed embryonic cuttlefish (*Sepia officinalis*) to the odour of a predator and tested both the responses of the embryos to this stimulus, and the latent effects of both long (approximately 3 weeks)- and short (a few days)- exposure on the behaviour of newly-hatched juveniles, in particular the efficiency of cryptic behaviour on uniform and sandy substrates. Exposure to novel odours, whether they were predators or non-predators, increased the ventilation rate of embryos. This may be adaptive, because it helps an individual survive first encounters with unknown potential dangers before they have opportunity to collect information about a novel stimulus. Long-term exposure to predator odour increased the camouflage efficiencies of juveniles on uniform substrates. On sandy substrate, the exposure did not affect camouflage, but increased the extent of sand digging behaviour. Juveniles were also larger in size at hatching when exposed to predators compared to those that were not. These results were not seen in individuals with only short-term exposure to predator. Short-term exposure also had no effect on camouflage efficiencies on uniform or sandy substrates, or on sand digging behaviour. The results of my thesis indicate that high predation risk during embryonic development induces behavioural and morphological changes in camouflage expression and body size in cuttlefish hatchlings. The behavioural plasticity may provide survival benefits for newly hatched individuals, but may come at a cost in terms of body size. Such behavioural and morphological plasticity may have an impact on predator-prey dynamics and organization of communities.

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Table of Contents

List of Figures

Chapter 1: Introduction

1.1. Selective force of predation on young

There is strong selection on prey to be efficient and effective at detecting and evading predators (Lima and Dill 1990; Ferrari et al. 2010), since the cost of not successfully avoiding predators is death. A diversity of mechanisms allow prey to display efficient responses to chemical, visual, or tactile cues from predators, such as changes in behaviour, life history, and morphology (Benard 2014). For example, common frog tadpoles (*Rana Temporaria*) respond to cues from predators by altering their body shape through a deepening of their tail fin, which may improve their ability to flee from predators (Laurila et al. 2001). These mechanisms may be particularly beneficial for young, especially for those that lack parental care; most mortality occurs early in life because juveniles are often more vulnerable due to their smaller relative size, limited body strength or constrained escape potential. Thus, selection should favour the early development of successful antipredator behaviour (Fuiman and Magurran 1994).

Early successful antipredator behaviour may be achieved through genetic predisposition for predator recognition, non-genetic maternal effects, or learning to recognize threatening stimuli before or shortly after birth (Stratmann and Taborsky 2014). For example, it has been shown that the ability of salamander larvae and tadpoles to recognize chemical cues produced by predators may be genetically determined (Petranka et al. 1987). In addition, larval streamside salamanders (*Ambystoma barbouri*) exposed to predatory flatworms or sunfish hatch later, at a larger size, and at a more advanced developmental stage than those grown in the absence of predators, thereby increasing their probability of survival through the vulnerable early stages (Sih and Moore 1993). A non-genetic maternal effect is a situation where the phenotype of an organism is determined not only by the environment it experiences, but also by the environment of its mother. For example, in some species, larger females (due to increased food intake) produce larger young, which may be less susceptible to predation than smaller young (Tollrian 1995).Young prey may also learn to recognize cues from predators using personal and/or social information or may learn to respond as a result of surviving their first encounter with a novel predatory species (Ferrari and Chivers 2011). Since the risk of encountering a predator changes in time and space, the best way to adaptively respond to predator presence or absence is to constantly update information regarding the relative risk associated with a given predator (Ferrari and Chivers 2009c). Stratmann and Taborsky (2014) suggest that environmental sampling of predation risk by

individuals combined with an innate (i.e., without any prior exposure) predisposition to correctly identify predators appears to prepare young best for the environment in which they grow up as juveniles and has been seen in cichlids (*Simochromis pleurospilus*) (Stratmann and Taborsky 2014). Learning to adaptively respond to novel predators has been demonstrated to significantly increase an individual's survival during subsequent predator encounters (Berejikian et al. 1999; Mirza and Chivers 2000). For example, Mirza and Chivers (2000) showed that brook trout (*Salvelinus fontinali*) trained to recognize a predator were better able to later evade predators during staged encounters than non-trained brook trout.

The ability to distinguish predators from non-predators can also decrease the amount of energy spent in unnecessary antipredator behaviour and allow the animal to spend more time and energy in other activities, such as foraging. This ability can be seen in response to risk experienced by prey as embryos. For example when eggs of ringed salamanders (*Ambystoma annulatum*) are exposed to chemical cues from predators, post-hatching larvae show reduced activity and greater shelter-seeking behaviour, whereas larvae exposed to non-predator cues do not (Mathis et al. 2008). Because the early life stages are often the most vulnerable to predation, particularly (1) in animals lacking parental care (Laurila et al. 2001), (2) when predation risk is variable in space and time, and (3) when cues of predation are reliable, one would predict that plastic predation-induced responses in these organisms will be present at a very early age.

1.2. Embryonic experience

The environment in which embryonic development occurs is known to affect the expression of subsequent behaviour (Bridges and Gutzke 1996). Studies have shown that embryos can retain information gained from chemical (Sneddon et al. 1998), auditory (Hepper 1996), and visual (Darmaillacq et al. 2008) cues into post-embryonic life stages. In past studies, learning by embryos has been primarily associated with maternal/auditory recognition or food preference (Hepper 1996; Sneddon et al. 1998). For example, Sneddon et al. (1998) showed that chicken (*Gallus gallus domesticus*) embryos exposed to strawberry smell were more likely to have an olfactory and gustatory preference for strawberry two days after hatching than those that were not exposed during the embryonic stage. Most research on the effects of exposure to stressful stimuli during embryonic development has focused on post-embryonic behaviour that appears to be abnormal or maladaptive (Vallee et al. 1997; Braastad 1998; Buitelaar et al. 2003). Prenatally stressed animals, such as rats (*Rattus*

norvegicus) are reported to show delayed motor development, reduced exploratory and play behaviour, and impairments of learning ability, social behaviour, and sexual and maternal behaviour (Vallee et al. 1997; Braastad 1998). Rhesus monkeys (*Macaca mulatta*) prenatally stressed with daily unpredictable noise stimuli show reduced muscle tone, poorer coordination, slower response speeds, delayed self-feeding (Schneider 1992). However, a biased underestimate of the adaptive potential of such effects, induced by what is known as Maternally-Derived Stress (MDS), is possible if they are not viewed within an ecologically relevant or a life-history optimization framework (Sheriff and Love 2013). Phenotypic offspring responses to MDS exposure can be physiological, behavioral and morphological, and are commonly not mutually exclusive (Sheriff and Love 2013). Giesing et al. (2011) found that stickleback (*Gasterosteus aculeatus*) females under greater predation risk produced eggs with greater stress hormone (glucocorticoid) levels and higher oxygen consumption, and as juveniles, the offspring exhibited tighter shoaling behaviour even before being exposed to a threat compared to control offspring.

In recent years, more studies have also focused on the adaptive value of embryonic exposure to stressful stimuli, such as predator cues (Mathis et al. 2008; Ferrari et al. 2010; Ferrari and Chivers 2011), and a growing number of studies suggest that the development of antipredator behaviours may depend on the environment that animals experience during ontogeny (Turner et al. 2006). For example, studies have shown that fish and amphibian species, such as fathead minnows (*Pimephales promelas*) (Ferrari and Chivers 2006) and ringed salamanders (Mathis et al. 2008), can develop predator and non-predator recognition during embryonic development using conspecific alarm cues (Mathis et al. 2008; Ferrari and Chivers 2009a and b). In addition, individuals reared in a predator-free environment may respond to a predator cue differently than those reared with predators (Bridges and Gutzke 1996). For example, salamander larvae (*Ambystoma texanum*) reared in habitats containing fish seek refugia in response to fish chemical cues, whereas larvae reared without fish do not (Kats 1988). The basis for predator-avoidance behaviour among individuals and populations may reflect more strongly either the heritable basis of behaviour or the effects of living in a predator-rich or predator-free environment (Bridges and Gutzke 1996). Prenatal sensory learning may be crucial to an individual's survival (Hepper1996; Darmaillacq et al. 2008), since it allows for behavioural plasticity, which may be necessary for increasing an individual's fitness into variable environments since present conditions may be a good short-term indicator of future conditions. Essentially, embryonic experience allows young to develop behaviours that are more suitably adapted to current environmental conditions and

thus give them a greater chance at survival. Embryonic exposure may be particularly important for juvenile survival in species that lack parental care. This is because hatchlings are on their own to forage for food and protect themselves against predators. Thus, information they can process early on in life (i.e. during embryonic development) about their surroundings could be beneficial.

1.3. Study system

One prey species, the common cuttlefish (*Sepia officinalis*), was used in my experiments. The common cuttlefish (referred to herein as cuttlefish) is a necto-benthic (swim actively just above the bottom in a body of water) species of coleoid cephalopod occurring predominantly on sandy bottoms from the coastline (2-3m depth) to approximately 200m depth (Guerra 2006). Their geographical distribution covers the Mediterranean Sea and the waters of the Eastern Atlantic from southern Norway and northern England to the north-western coast of Africa. Spawning occurs in shallow water, with peaks at water temperatures from 13 to 15°C (Jereb and Roper 2005). In the North Eastern Atlantic, this occurs between April and July. Eggs, which are 8-10 mm in diameter and blackened with ink at the time of laying are attached in grape-like clusters to seaweed, shells, debris and other substrates. They hatch after 30 to 90 days, depending on water temperature. Growth rate of juveniles varies directly with temperature and inversely with size of hatchlings at birth (Jereb and Roper 2005).

Cuttlefish hatch as fully functioning precocial individuals that are completely autonomous, with similar behaviour and morphology to adults, and their capacity for learning is well documented (Agin et al. 1998; Dickel et al. 2000; Darmaillacq et al 2004; 2006; 2008; Guibe et al. 2010; 2012). Studies that have looked at embryonic learning in cuttlefish focused on visual imprinting of prey and prey preference (Darmaillacq et al. 2004; 2006; 2008; Guibe et al. 2010; 2012). For instance, cuttlefish's preference for shrimp can be changed by early visual learning during embryonic and postembryonic life. This change in preference has been related to a form of food imprinting as well as evidence of behavioural plasticity within these species. In addition, Guibe et al. (2012) found that juvenile cuttlefish previously pre- or post-natally exposed to white crabs preferred black crabs to shrimp. This shows that cuttlefish embryos are able to learn several different characteristics of their prey at once. These results indicate for the first time that prey generalization occurs as early as the embryonic stages in cuttlefish. Such cognitive abilities could confer important adaptive advantages in processing

information about prey likely to be available in the egg-laying environment at hatching and during dispersal of juveniles.

