RESEARCH ARTICLE

Feel, smell and see in an egg: emergence of perception and learning in an immature invertebrate, the cuttlefish embryo

Sébastien Romagny[1], Anne-Sophie Darmaillacq[2], Mathieu Guibé[2], Cécile Bellanger[2] and Ludovic Dickel[2,*]

[1]Equipe d'Ethologie et de Psychobiologie Sensorielle, Centre des Sciences du Goût et de l'Alimentation, UMR 6265 CNRS/Université de Bourgogne/INRA, F-21000 Dijon, France and [2]Université de Caen Basse-Normandie, Groupe Mémoire et Plasticité Comportementale, EA 4259, F-14032 Caen, France

*Author for correspondence (ludovic.dickel@unicaen.fr)

SUMMARY

It is now well established that prenatal sensory experience affects development itself and has long-term consequences in terms of postnatal behavior. This study focused on the functionality of the sensory system in cuttlefish *in ovo*. Embryos of stage 23, 25 and 30 received a tactile, chemical or visual stimulus. An increase of mantle contraction rhythm was taken to indicate a behavioral response to the stimulus. We clearly demonstrated that tactile and chemical systems are functional from stage 23, whereas the visual system is functional only from stage 25. At stage 25 and 30, embryos were also exposed to a repeated light stimulus. Stage 30 embryos were capable of habituation, showing a progressive decrease in contractions across stimulations. This process was not due to fatigue as we observed response recovery after a dishabituation tactile stimulus. This study is the first to show that cuttlefish embryos behaviorally respond to stimuli of different modalities and that the visual system is the last to become functional during embryonic development, as in vertebrate embryos. It also provides new evidence that the memory system develops *in ovo* in cuttlefish.

Key words: cuttlefish, embryo, sensory development, habituation.

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INTRODUCTION

The present work focused on the embryonic onset of sensory systems and embryonic learning abilities in an invertebrate: the cuttlefish (Mollusk, Cephalopod). The sequence of the development of sensory systems has been shown to be the same in all vertebrates (Gottlieb, 1971). The development of sensory systems depends on the environmental input and early experience of animals (Sleigh et al., 1996; Foushée and Lickliter, 2002). Developing neuro-motor and sensory systems are capable of functioning long before they have completed their maturation. Because all experiences of an animal are founded on its ability to sense changes in its environment, measurement of the embryo's sensory capacities delimits its potential for modifying its behavior through experience (Smotherman and Robinson, 1992). One of the dominant aims of comparative psychology is to identify similarities in basic behavioral processes in different species, and to attribute the source of behavioral similarities either to adaptation to common pressures in the environment, often referred to as analogy or convergence, or to shared evolutionary heritage, referred to as homology (Ridley, 1983). Comparative studies of early behavior in different species might be a useful approach to study the evolutionary causes of sensory system development (Gottlieb, 1976; Lecanuet et al., 1995; Lickliter and Harshaw, 2010). However, to our knowledge, no study has yet been carried out on the development of sensory systems in invertebrates, and the first aim of this study was to bridge this gap.

In vertebrates, it is known that the functionality of sensory systems (i.e. the capability of sensory systems to elicit behavioral responses to the appropriate type of stimuli) (Lickliter, 2005) is paired with 'immature' forms of prenatal simple learning such as habituation (Schaal, 1988; Shalev et al., 1989; Smotherman and Robinson, 1992; van Heteren et al., 2001). In invertebrates, very few studies have shown prenatal learning and this question is still debated (Isingrini et al., 1985); this constitutes the second aim of this study.

In *Sepia*, eggs and hatchlings do not benefit from parental care. This allows for a precise experimental control of embryos' and juveniles' experiential history and direct manipulations during the perinatal period of development. The different stages of morphological embryonic development have already been described (Lemaire, 1970). Here, we first aimed at determining the appearance of a behavioral response to tactile, chemical and visual stimuli in cuttlefish embryos. As in several other invertebrates (Corner and Bour, 1984), movements occur *in ovo* in cuttlefish, the most observable behavior being mantle contractions. Mantle contractions are a pumping action involving the entire musculature (Corner, 1977). In adult cuttlefish, variations in the frequency of mantle contractions (Packard and Trueman, 1974) have been shown to be a reliable and measurable response to odors (Boal and Golden, 1999) and visual stimuli (King and Adamo, 2006).