Cuttlefish eggs, initially laid with a deposit of ink, which is thought to aid in camouflage, becomes translucent and increases in permeability a few weeks before hatching due to dilation of the egg capsule (Romagny et al. 2012). In addition, the eyes are fully developed by this stage in development (Lemaire 1970) and can detect and process external visual cues (Darmaillacq et al. 2008; Guibe et al. 2012; Romagny et al. 2012). There are a total of 30 stages of embryonic development that have been defined in cuttlefish (Lemare 1970), separated by the development of mainly morphological features, but include some behavioural features as well. At stage 23 (about 4 weeks before hatching, at 18 ± 2ºC (SD)), the first spontaneous mantle contractions can be observed, and at stage 25 (3 weeks before hatching) the first pigments in the retina are observed. Stage 30 (about 1 week before hatching) is the last stage before hatching. Romagny et al. (2012) clearly demonstrated that tactile and chemical systems are functional from stage 23, whereas the visual system is functional only from stage 25 (Romagny et al. 2012). The most observable movements that occur *in ovo* are mantle contractions, as well as ventilatory contractions (King and Adamo 2006). Mantle contractions are a pumping action involving the entire mantle musculature in response to salient stimuli, whereas ventilatory contractions only involve contraction of the gill folds, and can be used as an estimation of respiration (Romagny et al. 2012). In adult cuttlefish, variation in the frequency of mantle contractions, as well as in respiration, have been shown to be a reliable and measurable response to salient chemical stimuli (Boal and Golden 1999). Chemoreceptors in cuttlefish are found in olfactory organs just below and behind the eye, as well on the suckers of the arms.

Cuttlefish are well known for expressing a variety of cryptic chromatic patterns, including in antipredator contexts. Langridge (2007) organized cuttlefish defensive behaviour into two broad categories: primary and secondary. Primary defensive behaviour includes deceptive resemblance and cryptic colouration, as well as sand digging when on sandy substrates and requires no exposure to predator cues. Secondary defence is used when a predator is approaching and is thought to startle or intimidate the approaching predator (Langridge et al 2007). Thus primary defence can be thought of as predator avoidance behaviour, and secondary defence as antipredator response behaviour. In this study, I focus on primary defence in juveniles. In cuttlefish, crypsis is achieved through homochromy, which is comprised of matching the brightness, pattern, and texture of the background, and includes specific and complex postural and chromatic components to mimic objects in its

environment, such as stones and algae, and other substrates (Hanlon & Messenger 1988). In primary defence, this cryptic colouration is aided by lying motionless on top of a substrate. There are four basic categories for cryptic colouring (Hanlon and Messenger 1988), which are categorized as follows:

(i) Uniform – There is little or no variation in contrast and brightness compared to the background. This display is generally observed on open uniform substrates.

(ii) Stipple – The overall pattern is uniform, with the expansion of numerous small dark chromatophores and conspicuous, well-defined risen white spots localized on specific body parts.

(iii) Mottle – There are patches of dark chromatophores of irregular shapes and varying sizes. It is sometimes expressed only with yellow chromatophores, resulting in a lighter mottle pattern.

(iv) Disruptive – Characterized by bold transverse or longitudinal chromatic components. This serves to break the natural outline of the body on a variegated substrate, essentially causing the cuttlefish to no longer look like a cuttlefish (Poirier et al. 2005).

Newly hatched cuttlefish preferentially show a disruptive pattern, even when placed on a uniform background (Poirier et al. 2005), and very few hatchlings express sand digging behaviour before 3-6 days after hatching (Poirier et al. 2004). However, young cuttlefish improve their sand digging ability and progressively get better at adapting their body patterns to uniform backgrounds throughout postembryonic development (Poirier et al. 2004; 2005). Cuttlefish express a deimatic display, characterized by a pair of dark posterior mantle spots as well as darkening of the pupil and eye ring, and the fin line (Langridge 2007), to smaller, visual predators, such as juvenile sea bass. The display is not shown in response to larger visual predators (Langridge 2009). Naive juvenile cuttlefish also respond to different predators in different ways (Langridge et al. 2007, Langridge 2009). For example, naive juveniles respond to sea bass with a different visual signal than in response to dogfish and crab (Langridge et al. 2007). These predator-specific responses by juveniles that had no previous exposure to predators suggest that cuttlefish display a degree of innate predator recognition.

Currently, few studies have looked at the effects of embryonic exposure to predators on post-hatching behaviour in cuttlefish (Guibe et al. 2012). Most of the studies that have focused response of embryos to visual stimuli have not used actual predators, but have used non-relevant objects such as styrofoam cut-outs (King and Adamo 2006). In addition, it has

not been clearly shown if cuttlefish can learn to recognize a novel predator during embryonic development. Cuttlefish have many predators (sharks, fish, dolphins, and other cuttlefish), so developing effective antipredator behaviour early on in life may provide adaptive advantages and prolong survival.

1.4. Research objectives

My overall objective was to examine whether the embryos are able to assess predation risk. In my thesis, I present a series of experiments divided into two data chapters, showing how embryos respond to direct predator cues, and how long- and short-term embryonic exposure to these cues affects camouflage ability in hatchlings. I used cuttlefish embryos and juveniles to answer the following questions:

Do embryos respond to predator odour? Previous work has shown that embryos do respond to visual, tactile, and chemical stimuli at certain stages of development (Romagny et al. 2012), however, it is unclear whether embryos are able to distinguish between predators and non-predators at these stages. In Chapter 2, I tested whether cuttlefish embryos increased their ventilation rate in response to predator odours. Increased respiration may improve the movement of water across the gill-folds and increase the sampling rate of the odour by the embryo. Increased respiration may also be indicative of a stress response. Since juvenile cuttlefish do show some levels of innate predator recognition, I predicted that embryos exposed to predator odours would increase their respiration more than embryos exposed to control odours.

Does embryonic exposure to predator odours affect predator avoidance behaviour in hatchlings? In Chapter 3, I investigated if different predator-related embryonic experiences would affect the subsequent behaviours of newly-hatched cuttlefish. I ran a 2-way ANOVA for analysis, where I exposed embryos to one of four cues (cues from predators fed a conspecific diet, cues from predators fed a heterospecific diet, cues from a non-predator, or a blank water control), for two durations (short-term exposure of 1-3 days or long-term exposure of 19-21 days). I then tested the primary defensive behaviour of hatchlings by testing their camouflage expression on a variety of backgrounds without the presence of any odour cues. The long-term exposure to predator odour throughout the incubation period could perhaps appear as a predictor of a high-predation environment. Juveniles could thus behave as if a threat was potentially around even without chemical

evidence of a predator nearby. If individuals are constantly updating information on predation risk, one would expect to see similar effects on behaviour when exposed to predator odour for only a few days prior to hatching. However, short-term exposure to predator odour may not be enough to reduce uncertainty of a high-predation environment, and so juveniles may not employ much effort in defence without a direct threat present.

1.5. Anticipated significance

My research is aimed at understanding fundamental questions about predation risk assessment. Many studies have investigated predation risk assessment and embryonic learning in a wide variety of species. However, very few of these studies have been done on invertebrates, which is the focus of my thesis. I investigate whether prey process information during embryonic development and use it to adjust their behaviour during post-embryonic development.

Chapter 2: Recognition in the egg: the direct response of cuttlefish embryos to predator odour

2.1. Introduction

Since neonates are often the age-class most susceptible to predation, there should be strong selective pressure on prey for early development of successful antipredator behaviour (Fuiman and Magurran 1994). The ability to assess predation risk as early as the embryonic stages may increase an individual's survival (Ferrari et al. 2010), as it would allow young individuals to be better adapted to current predation risk. This is because even in variable environments present conditions are often a good short-term indicator of future conditions. In species that lack parental care, the environment embryos develop in may be particularly important for juvenile survival, in addition to an innate ability to detect predators.

Predator recognition and avoidance in aquatic ecosystems often involves detection of olfactory cues from predators (Wisenden 2000; Kelley & Magurran 2003). Thus, chemical cues may play an important role in embryonic development in cuttlefish, since tactile and chemosensory systems develop at an even earlier stage than vision. This makes sense from a biological perspective, since tactile and chemosensory stimuli can permeate through the inked egg more easily than visual stimuli (Romagny et al. 2012). Though viewed as a primarily visual species, cuttlefish also respond to biologically important chemical cues through increased ventilation (Boal and Golden 1999; King and Adamo 2006), and embryos have also been shown to increase mantle contractions in response to chemical stimuli at certain stages of development (Romagny et al. 2012). However, to my knowledge, no study has been carried out on whether embryos also increase their respiration in response to predator odours.

In the following study, I investigated the ability of cuttlefish embryos to respond to predators odours. The goal of the experiment was to determine whether the embryos increased their ventilation in response to predator odours more than in response to non-predator odours. I hypothesized that a greater increase in ventilation rate would be seen in embryos exposed to predator odours compared to embryos exposed either control odour, since embryos have been shown to respond to important chemical stimuli and naive juveniles do some levels of innate predator recognition I exposed embryos to two types of predator odours, one of predators fed with conspecifics and the other of predators fed with commercial fish pellets. Prey animals often exhibit higher antipredator responses to chemical cues of predators fed conspecifics of the prey than those fed another diet (Chivers & Mirza 2001). I

compared the embryos' change in ventilation rate from before and after exposure to the odours with the change in ventilation rate of embryos exposed to seawater (control) and water containing sea urchin odour (control). I included sea urchin odour as a control to make sure any increase in ventilation was due to exposure to predator odour, and not just any novel odour.

2.2. Methodology

Test Species

I used cuttlefish eggs laid by wild females, which were obtained from trawling off Luc-sur-Mer, Calvados, France, and kept at the Centre de Recherches en Environnement Côtier (CREC), Luc-sur-Mer, France in May and June 2013. Eggs, initially laid in clusters by approximately 10 to 13 females to plastic meshes available in the traps, were separated and put randomly in circular shallow sieves (25cm x 11cm) to ensure optimum developmental conditions. Tanks were supplied with running oxygenated sea water at a temperature of 18°C ± 2°C (mean ± SD).The photoperiod was adjusted to a 16:8 h light:dark cycle. I used eggs in late stages (stage 25) of development for my experiment, to ensure that embryos could detect external chemical cues. Late stages of cuttlefish embryo development were confirmed by the dark red colour of the eyes, which can be seen through the egg capsule. Eggs selected for the experiment were chosen at random. All experiments took place at CREC, Luc-sur-Mer, France.

Stimulus Collection

Four stimuli were used for chemical exposure: (1) odour of European seabass (*Dicentrarchus labrax*), a natural predator (Guerra et al. 2006) fed with cuttlefish embryos and their yolk sack (PE); (2) odour of seabass fed with standard commercial fish pellets (PP); (3) Sea urchin (*Paracentrotus lividus*) odour fed with brown algae (SU); (4) Seawater odour (SW). The sea bass and sea urchins were obtained from the CREC.

Seabass odour

Prey animals often exhibit antipredator responses to chemical cues of predators fed conspecifics of the prey, but not those fed another diet (Chivers & Mirza 2001). Thus, two types of predator odour were used. Four arbitrarily chosen seabass (length: mean ± S.D. =15.2 ± 1.2 cm) were placed in 144-L opaque flow-through tanks (water circulation: 1.5

L/min) containing two fish per tank. Each tank contained an air stone to supply constant oxygen and the water was kept at 18°C ± 2°. To ensure sufficient odour in the water, the fish were starved for approximately 24 hours and then fed either pellets or embryos ad libitum for a minimum of 5 days before using the odour in experiments. Fresh (i.e., not frozen) odour was used for each experiment.

Sea urchin odour

Four sea urchins (diameter: mean ± S.D. =10.16 ± 0.43 cm) were placed in a 144-L opaque tank, supplied with running oxygenated sea water at 18°C ± 2° (water circulation: 1.5 L/min). Urchins were fed brown algae (collected from the beaches of Luc-sur-Mer) *ad libitum* for a minimum of 5 days before using the odour in experiments.

Experimental Protocol

To test whether embryos respond to predator odour, changes in their ventilation rate were measured. Embryos were kept in the stock tanks (N = 38) and not exposed to any predator or sea urchin odour prior to the experiment. Trials were conducted in the morning and early evening, when activity levels of cuttlefish are known to be approximately the same (Ludovic Dickel, personal communication). Before the start of each trial, the outer envelope of the egg was carefully removed so ventilation (gill-fold) contractions could be viewed through a dissecting microscope. After the outer envelope of the eggs was removed, the eggs were allowed to acclimate in individual cylindrical tanks (5 x 5 x 6 cm) containing the original stock tank sea water for approximately 15-30 minutes. Sheets of cardboard were placed around the tanks and the lights in the experimental room were kept off to prevent exposure to any visual disturbances. This acclimation before the beginning of a trial was needed since handling and removing the envelope causes disturbance to the embryo. At the start of a trial, one egg was placed in a 20-ml glass beaker containing 10 ml of stock tank sea water and put under the dissecting microscope using a bottom-source light. The embryo was allowed to settle for approximately five minutes after being exposed to the light. I then video recorded and counted the embryo's gill-fold contractions for five minutes (prestimulus ventilation rate). The seawater was then removed from the beaker with a pipette and replaced with 10ml of one of the four stimuli (PE, PP, SU, SW). Treatments were randomized, and when possible, I was blind to the stimulus presented. I resumed counting gill-fold contractions immediately after addition of the stimulus for 25 consecutive minutes, divided in 5, 5-min periods. The mean rate for each 5-minute period was calculated to compare it to the

pre-stimulus baseline. The mean change in ventilation rate 15 minutes after injection of the stimulus (15-20 min period) from the pre-stimulus baseline was calculated and used for statistical analysis. Fifteen minutes was chosen because previous experiments show that the strongest response to pertinent chemical stimuli occurs about 15 minutes after exposure. This delay is related to the permeability of the egg membrane to chemicals. The response wanes after 20 minutes, a result of motor fatigue, adaptation or degradation of the odour (Romagny et al. 2012). My preliminary trials were consistent with these findings (see Figure 2.1).

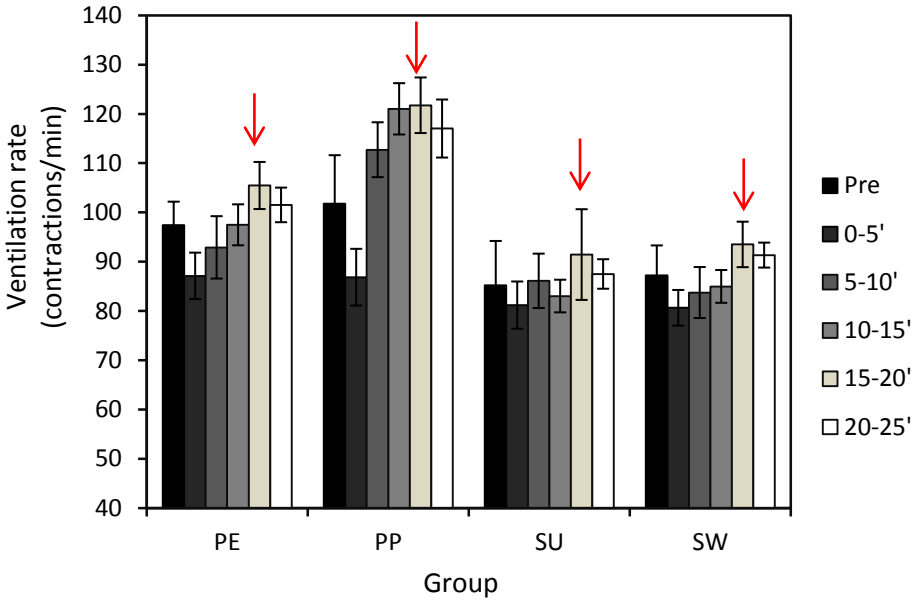

Figure 2.1. Mean ± S.E. ventilation rate of cuttlefish embryos for each five-minute period pre- and post-stimulus injection of seawater containing odours of predators fed with embryos (PE) (n=10); predators fed with pellets (PP) (n=8); sea urchins (SU) (n=10); sea water (SW) (n=10). The red arrows indicate during which time interval the strongest response occurred.

Statistical Analysis

For each odour, I calculated the mean change in ventilation rate 15 minutes after injection of the stimulus from the pre-stimulus baseline. The pre-stimulus baseline was consistent across treatments ($F_{3, 34} = 1.086$, $p = 0.37$), so I was able to compare the absolute change in ventilation rate across treatments. Data were parametric so I used a one-way ANOVA to test whether there were any differences in changes in ventilation rate among the four groups, followed by Tukey's HSD for post-hoc tests.

I also observed that there was a decrease in ventilation rate between the pre-stimulus period and the first five-minute period following injection (see Figure 2.1). This may be

because longer time was needed to allow the embryos to settle in the experimental set-up and so the decrease in ventilation may reflect the embryos becoming calmer. Thus, I also calculated the change in ventilation rate between the first five-minute period and the 15-20 min period after injection of the stimulus. This data did not meet equal variance assumptions for a parametric test, so I analyzed the effect of treatment on the response variable using a Kruskal-Wallis test followed by two-tailed Mann-Whitney U tests. To correct for type 1 error, the level of rejection was set at 0.008 following a Bonferroni adjustment.

2.3. Results

The ANOVA revealed a significant effect of treatment on the change in ventilation rate ($F_{3, 34} = 4.30$, $p = 0.011$). Tukey's HSD showed that the change in ventilation rate was significantly higher in PP than both SU ($p = 0.014$) and SW ($p = 0.025$). PE did not differ from any of the other groups.

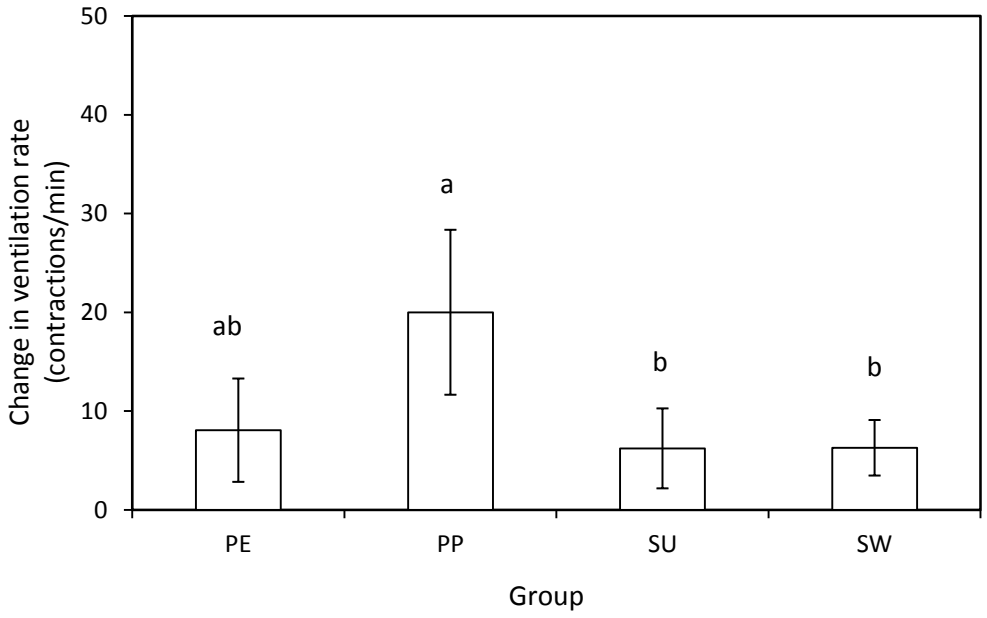

Figure 2.2. Mean (± S.E.) change from the pre-stimulus baseline in ventilation rate for cuttlefish embryos exposed to seawater containing odours of predators fed with embryos (PE) (n=10); predators fed with pellets (PP) (n=8); sea urchins (SU) (n=10); sea water (SW) (n=10). The different letters above the bars indicates groups that are statistically different from each other at alpha = 0.05.

However, the Kruskal-Wallis test also revealed an effect of treatment on the change in ventilation rate between the first five-minute period following injection of the stimulus and

after 15 minutes ($\chi^2_3 = 10.54$, p = 0.015). The results of the Mann-Whitney U tests are shown in Figure 2.3 below.

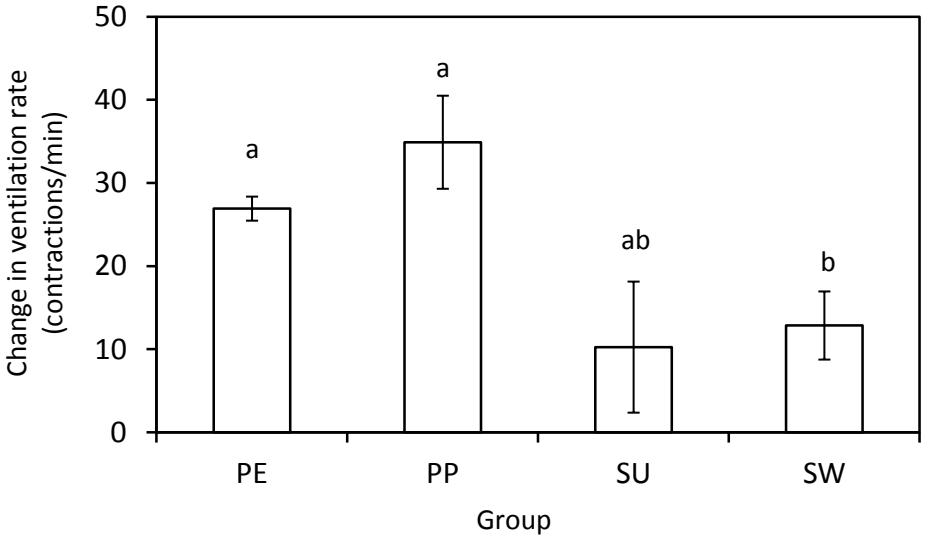

Figure 2.3. Mean (± S.E.) change in ventilation rate from the first five-minute period following exposure to fifteen minutes following exposure, in cuttlefish embryos exposed to seawater containing odours of predators fed with embryos (PE) (n=10); predators fed with pellets (PP) (n=8); sea urchins (SU) (n=10); sea water (SW) (n=10). The different letters above the bars indicates groups that are statistically different from each other at alpha = 0.008.