Despite the black coloration of the egg capsule, recent studies on cuttlefish have shown that visual information could reach the embryo and that visual or chemical exposure to potential prey induced a change in hatchlings' natural prey preference (Darmaillacq et al., 2008; Guibé et al., 2010). However, there is still no direct evidence of embryonic learning. Here, we also studied the motor response of cuttlefish *in ovo* to a repeated visual stimulus (simple learning) at different stages of embryonic development.

MATERIALS AND METHODS
Subjects

Eggs of *Sepia officinalis* Linnaeus 1758 were collected on cuttlefish traps in the vicinity of Luc-sur-Mer (Calvados, France) in April and May 2010 and in April 2011. All experiments were conducted at the Centre de Recherches en Environnement Côtier (CREC, Luc-sur-Mer, France). Eggs were kept in plastic tanks with circulating seawater at 18±2°C, with a natural light–dark cycle. We studied embryonic stages (E)23, 25 and 30 (Lemaire, 1970). At stage 23 (about 4 weeks before hatching at 18°C) 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. Experiments were carried out in agreement with French regulations. E23 and E25 embryos were anesthetized in a 2% solution of ethanol and killed after the experiments. E30 embryos were hatched immediately after the experiments and hatchlings were released into the sea at low tide, in natural pools where prey of suitable size were abundant.

General procedure

The outer layers of the eggs were gently removed in order to make mantle contractions of the embryos easily visible under a binocular microscope. The ambient lights of the experimental room were switched off to create a dimly lit environment (10 lx) sufficient to count mantle contractions. The eggs were isolated in individual pillboxes (4 cm in diameter) in 10 ml total volume of seawater at 18°C. The experiments began 15 min after the removal of the outer egg layers. Full mantle contractions (contraction of radial and circular muscles of the mantle) (Packard and Trueman, 1974) were counted under a binocular microscope over a period of 150 s, before stimulation and then during (light or chemical) or after (tactile) stimulation and expressed as number of contractions per minute (contractions min^{-1}). They were then expressed as the difference Δ contractions min^{-1} between the number of contractions after and before the stimulus. Embryos were each tested only once. All experiments were carried out between 08:00 h and 18:00 h, and the different stimuli were randomly assigned to embryos during the day.

Experiment 1: onset of tactile, chemical and visual systems
Methods
Tactile stimulus

Embryos (E23, N_{T23}=10; E25, N_{T25}=10; E30, N_{T30}=12) were lightly pricked once with a blunt needle (0.3 mm diameter) through the membrane of the egg. The location of the prick, at the posterior end of the ventral mantle, was the same for all the embryos. The strength and duration of the prick were controlled: the prick was stopped within 1 s when the embryo moved about 2 mm in the perivitelline fluid. The perivitelline fluid density ensured the same prick pressure for all stimulations at each embryonic stage. In control groups (*N*=10 for each stage), the needle was passed through the membrane but the embryos did not receive any prick.

Chemical stimulus

Chemical cues were introduced into the pillbox by siphoning off the total volume of initial water and replacing it with 10 ml of water with odor of a natural predator: the European sea bass, *Dicentrarchus labrax* (Linnaeus 1758) (Hanlon and Messenger, 1996; Guerra, 2006). The fish was caught in the vicinity of Luc-sur-Mer (France) and maintained in an aquarium (2×1.5×0.5 m) in the CREC, to provide the chemical stimuli. The water in the aquarium was renewed by circulation at 1 l min^{-1}. Odor-containing water was taken

directly from the aquarium and transferred to the experimental pillbox. Cuttlefish mantle contractions (E23, N_{C23}=10; E25, N_{C25}=10; E30, N_{C30}=15) were counted before the presentation of the stimulus, immediately after addition and 15 min after addition of the odor-containing water (note that the time of 15 min was determined in preliminary observations using the same counting method for a period of 1 h 30 min; see Fig. 1). Control individuals (*N*=10) were tested under the same conditions with normal seawater.

Visual stimulus

Embryos (E23, N_{V23}=10; E25, N_{V25}=13; E30, N_{V30}=16) were illuminated by a cold light (100,000 lx) placed above the box for 150 s. This intensity corresponds to the light of a sunny day in the field, at low tide for eggs laid in shallow water. The light was applied at a distance of 2 cm from the embryo. Control embryos (*N*=10 for each stage) did not receive the light stimulus.