2.4. Discussion

My experiment confirms that cuttlefish embryos are capable of responding to ecologically salient chemical cues. When comparing the change ventilation rate from pre-stimulus baseline, only embryos exposed to odours of predators fed fish pellets responded more strongly than controls. However, the ventilation rate during the pre-stimulus period was higher than during the first five-minute period following exposure to the stimulus, which may suggest that five minutes was not long enough for the embryos to acclimate to the experimental setup. When I compared the change in ventilation from the first five-minute period, both predator groups had significantly larger increases in ventilation compared to the seawater control, but not to the sea urchin group. It is possible that embryos respond to novel odour, regardless of its threat potential. Boal and Golden (1999) showed that adult cuttlefish do respond to novel odour as well as predator odour. Strong neophobic responses can be

adaptive, because they can help an individual survive first encounters with unknown potential dangers before they have opportunity to collect information about a novel stimulus (Stratmann and Taborsky 2014; Brown et al. 2013).

Embryos had a higher increase in ventilation in response to odours of predators fed with commercial fish pellets than any of the odours. The exact composition of the pellets is not known, however it likely includes components of crustaceans, including shrimp, which are natural prey for both seabass and cuttlefish (Guibe et al. 2012). It is therefore possible embryos could have also been responding to chemical elements of the predator diet, as well as the predator odour itself. Boal and Golden (1999) showed that adult cuttlefish responded more strongly to the odour of prey than any odour, which corresponds with my results. Thus, the fish pellet diet may have confounded my overall findings.

Chapter 3: Effects of embryonic exposure to predator odour on post-hatching camouflage efficiency in juvenile cuttlefish

3.1. Introduction

When environmental conditions change in time and space, behavioural plasticity allows young to develop behaviours that are more suitably adapted to current environmental conditions, which may increase an individual's fitness since present conditions are often a good short-term indicator of future conditions. Many studies show that exposure to predator stress during embryonic development or the early juvenile period increases antipredator behaviour (Jonsson and Jonsson 2014). For example, experience of ringed salamander embryos with predator cues increases shelter-seeking behaviour and reduced foraging in newly hatched larvae (Mathis et al. 2008).

In addition to affecting behaviour, predator stress during embryonic development may also influence morphological traits. Predator-induced changes in morphology may be adaptive, such as the deepening of tail fins in common frog tadpoles, which increases thrust ability (Laurila et al. 2001). In some instances, changes in morphology may not appear to be adaptive. For example, green frog (*Rana clamitans*) embryos hatch at a smaller size when exposed to egg predators during development, which may lead to reduced fitness (Ireland et al. 2007), and so some morphological changes may appear to be a cost of increased antipredator behaviour. The smaller size at hatching in green frogs is due to early hatching, which may increase survival for embryos, but may in turn decrease survival as larvae, if there are larval predators present (Ireland et al. 2007). Though studies have shown that cuttlefish embryos are able to learn about their prey during embryonic development (Darmaillacq et al. 2008), no studies have shown whether they are able to learn about their predators before hatching.

Cuttlefish are well known for expressing a variety of cryptic chromatic patterns in avoiding predators and can transition between these various patterns in a matter of milliseconds. They have evolved highly effective homochromy, and can match the pattern, texture and brightness of their background effectively. For deceptive resemblance, the cuttlefish uses specific and complex postural and chromatic components to mimic objects in its environment (Hanlon & Messenger 1988). Primary defensive behaviour also involves lying motionless on top of a substrate or sand digging when on a sandy substrate. Young cuttlefish have a smaller and simpler repertoire of patterns than adults and few hatchlings

display sand digging, however, they improve their sand digging ability and progressively get better at adapting their body patterns to uniform backgrounds throughout postembryonic development (Poirier et al. 2004; 2005).

In the following studies, I investigated whether embryonic exposure to predator odours affects camouflage expression in juvenile cuttlefish. The goal of Experiment 2 was to determine if camouflage expression differed among juveniles after three weeks of exposure to predator odour during embryonic development and the goal of Experiment 3 was to determine if camouflage expression would differ after only a few days of exposure to predator odour. In Experiment 2 I exposed embryos constantly to an odour from stage 25 until hatching, while in Experiment 3 I exposed embryos constantly to an odour cue for only a few days before hatching. I did this by incubating them in water containing their given odour, whether it was predator or non-predator. Once an embryo hatched, I tested the camouflage expression of the individual using two different substrates: uniform and sand. Only primary defence behaviour was tested and juveniles were not exposed to any odours during trials. I hypothesized that those that received long-term embryonic exposure to predator odour would conceal themselves more effectively than those that were not exposed to predator odour, since the exposure to predator odour throughout the incubation period could perhaps appear as a predictor of a high-predation environment. Juveniles could thus behave as if a threat was potentially around even without chemical evidence of a predator nearby (Darmaillacq et al. 2008). It is possible that this effect would also be seen in those with short-term exposure to predators, since green frog (*Rana Clamitans*) embryos exhibit predator-specific phenotypic responses after only 3 days of exposure to predator odour (Ireland et al. 2007). However, short-term exposure to predator odour may not be indicative of a high-predation environment and so juveniles may not increase their camouflage expression without detection of a predator nearby.

3.2. Methodology

Test Species and stimulus preparation

Experiment 2 was performed simultaneously with Experiment 1 (see Chapter 2). Thus, the origin of the cuttlefish embryos and the stimulus preparations were identical to that of Experiment 1. For Experiment 3, cuttlefish eggs deposited by approximately seven different mothers were collected from traps in June 2013 and so originated from different

mothers from that of those used in Experiments 1 and 2. The stimulus preparations were identical to that of the first two experiments.

Embryo Incubation

Eggs (N = 77) were divided into four groups and suspended in a circular sieve (11x25 cm) which was placed in a perforated grey PVC tube that acted as a barrier between the eggs and the sea bass and sea urchins, to ensure only chemical and no visual exposure to the stimulus. The circular sieve along with the PVC tube was placed in the 144-L opaque flow-through tanks containing oxygenated sea water at 18º ± 2ºC and their respective cue donor to obtain the 4 treatment groups (PE, PP, SU, SW). Newly hatched juveniles were collected in the very early hours each morning, since cuttlefish normally hatch during hours of darkness (Ludovic Dickel, personal communication) to limit the exposure after hatching. The dorsal mantle length of each individual, which is an approximation of body size, was measured at time of collection. Juveniles were kept in individual opaque tanks (4x11x8 cm) with running oxygenated seawater containing no odour until the days of testing.

3.2.1 Juveniles increase camouflage efficiency following long-term embryonic exposure to predator odour

Experimental Protocol

Embryos were exposed to their respective stimulus/odour for approximately 3 weeks prior to hatching (embryos varied slightly in time of hatching by 1-3 days). The variation in hatching is likely due to the fact that the eggs were laid on different days since they came from different females. Juveniles were tested twice (i.e., in two different assays), and were returned to their holding tanks between trials.

This experiment consisted of two phases: trials testing camouflage on a uniform substrate followed by trials testing camouflage on a sandy substrate. Tests on uniform substrate were conducted on day 2 after hatching and tests on sandy substrates were conducted on day 5 after hatching. Tests on sand were only tested on day 5, since sand digging is very rarely seen in new hatchlings before 3-6 days (Ludovic Dickel, personal communication; Poirier et al. 2004).

Experimental setup

The experimental set up consisted of a round grey PVC compartment (size: 6 cm diameter x 2.5 cm depth), that was provided with running oxygenated seawater. For the uniform trials, the bottom of the compartments was left bare. For the sandy substrate trials, however, the bottom of the compartments was covered with 1 cm layer of sand, which was enough for the juveniles to completely bury themselves, should they decide to. A light source was placed above the compartment so that each trial received the same amount of light. For both tests, a video recording was used to allow the juveniles to settle on the substrates without a human observer present. Three colour pictures (from the video recording) were taken per individual: (1) a few seconds after introduction; (2) 1 h after introduction; (3) 2 h after the introduction. Pictures were converted into greyscale images using ImageJ© (imagej.nih.gov/ij). All data collection and calculations were carried via the ImageJ software. To ensure I did not introduce a bias in my body outlining technique, I was blind to the treatment while analysing the pictures. Details on data collection are provided below for each assay:

Uniform substrate trials

The homochromy (i.e., background resemblance) between the cuttlefish mantle and the substrate was evaluated according to two criteria: (1) the brightness matching of the animal's mantle compared to the background luminance according to the mean grey level (GL) (Poirier et al. 2005), and (2) the heterogeneity of the uniform pattern. For each picture, to measure the brightness matching ability, the grey level (GL) of the cuttlefish mantle was calculated and then compared to the GL of the background (Figure 3.1a). The value was expressed as the percentage of the grey level of the test background. Thus, the closer to 100% the score was, the better the cuttlefish matched the background luminance, hence the better the camouflage of the cuttlefish. In addition, the heterogeneity of the pattern exhibited on the cuttlefish mantle was also evaluated using an index of heterogeneity (HI) calculated according to:

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

Where:

N = total number of pixels that composed the cuttlefish mantle on the image

x = grey level of each pixel that composed the cuttlefish mantle on the image

$\bar{x}$ = mean grey level of all pixels that composed the cuttlefish mantle on the image

Thus, the closer to 0 the index was, the less heterogeneous was the uniform pattern, and therefore the better was the camouflage (Figure 3.1b).

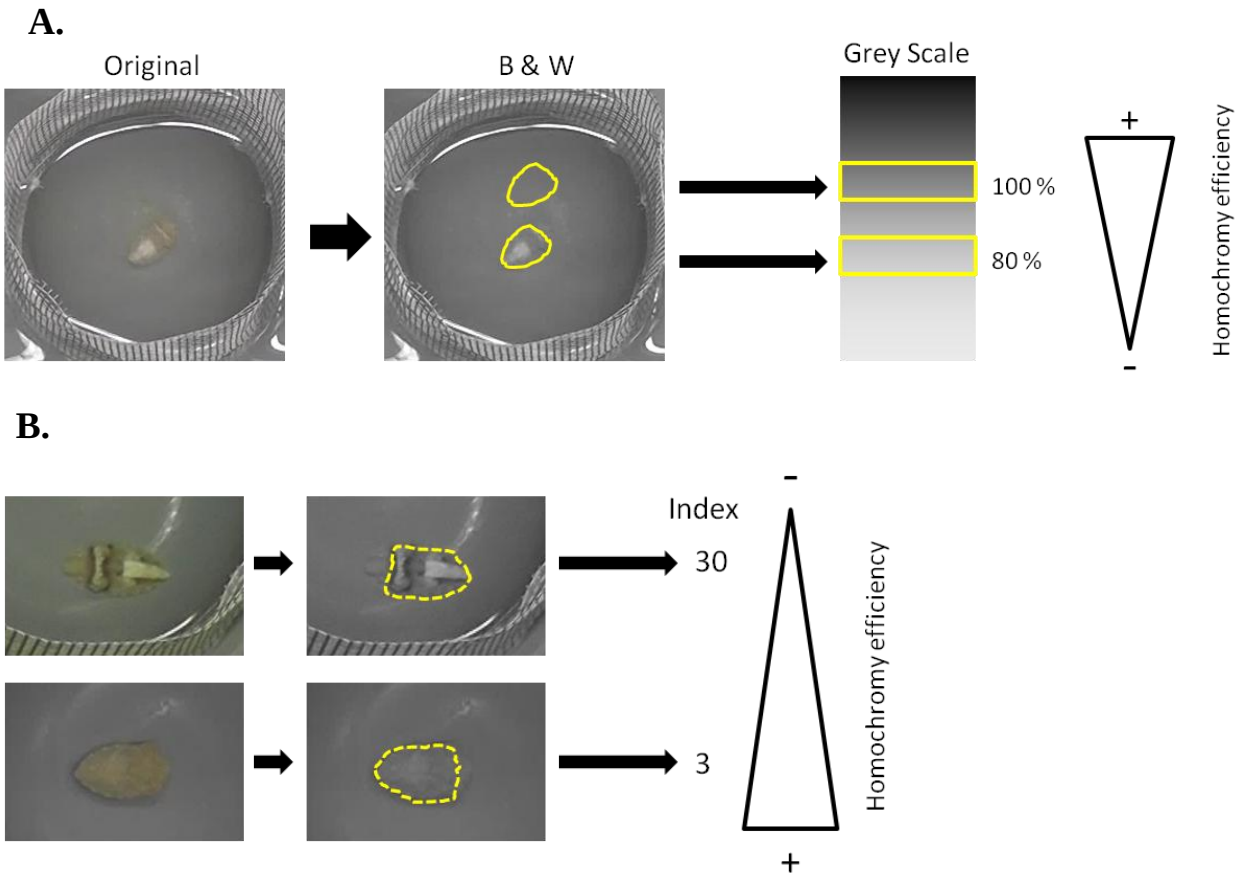

Figure 3.1. Schematic representation of the analysis procedure for the uniform body patterning efficiency of cuttlefish. The homochromy level between the cuttlefish mantle and the uniform grey substrate was determined using (A) the mean grey level (method based on Poirier et al. 2005), and (B) the heterogeneity index of the uniform body pattern.

Sandy substrate trials

First, the number of buried *versus* unburied animals was compared between the four groups after 1 h and again after 2 h. Animals were considered buried when the mantle was partially (i.e., a part of the mantle buried) or completely (i.e., only the eyes visible) covered by sand, and unburied when cuttlefish settled on top of the sand. The efficiency of burying behaviour was assessed for each picture according to two criteria: (1) the percentage of the

animal's body buried in sand; and (2) the homochromy between the part of the body visible and the sandy background when the animals were only partially or not buried. The area of the unburied part of the body was compared to the area representing the total cuttlefish surface (i.e., 100%) in order to obtain the percentage of body covered by sand (Figure 3.2). The closer to 100% the score was, the greatest body area was covered by sand, hence, the more efficient the sand digging behaviour was. When the animal was only partially or not buried at all, the GL of the cuttlefish mantle was calculated and compared to the GL of the background, just as in the tests on the uniform background (see Figure 3.1a).

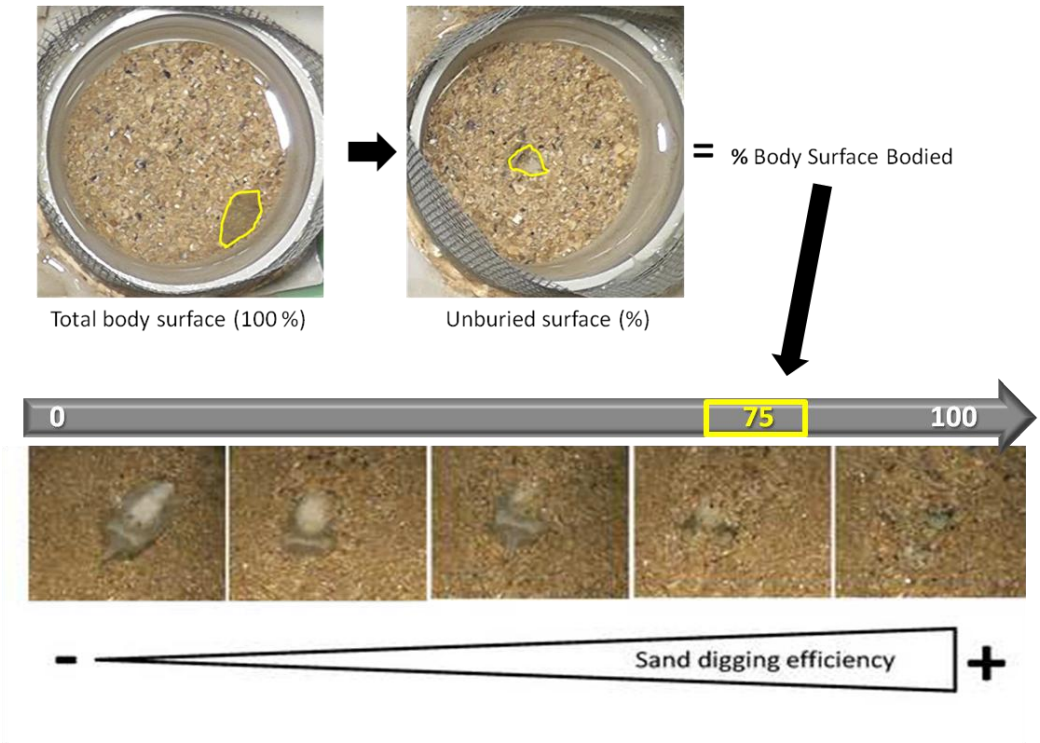

Figure 3.2 Schematic representation of the analysis procedure for the sand digging behaviour efficiency of cuttlefish by the percentage of the animal body buried in sand.

Statistical analysis

The average dorsal mantle length (cm) at hatching was compared among the four groups. The data met parametric assumptions, thus were analysed using a one-way Analysis of Variance test (ANOVA). Data on GL and HI from the uniform substrate assay and GL and burying efficiency for the sandy substrate assays also met parametric assumptions, and thus, were analysed using a Repeated-Measures ANOVA (RM ANOVA) assuming sphericity, to determine whether the treatment effects differed among the four groups, and whether the effects changed over time. For the data sets that lacked sphericity, I used the Huynh-Feldt

adjustment (more conservative results). To determine whether the proportion of individuals that buried in sand was different among the groups after one hr and after two hr, I used a Fisher's Exact test, since some of the expected cells were <5.

3.2.2 Juveniles increase camouflage efficiency following short-term embryonic exposure to predator odour

Experimental Protocol

Embryos (N = 73) were exposed to their respective stimulus/odour for approximately 1-3 days prior to hatching, since embryos varied on time of hatching. The experimental protocol and set up, as well as all measurements made from pictures taken from the video recordings of the trials, were identical to Experiment 2.

Statistical Analysis

The average dorsal mantle length (cm) at hatching was measured for the four groups. The data did not meet equal variance assumptions, thus I analyzed the effect of treatment on body size at hatching using a Kruskal-Wallis test. Data from both the uniform substrate and the sandy substrate trials met parametric assumptions and so I conducted RM ANOVA to determine whether the treatment had an effect on the four groups over time. I also used a RM ANOVA to compare the heterogeneity index over time between the groups in the uniform substrate trials and to compare the percentage of body surface buried in sand, since data met parametric assumptions. For the data sets that lacked sphericity, I used the Huynh-Feldt adjustment (more conservative estimate). To determine whether the proportion of individuals that buried in sand was different among the groups after one hour and after two hours, I used a Fisher's Exact test.

3.3. Results

Experiment 2

Body Size

The ANOVA revealed that DML differed significantly among the groups at hatching ($F_{3, 76} = 9.3$, $p < 0.001$). The results of Tukey post-hoc comparisons are illustrated in Figure 3.3.

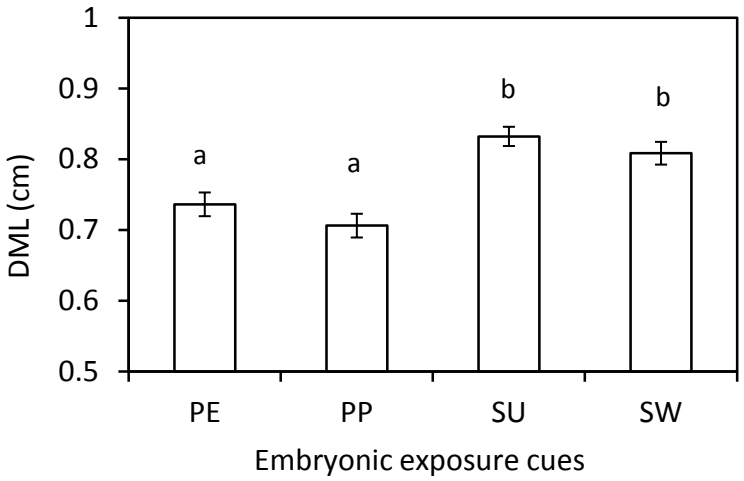

Figure 3.3. Mean (± S.E) dorsal mantle length (DML) of cuttlefish at hatching. The cuttlefish were exposed to odours of sea bass fed with embryos (PE) (n = 19), sea bass fed with pellets (PP) (n = 19), sea urchin (SU) (n = 20), or seawater (SW) (n = 19) for approximately 3 weeks as embryos. The different letters above the bars indicates groups that are statistically different from each other at alpha = 0.05.

Uniform substrate trials

The RM ANOVA did not show an interaction between group and time on brightness matching (; $F_{6, 152} = 10.4$, $p = 0.77$) of juveniles on the uniform background. However, all groups increased their brightness matching over time ($F_{2, 152} = 14.95$, $p < 0.001$, Figure 3.4) and groups also differed in their brightness matching ($F_{3, 76} = 14.57$, $p< 0.001$). Subsequent Tukey's HSD post-hoc comparisons revealed that the PP and PE groups had a significantly higher brightness matching ability than both of the control groups ($p < 0.001$).