Statistics

For each sensory modality and each embryonic stage, the mean basal number of counts per minute was compared with the mean number during or after presentation of the stimulus with a permutation test for paired samples, using StatXact 7.0 (Cytel Software, www.cytel.com) with α=0.05.

Experiment 2: habituation to repeated light stimuli
Methods

The results of experiment 1 revealed that embryos did not respond to a light stimulus before stage 25 (see Results); therefore, only E25 (N_{25}=10) and E30 (N_{30}=10) were tested in this experiment. Embryos were exposed to a light stimulus (100,000 lx for 150 s) three consecutive times with interstimulus intervals (ISI) of 30 min (preliminary trials showed that 15 min were needed for the recovery of the basal mantle contraction rate). Five minutes after the presentation of the third stimulus, embryos were pricked once at the posterior end of the ventral mantle; the prick was used as a dishabituation stimulus. Preliminary results showed that the embryo recovered its basal mantle contraction rate about 20 min after the prick. Twenty-five minutes after the prick, embryos were exposed to a second set of three consecutive light stimuli. Mantle contractions were counted 150 s before and during each stimulus presentation.
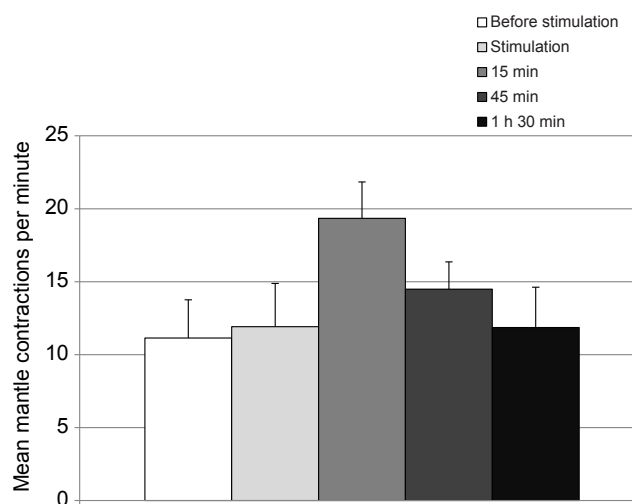

Fig. 1. Behavioral responses to a chemical stimulus. Mean (+ s.e.m.) mantle contractions per minute for developmental stage 25, before and up to 1 h 30 min after the addition of water containing predator odor.

Embryos were thus exposed to a total of six light stimuli. Two control groups were tested: (i) a 'light control' group (LC; N_{LC}=10 for each stage) in which embryos were tested under basal light conditions (10 lx) and received the prick; and (ii) a 'dishabituation control' group (DC; N_{DC}=10 for each stage) in which embryos were tested with the light stimuli but were not pricked. ISI was the same in the LC and DC groups.

If no habituation was observed for an ISI of 30 min, new embryos were tested with the above method but with an ISI of 15 min.

Statistics

For each of the six presentations of the light stimulus, the results are expressed with the following 'mantle contractions' index I:

$$I_{n\text{th stimulus}} = \text{no. mantle contractions during } n\text{th stimulus} - \text{basal no. mantle contractions before } n\text{th stimulus} . \quad (1)$$

The Friedman test was used to detect any significant differences across stimulus presentations. Then, for each group, I_1 versus I_3, I_3 versus I_4 and I_4 versus I_6 were compared using a permutation test for paired samples (α=0.05) with Bonferroni's sequential correction in cases of multiple comparisons.

All analyses were done with StatXact v7.0 (Cytel Software).

RESULTS
Experiment 1
Responses to a tactile stimulus

In E23, E25 and E30, the number of contractions per minute significantly increased just after the prick (permutation test for paired samples, N_{T23}=10, P=0.01; N_{T25}=10, P=0.0019; N_{T30}=12, P=0.0004) (Table 1).

Responses to a chemical stimulus

Addition of blank seawater (without odor) had no effect on the number of contractions per minute (permutation test for paired samples, N_{C23}=10, N_{C25}=10, N_{C30}=15, P>0.05) (Table 1). There was no time effect on the number of contractions per minute in the control group during the experiment at all stages (Friedman test, E23: N=10, P>0.05; E25: N=11, P>0.05; E30: N=11, P>0.05).