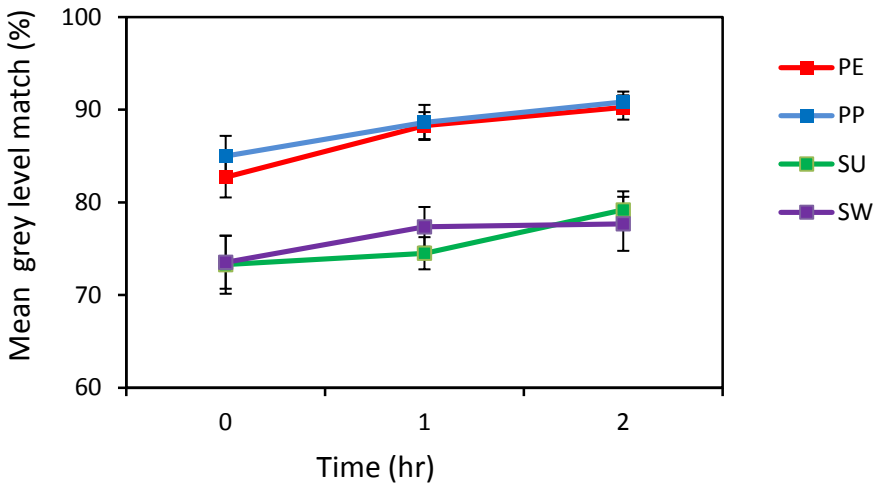

Figure 3.4. Mean (± S.E) grey level percentage of four groups of cuttlefish tested on a uniform background at the beginning of the test, after one hour, and after two hours. The cuttlefish were exposed, as embryos, to the odour of predators fed with embryos (PE) (n = 19), of predators fed with pellets (PP) (n = 19), sea urchin odour (SU) (n = 20), and seawater (SW) (n = 19), for approximately 3 weeks.

The RM ANOVA did not reveal an interaction between group and time on heterogeneity index (HI) ($F_{6, 152} = 0.60$, $p = 0.73$). In all groups, HI decreased with time ($F_{2,152} = 5.03$, $p = 0.008$), and although PE and PP had on average a lower heterogeneity index than the controls (Figure 3.5), groups did not differ significantly from each other ($F_{3, 76} = 1.45$, $p = 0.23$) in their HI.

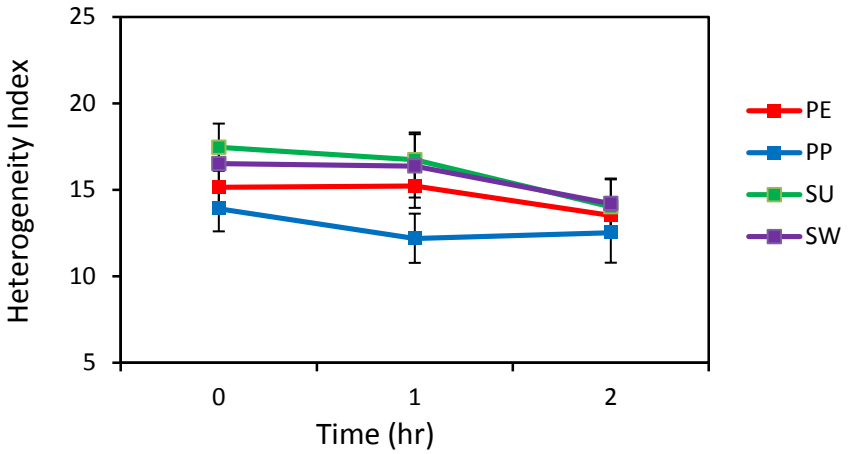

Figure 3.5. Mean (± S.E) heterogeneity index of four groups of cuttlefish tested on a uniform background at the beginning of the test, after one hour, and after two hours. The cuttlefish were exposed, as embryos, to the odour of predators fed with embryos (PE) (n = 19), of predators fed with pellets (PP) (n = 19), sea urchin odour (SU) (n = 20), and seawater (SW) (n = 19), for approximately 3 weeks).

Sandy substrate trials

Time affected the brightness matching differently between the four groups (time x group interaction: $F_{4.9, 98} = 3.89$, p=0.004). Tukey's HSD post-hoc comparisons showed that there were no differences between the groups at time zero (p = 0.077). After one hour, SW was significantly lower than SU (p= 0.008) and PP (p=0.031), but not PE (p = 0.085). After 2 hours, there were no significant difference between the groups (p = 0.15; Figure 3.6).

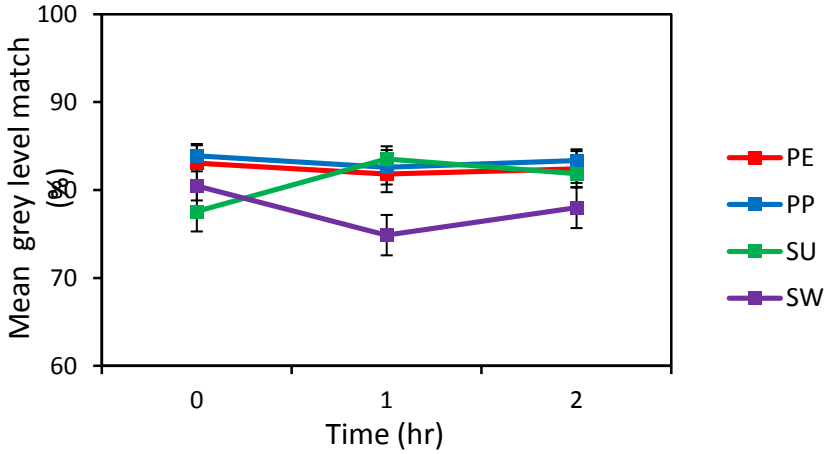

Figure 3.6. Mean (± S.E) grey level percentage match of four groups of cuttlefish tested on sand at the beginning of the test, after one hour, and after two hours. The cuttlefish were exposed, as embryos, to the odour of predators fed with embryos (PE) (n = 19), of predators fed with pellets (PP) (n = 19), sea urchin odour (SU) (n = 20), and seawater (SW) (n = 19), for approximately 3 weeks).

The proportion of cuttlefish that buried compared to those that did not bury were significantly different among the groups after one hour, with PE and PP having higher proportions than SU and SW (*Fisher's Exact test* p = 0.011), but not after two (*Fisher's Exact test* p = 0.091).With regards to how much of the body surface was buried, no interaction between time and group was found ($F_{3, 72} = 0.55$, p=0.65). All groups increased the percentage of body covered over time ($F_{1, 72} = 4.95$, p = 0.029), and PE and PP had significantly higher percentage of mantle covered than both controls ($F_{3, 72} = 12.76$, p < 0.001; Figure 3.7). I also compared the brightness matching ability on sand of those that never buried and those that did bury using a RM ANOVA and there was no significant difference between them ($F_{3, 72} = 0.74$, p = 0.45).

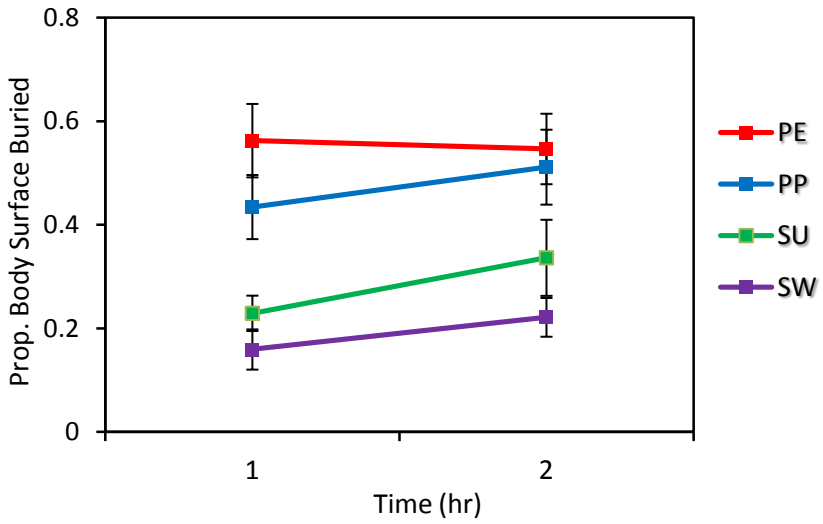

Figure 3.7. Proportion (± S.E) of body surface buried in sand of four groups of cuttlefish tested after one hour and after two hours.The cuttlefish were exposed, as embryos, to the odour of predators fed with embryos (PE) (n = 19), of predators fed with pellets (PP) (n = 19), sea urchin odour (SU) (n = 20), and seawater (SW) (n = 19), for approximately 3 weeks).

Experiment 3

Body Size

The Kruskal-Wallis test revealed that DML did not differ significantly among the groups at hatching ($\chi^2_3 = 5.3$, p = 0.15; Figure 3.8).

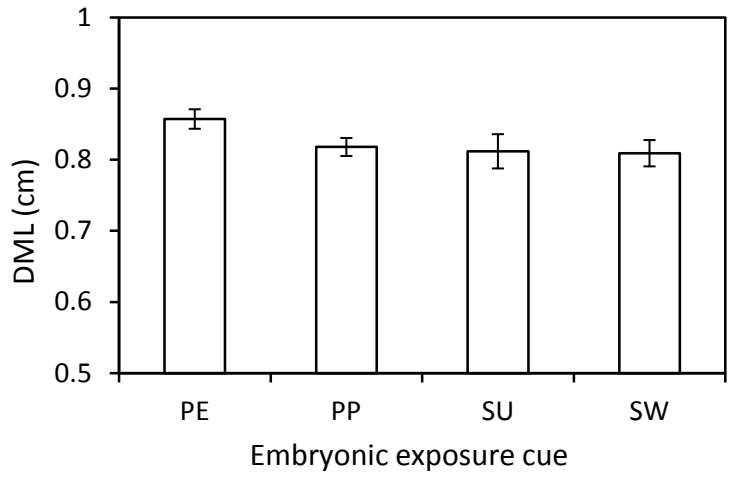

Figure 3.8. Mean (± S.E) dorsal mantle length (DML) of cuttlefish at hatching. The cuttlefish were exposed to odours of sea bass fed with embryos (PE) (n = 13), sea bass fed with pellets (PP) (n = 23), sea urchin (SU) (n = 16), or seawater (SW) (n = 21) for 1-3 days as embryos .

Uniform substrate trials

The RM ANOVA revealed an interaction between group and time on brightness matching ($F_{5.8, 134.0} = 2.90$, $p = 0.012$) and Tukey's HSD showed that PE and PP were significantly higher than SW ($p = 0.05$ and $p = 0.027$ respectively) at the beginning of the trial (time zero), but were not significantly different from SU ($p = 0.52$ and $p = 0.57$ respectively). None of the groups differed significantly after 1 hour or 2 hours ($p > 0.3$ for all post-hoc tests - Figure 3.9).

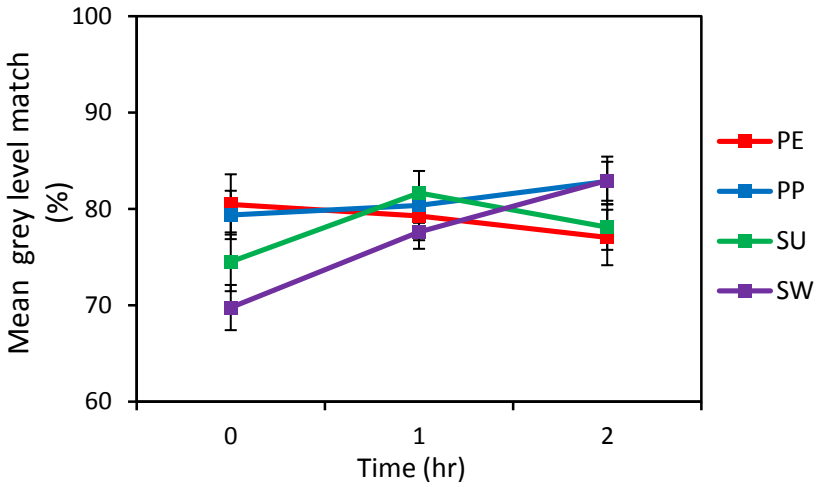

Figure 3.9. Mean (± S.E) grey level percentage of four groups of cuttlefish tested on a uniform background at the beginning of the test, after one hour, and after two hours. The cuttlefish were exposed to odours of sea bass fed with embryos (PE) (n = 13), sea bass fed with pellets (PP) (n = 23), sea urchin (SU) (n = 16), or seawater (SW) (n = 21) for 1-3 days as embryos.