In E23, E25 and E30, the number of contractions per minute increased significantly 15 min after the addition of the predator odor (permutation test for paired samples and sequential Bonferroni correction, N_{C23}=10, P<0.01; N_{C25}=10, P<0.01; N_{C30}=15, P<0.01).

Responses to a visual stimulus

In E23, there was no difference in the number of contractions per minute before and during the presentation of the stimulus (permutation test for paired samples, N_{V23}=10, P>0.05) (Table 1). In contrast, the number of contractions per minute significantly increased during the presentation of the visual stimulus in E25 and E30 (permutation test for paired samples, N_{V25}=13, P<0.01; N_{V30}=16, P<0.01).

Experiment 2

In E25, there was no change in the I index in the course of the repeated stimulation for any group (Friedman test, N_{25}=10, N_{DC}=10, N_{LC}=10, P>0.05) with both 30 min ISI (Fig. 2) and 15 min ISI (Friedman test: N'_{25}=8, P>0.05, Fig. 3).

In E30 (Fig. 4), I_3 (3.40±0.64) was significantly lower than I_1 (7.80±1.21, permutation test for paired samples, N_{30}=10, P=0.0039). After the dishabituation stimulus, I_4 (6.20±0.70) was significantly higher than I_3 (3.40±0.64, exact permutation test for paired samples, N_{30}=10, P=0.0039). I_6 (3.50±0.40) was significantly lower than I_4 (6.20±0.70, exact permutation test for paired samples, N_{30}=10, P=0.0039).

In the light control group, the I index did not change in the course of the experiment (Friedman exact test: 5.29, N_{LC}=10, P=0.32). In the dishabituation control group, I_3 (3.00±0.44) was significantly lower than I_1 (7.11±0.79, permutation test for paired samples, N_{DC}=9, P=0.0078). There was no significant difference between I_3 (3.00±0.44) and I_4 (2.78±0.46, permutation test for paired samples, N_{DC}=9, P=0.875) or between I_4 (2.78±0.46) and I_6 (3.22±0.43, permutation test for paired samples, N_{DC}=9, P=0.56).

DISCUSSION
Experiment 1

We first showed that embryos behaviorally respond to pricks on the ventral mantle as early as stage 23; this demonstrates that at least some of the mechanoreceptors of the mantle are functional at this embryonic stage. This confirms the hypothesis of Baratte and Bonnaud that, from histological observations, the sensory catecholamine-containing neurons of the mantle's epithelium are differentiated and potentially functional from stage 21 (Baratte and Bonnaud, 2009). These primary sensory neurons probably mediate mechanoreception in mollusks (Croll, 1983).

From stage 23, cuttlefish embryos also respond to sea bass odor. In Coleoid cephalopods (octopus, squid and cuttlefish), chemoreceptors were described on sucker-edge epithelium and on the lips (Graziadei, 1964; Emery, 1975a; Emery, 1975b). In cuttlefish, some chemical compounds pass through the membrane of the egg during the late stages of embryonic development (Lacoue-Labarthe, 2007). Chemical cues from sea bass are a relevant stimulus for embryos but our data do not allow us to determine which types of chemoreceptor are involved in the perception of predator odor. The present work shows that the number of contractions per minute increased at E23, E25 and E30, 15 min after this predator odor was added to the pillbox. In adult cuttlefish (Boal

Table 1. Mean (±s.e.m.) mantle contractions per minute for developmental stages (E)23, 25 and 30 before and after tactile, chemical or visual stimulation

	E23		E25		E30	
	Before stimulation	After stimulation	Before stimulation	After stimulation	Before stimulation	After stimulation
Tactile	0.9±0.4	5.9±1.6*	39.1±5.6	62.4±6.6*	46±3.0	83.5±6.7*
Tactile control	0.88±0.21	0.85±0.16	9.96±1.7	9.6±1.15	0.68±0.17	0.44±0.13
Chemical	11.14±2.6	19.34±2.5*	11.1±2.5	19.3±2.5*	20.2±6.8	31.47±11.36*
Chemical control	0.82±0.24	0.71±0.21	9.49±2.07	8.76±1.45	5.88±2.07	6.08±2.07
Visual	1.12±0.5	1.12±0.5	7.7±1.7	13.3±2.4*	7.2±6.3	30.9±10.8*
Visual control	0.88±0.21	0.85±0.16	9.96±1.7	9.6±1.15	0.68±0.17	0.44±0.13

*Exact permutation test for paired samples between the number of counts per minute before and after stimulation with α=0.05.
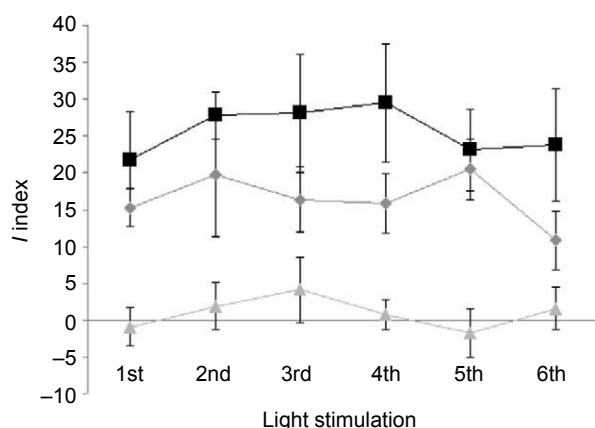
Fig. 2. Habituation to repeated light stimuli at stage 25 with an interstimulus interval (ISI) of 30 min. Mean (± s.e.m.) difference in mantle contractions (*I* index) between basal rate and stimulation rate (ISI 30 min) at stage 25 for the lit and pricked group (diamonds), the unlit but pricked group (triangles) and the lit but not pricked group (squares). Pricks were applied between the third and the fourth stimulation.

and Golden, 1999), reaction to odors is immediate. Here, the 15 min delay in response could be explained by the permeability of the egg membrane to chemicals. The progressive decrease of the response might be the result of motor fatigue, habituation or degradation of the odor. It has been demonstrated that the prey preferences of newly hatched cuttlefish can change after a late stage (>25) embryonic exposure to prey odors (Guibé et al., 2010). Thus it would be interesting to investigate whether embryonic exposure to odors before the visual system becomes functional (before stage 25), could elicit any change in visual prey preference.

In this study, we showed that embryos are able to behaviorally respond to sudden light at stages 25 and 30 but not at stage 23. Yamamoto suggested that *Sepiella japonica* becomes photosensitive a long time before hatching (Yamamoto, 1985). Here, we show that the visual system is functional as soon as the first pigments appear, at stage 25 (Lemaire, 1970). However, we cannot exclude the possibility that embryos can potentially perceive light *via* extraocular photoreceptors (Mäthger et al., 2010; Sundermann, 1990). However, the lumina of ectodermal vesicle organs has not completed its
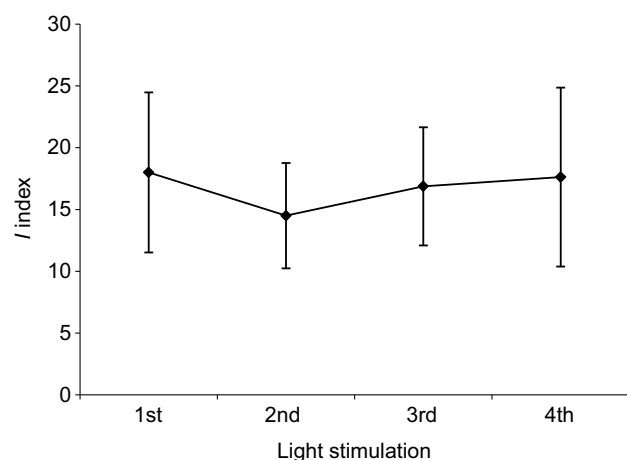