With regards to the heterogeneity index, no interaction between group and time was found ($F_{5.7,\ 131.1} = 1.27$, $p = 0.28$), and there were no difference between the groups ($F_{3,\ 69} = 1.11$, $p = 0.35$). However, all groups did decrease their heterogeneity index over time ($F_{1.9,\ 131.1} = 48.5$, $p < 0.001$; Figure 3.10).

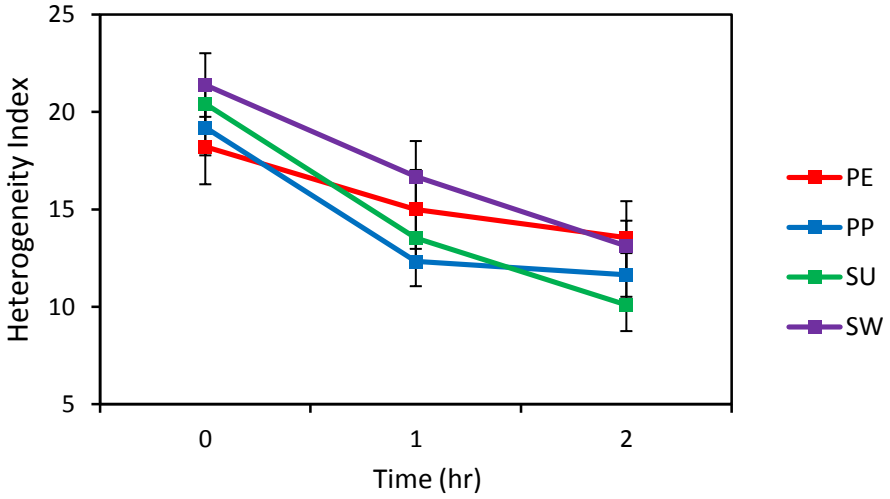

Figure 3.10. Mean (± S.E) heterogeneity index of four groups of cuttlefish tested on a uniform background at the beginning of the test, after one hour, and after two hours. The cuttlefish were exposed to odours of sea bass fed with embryos (PE) (n = 13), sea bass fed with pellets (PP) (n = 23), sea urchin (SU) (n = 16), or seawater (SW) (n = 21) for 1-3 days as embryos.

Sandy substrate trials

The RM ANOVA did not reveal an interaction between time and group when measuring brightness matching ability on sand ($F_{5.2, 134.0} = 0.90$, $p = 0.49$) and embryos did not increase their brightness matching ability over time ($F_{1.7, 134.0} = 2.33$, $p = 0.11$) and there was also no difference between groups ($F_{3, 69} = 1.53$, $p = 0.22$; Figure 3.11).

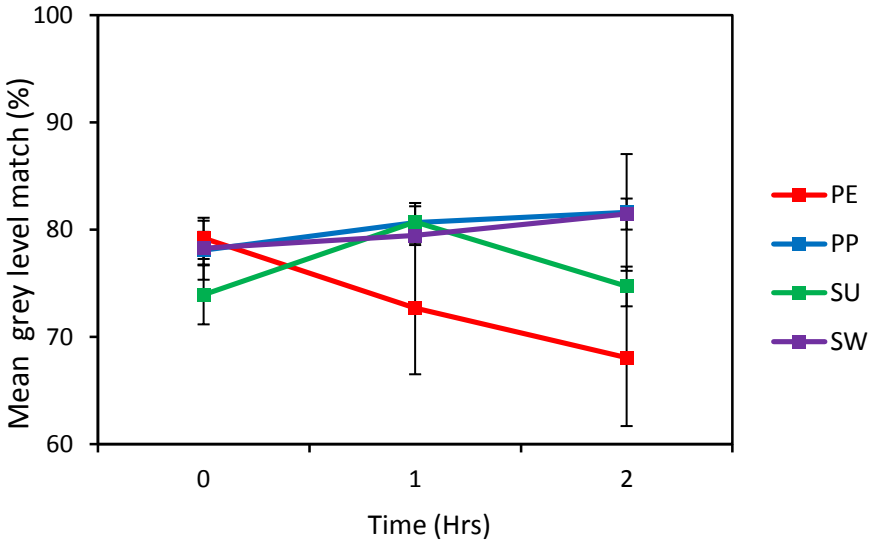

Figure 3.11. Mean (± S.E) mean grey level match of four groups of cuttlefish tested on sand at the beginning of the test, after one hour, and after two hours. The cuttlefish were exposed to odours of sea bass fed with embryos (PE) (n = 13), sea bass fed with pellets (PP) (n = 23), sea urchin (SU) (n = 16), or seawater (SW) (n = 21) for 1-3 days as embryos.

Burying in sand was observed in all groups (Figure 3.12). However, the proportion of those that buried compared to those that did not bury were not significantly different among the groups after one hour ($\chi^2_3 = 1.29$, $p = 0.73$), or after two ($\chi^2_3 = 1.67$, $p = 0.64$). There was also no significant difference in the amount of body surface buried between the groups ($F_{3, 69} = 2.57$, $p = 0.06$) and groups did not increase the amount of body surface buried over time ($F_{1, 69} = 0.05$, $p = 0.82$).

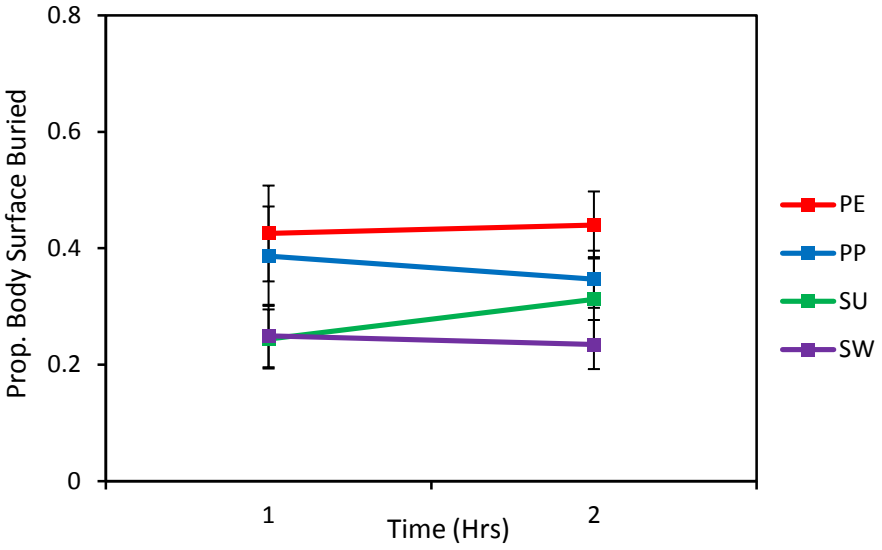

Figure 3.12. Proportion (± S.E) of body surface buried in sand of four groups of cuttlefish tested after one hour and after two hours. The cuttlefish were exposed to odours of sea bass fed with embryos (PE) (n = 13), sea bass fed with pellets (PP) (n = 23), sea urchin (SU) (n = 16), or seawater (SW) (n = 21) for 1-3 days as embryos.

3.4. Discussion

The results of Experiment 2 and 3 demonstrated the correlation between chemical cues and embryonic experience and the development of primary predator defense. Experiment 2 showed that when exposed to predator odour over a long period of time (i.e., about 3 weeks) during embryonic development, juveniles conceal themselves more efficiently than those not exposed to predator odours. This was seen on a grey uniform pattern, where juveniles exposed to predators had on average higher homochromy efficiency in brightness matching at the beginning through to the end of each trial The brightness matching ability of the cuttlefish mantle significantly increased over the 2-hr test period for all groups. On sandy substrates, only the groups exposed to the seawater control remained lower than the other groups in their homochromy efficiency, and there were no differences among the groups at the beginning or the end of the trial. However, though all groups increased the percentage of body covered over time, a higher proportion of the predator exposed groups buried in sand compared to those in the non-predator groups and had more of their mantle covered throughout the 2-hour period. These results suggest that, on sand, brightness matching may not be as important, as sand digging would be more effective in concealing an individual from predators.

The results of the uniform and the sandy substrates tests demonstrate that embryonic chemical experience of a potential threat may better concealment of juvenile cuttlefish in their environment. This agrees with many other studies that show embryonic exposure to predators increases antipredator behaviour in juveniles (Mathis et al. 2008; Ferrari et al. 2010; Jonsson and Jonsson 2014). These results show that cuttlefish embryonic experience influences the development of antipredator behaviour, which may be benefit juvenile survival if embryonic experience is a good predictor of future risk. In Experiment 3, where embryos were only exposed to their given stimuli over a short period of time (i.e., 1 to 3 days), the effects of predator cues on primary defense were not seen. With regards to the uniform tests, the predator groups showed a higher brightness matching ability than controls at the beginning of the trials, but there were no differences after one or after two hr. The heterogeneity index did not differ between the groups at any time within the trials. In addition, no differences in brightness matching ability or sand digging were found among the groups and the predator groups did not have a higher proportion of their body surface buried during the trials on sand. These results demonstrate that embryos may need a certain amount of exposure to the threat of predators in order to assess the predation risk as high, or to elicit stronger predator avoidance behaviour. It is generally optimal to delay costly phenotypic adjustments until sufficient information has been collected about the state of the environment (Fischer et al. 2014). Plasticity is especially adaptive when environments are variable and when sufficiently reliable environmental cues are available (Fischer et al. 2014). One to three days exposure right before hatching may not provide embryos with enough information about their environment and may not indicate a high-risk environment. Thus, hatchlings may behave as if they are in a low-risk environment, which may reflect a semi-permanent adaptation until directly exposed to a threat. In Experiment 1 and 2, hatchlings were not exposed to predator odours before or during the time of testing and were so were likely relying on the information collected as embryos.

The results of my experiments showed that embryos exposed to predator odours over long periods of time were smaller at hatching than those not exposed, and this effect was not seen in the experiment that involved individuals exposed to predators over a short period (few days) of time. These results, together with the results of juvenile testing, suggest a cost associated with increased camouflage expression. Smaller juveniles may be at a disadvantage because this would limit the size of shrimp or other prey items they could capture (personal observation). Whether this cost came from increased number or density of chromatophores at

hatching, or greater neural control of expansion and contraction of muscles associated with chromatophores is unknown, however it has been speculated that colour and pattern change for camouflage are energetically costly in cephalopods (Allen et al. 1999). Plasticity generally comes at a cost. Morphological adjustments are likely to be associated with high construction costs and may be difficult to reverse (Callahan et al. 2008). Limits to plasticity are also illustrated by the observation that many organisms are more responsive to environmental perturbations during some ages or life stages than during others (Jonsson and Jonsson 2014). For instance, bryozoans can grow defensive structures in response to chemical predator cues only early in their life (Fischer et al. 2014). It is not yet understood which factors determine the diverse patterns of age-dependent plasticity across species and traits that are observed in nature. In general, changes in plasticity in age are expected if an organism does not have perfect information early in development but can improve its estimate of the environmental state by integrating information accumulated over a long period of time (Fischer et al. 2014).

Chapter 4: General Discussion

4.1. Innate Predator Recognition

Effective early defences of prey towards predator presence or absence may be innate, transmitted through non-genetic parental effects, or acquired by early individual experience, and may act in combination with each other (Stramann and Taborsky 2014). Juvenile prey, because of their limited body strength, small size, or constrained escape potential, are more vulnerable to predation than adults, and so the early development of effective antipredator behaviour may receive particularly strong selective pressure (Fuiman and Magurran 1994). Substantial empirical work has demonstrated that many organisms induce defensive morphological as well as behavioural changes in response to the risk of predation (Benard 2014). Predator-induced behavioural and morphological change can provide the benefit of reduced risk, but can also incur costs, such as reduced growth rates and smaller body size (Benard 2014).

Whereas most prey species require learning to be able to recognize predator odour as threatening, several aquatic species display antipredator responses upon their first detection of the odour of some predators. Such responses have been shown in a variety of species, including freshwater snails and salmonid fishes, as well as larval toads and salamanders (Ferrari et al. 2010). In my study, I first investigated innate predator recognition by cuttlefish embryos. In Experiment 1, I showed that embryos can recognize ecologically salient chemical stimuli, including predators. However, because there was no difference between response to predators and response to non-predators (i.e sea urchins), I cannot conclude that cuttlefish embryos have the ability to innately recognize predator odours from non-threatening odours. In addition, there is a possible confound of dietary cues from the odour of sea bass fed with commercialized fish pellets. The contents of the fish pellets used to feed the sea bass were found to include components of similar prey of cuttlefish. If embryos were responding to the dietary cues rather than to the predator, these results corresponds to the results found in Boal and Golden (1999), in which adult cuttlefish increased their ventilation most strongly to the odour of prey and responded more weakly to the odours of predators, novel stimuli, conspecifics, and ink. Strong neophobic responses can be adaptive, because they can help to survive first encounters with unknown potential dangers before an individual had the opportunity to collect information about a novel stimulus (Stratmann and Taborsky 2014; Brown et al. 2013). It is also possible that rate of mantle contractions could be a more accurate measurement to determine predator detection in embryos (Romagny et al. 2012,

Anne-Sophie Darmaillacq, personal communication). Measurement of mantle contraction rate has been used on cuttlefish embryos to determine detection of stimuli (Romagny et al. 2012), and has been seen to increase in response to predator odour (Ludovic Dickel, personal communication). Mantle contractions could better represent a response to predators than increased respiration, since individuals may increase their respiration in response to either predator or diet cues. It is unlikely that mantle contractions would be involved in response to diet cues, since the mantle musculature is not involved in prey capture in cuttlefish (cuttlefish use arm strikes to capture prey) (Darmaillacq et al. 2004). Thus, using the change in mantle contraction rate could remove uncertainty in the confound of diet cues on response to predators. The results of Experiment 2 may further support the use of an alternative method to measure detection and recognition of stimuli in embryos. Oxygen consumption throughout embryonic development can affect how quickly and how much of the yolk sac is used up during this time, which in turn affects how long new hatchlings can survive without foraging (Boletzky 2003). So one can see how increasing ventilation over a long period of time would not be adaptive for an embryo, since this could decrease the chance of survival for a juvenile if it is not able to capture prey before its yolk sac is fully consumed. However, it does appear that exposure to predators (at least over a approximately 3 week period) during embryonic development does have some physiological effect on cuttlefish, since exposure to predator odour produced smaller hatchlings. It is not clear that the differences seen in the size of juveniles in Experiment 2 were due to differences in development (i.e., smaller individuals from the predator groups were "younger" than those in the control groups) or size alone. In some species, embryonic development can vary, producing individuals that leave the egg at different size and stages of development (Warkentin 1995), which can affect fitness because predation rates on hatchlings are often size or stage-dependent (Warkentin 2000). This is usually caused by some individuals hatching earlier than others. Though time of hatching did not vary in my experiment, the size differences observed could potentially be another example of hatching plasticity. I do not have any developmental data to support this hypothesis, however, it would be worth following up on in future studies.

4.2. Predation risk assessment by embryos

The early life stages are often the most vulnerable to predation, particularly in animals lacking parental care (e.g. Fuiman and Magurran 1994). Hence, when cues of predation are reliable (Laurila et al. 2001), one would predict that plastic predation-induced responses will be present at a very early age. In Experiments 2, I showed long-term exposure (approximately 3 weeks) to predator odour increased the camouflage expression in newly hatched juveniles. Those that were exposed to predator odours showed higher homochromy and sand digging efficiency than those that were not exposed to predator odours. This shows that the development of predator avoidance behaviour in cuttlefish may depend on the prenatal environment that they experience, which has also been found in both vertebrate and invertebrate species (Turner et al. 2006). Wild-caught snails, with previous exposure to predators, show a stronger response to predators than captive-reared snails that are naive to predators (Turner et al. 2006). Prey can assess predation risk through evaluating the probability of the various subcomponents involved in a predation event, which include detection, encounter, attack, and consumption (Lima and Dill 1990). Assessing each subcomponent allows animals to behaviourally control its risk, at least to some extent. Prey may estimate the rate of encounter between predator and prey via the frequency of direct or indirect predator cues (Lima and Dill 1990). The responses I documented in Experiments 2 and 3 are those in the absence of acute risk, thus only testing behaviour that would reduce the probability of detection by a predator. The results of these experiments suggest that growing up in a high-risk environment may require a permanent or semi-permanent level of vigilance and camouflage in cuttlefish in order to avoid a predator. For cuttlefish, these responses are likely to decrease the probability of detection by predators, thus aiding in survival. However this does not necessarily mean that individuals who spend less time in camouflage cannot respond to predation risk when predators are detected. Spending more time in camouflage may be costly, and hence, should only be done in high-risk environments.

However, not all species respond to predation risk assessed during development in the same way. For example, some embryos hatch early in response to egg predation, such as red-eyed tree frogs (*Agalychnis callidryas*) in response to social wasps (*Polybia rejecta*) (Warkentin 2000) or snakes, (*Leptodeira septentrionalis*) (Warkentin 1995), which are congruent with the scale of the risk. Individual embryos hatch in response to wasps, which take single eggs, whereas whole clutches hatch in response to snakes, which consume entire clutches (Warkentin 2000). Thus many prey species are able to respond appropriately to

gradation in mortality risks during embryonic development. Early experience to predators may also have long-term effects. For example, predator cues perceived by female mouthbrooding cichlids (*Eretmodus cyanostictus*) early in life increase egg mass, suggesting that these cues allow her to predict the predation risk for her offspring. In Experiment 3, camouflage expression in the absence of risk was not affected when embryos only received short-term exposure to predator odour. It is possible that in order to reduce uncertainty about the predation risk of their environment, cuttlefish embryos need a certain amount of exposure. Whether it is longer exposure duration, or overall stronger cue strength that is needed is unclear. Other studies have shown that in some species embryos exposed to increasing concentration of threatening cues during their embryonic development subsequently display stronger antipredator responses after hatching (Ferrari and Chivers 2010). Predation risk not only varies in intensity, but also in space and time. To respond adaptively to fluctuations in risk, prey must sample to gain information on the current presence or absence of predators (Sih 1992). However, changes in behavioural responses should also depend on the quality of prey information about the predation risk (Sih 1992). If most of their development occurs in the absence of predators, and only a few days in the presence of predators, it is possible this may lead embryos to perceive their surroundings as a low-risk environment. It is also possible that predator-induced behavioural plasticity in cuttlefish may be dependent on the timing of exposure rather than the duration. The timing of events during development may play an important role on subsequent morphology and behaviour (Bateson 1970). For example, starvation only has stunting effects on the size and other features of adult rats when it has occurred early in life (Bateson 1979). This suggests there may be periods during development that are more sensitive to factors such as stress, however, the extent of this is not known in cuttlefish.

4.3. Cost of predator-induced plasticity

Phenotypic plasticity caused by early experience plays a critical role in brain development and flexibility influencing cognition, behaviour, social skills, stress responsiveness and personality development (Jonsson and Jonsson 2014). Also, however, life-history traits, growth and age at developmental shifts are phenotypically plastic and can also be affected by exposure to predators (Jonsson and Jonsson 2014). This plasticity may be adaptive or non-adaptive (Warkentin 2005), and may or may not be a direct result of high predation risk. For example, juveniles of *Daphnia pulex* undergo morphological changes and

undergo life history shifts as defenses against predators (Tollrian 1995). Body size (both length and depth) and fecundity was increased in response to predator odour, but this resulted in an increased time to reach maturity. This increased time to maturity may not reflect a direct physiological cost of production of morphological defences, but a trade-off for larger body size (Tollrian 1995). Thus the decrease in body size found in juveniles exposed to predator odour in Experiment 2 may be not directly caused by this exposure, but rather was a trade-off for higher homochromy efficiency. Whether this trade-off resulted from a higher density or higher number of chromatophores, greater innervation of the chromatophore musculature, or greater neural control is unknown (as that was not measured in this study), and further studies on cost of predator-induced plasticity could benefit from such analysis.

4.4. Future studies

The results of my thesis indicate that high predation risk during embryonic development induces behavioural and morphological changes in camouflage expression and body size in cuttlefish hatchlings. The behavioural plasticity may provide survival benefits for newly hatched individuals, but may come at a cost in terms of body size. However, although there was a significant effect of chemical cues in this experiment, these are not the only cues present in a natural aquatic environment. In a natural setting, prey rely not only on chemical cues but on visual and tactile cues as well (Bridges and Gutzke 1996). Future studies could incorporate these cues to determine how they affect antipredator behaviour in cuttlefish. These studies might compare the effects of exposure to chemical cues alone and visual cues alone to the effects of exposure to chemical and visual cues combined. Experiments involving juvenile responses to direct predator cues (both visual and chemical) after exposure during embryonic development would also be beneficial in understanding the degree to which embryonic experience impacts antipredator behaviour in cuttlefish. In addition, I was not able to determine whether the differences found in camouflage expression and sand digging ability among the groups would result in differences in survival in wild, and currently no studies exist on the effect of variation in cuttlefish camouflage expression in actually avoiding detection by predators. Antipredator behaviours have important consequences for the predator-prey dynamics. Thus, knowledge of how antipredator behaviour is modified is important to better understanding organization of communities and ecosystems.

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