Fig. 3. Habituation to repeated light stimuli at stage 25 with an ISI of 15 min. Mean (± s.e.m.) difference in mantle contractions between basal rate and stimulation rate (ISI 15 min) at stage 25 for the lit and pricked group. Pricks were applied between the third and the fourth stimulation.
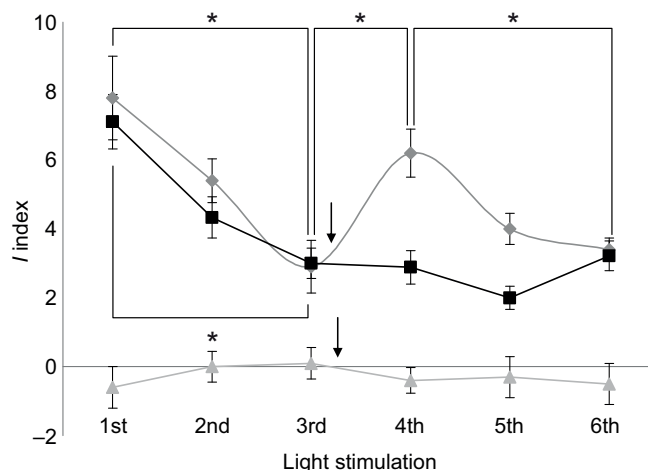

Fig. 4. Habituation to repeated light stimuli at stage 30 with an ISI of 30 min. Mean (± s.e.m.) difference in mantle contractions between basal rate and stimulation rate at stage 30 for the lit and pricked group (diamonds), the unlit but pricked group (triangles) and the lit but not pricked group (squares). Pricks were applied between the third and fourth stimulation. *Exact permutation test for paired samples between the contractions before and after stimulation with *P*<0.05.

maturation before hatching in cuttlefish (Sundermann, 1990) and the role of opsins in cuttlefish skin has not yet been determined (Mäthger et al., 2010).

Cuttlefish embryos develop in a visually buffered dark environment because of the presence of the black capsule round the eggs. As a result, light is typically attenuated prior to reaching the developing embryo. As the embryo develops, the egg capsule dilates and becomes more translucent in the latest stages of embryonic development. As a consequence, functionality of the visual system at stage 23 might not be relevant for embryos. The energy needed for visual integration would be spent only when it becomes relevant for an embryo to perceive visual elements from the outer environment. In the present experiment, as ink layers of the eggs were completely removed, the intensity of light passing through the membranes does not reflect natural conditions, especially for stage 23. However, there is indirect evidence that embryos can perceive visual information through the inked layers in the last stages of development [e.g. prey (Darmaillacq et al., 2008) and contrasts (Guibé et al., 2012)].

Experiment 2
At E30, repeated exposure to a light stimulus resulted in a decrease in the behavioral response to the stimulus. This decrease is a result of habituation, and is not due to fatigue as the tactile dishabituation stimulus caused a rapid recovery of the response. In contrast, there is no evidence of such learning at stage 25, with an ISI of 30 or even 15 min. This does not mean that habituation to repeated visual stimulus cannot occur at E25 with shorter ISI or more stimuli, but that habituation abilities strongly improve between E25 and E30. This is in accordance with the results of Rankin and Carew, which showed an improvement of habituation learning (i.e. fewer stimulus repetitions needed for habituation) between developmental stages 9 and 12 in *Aplysia californica* (Rankin and Carew, 1987).

General discussion
The present work clearly shows that cuttlefish embryos are able to perceive their environment through at least tactile, chemical and

visual sensory modalities. These data support previous studies that showed that late embryos can be familiarized to the sight or the odor of a prey (Darmaillacq et al., 2008; Guibé et al., 2010). The present study clearly shows that the visual system is functional from about 3 weeks before hatching (at stage 25). This allows embryos to collect large amounts of visual information from their surroundings, and potentially to adapt species-specific behavior after hatching [by identifying available prey (Darmaillacq et al., 2008) or potential threat (L.D., unpublished data).

In the present study, we have shown that, as is the case in vertebrate embryos (Gottlieb, 1971), behavioral responses to visual stimuli appear later in development than those to chemical and tactile stimuli. A common developmental sequence of sensory systems could suggest a unique evolutionary origin and/or a convergent evolution between cuttlefish and vertebrates. The delay in the onset of development between the visual systems and the chemosensory and tactile ones can be explained as being a way of lowering energy costs in developing sensory structures (Lickliter, 2005). Moreover, if sensory systems became functional simultaneously, the amount of information might be too large to allow normal development of each individual system (Turkewitz and Kenny, 1982). Similar environmental pressures could have led to the conservation of the same developmental sequence in vertebrates and cuttlefish. Further investigations in behavioral embryology in invertebrates are needed in order to determine whether the same sequence of the onset of development of sensory systems in cuttlefish and vertebrates corresponds to a general rule in the animal kingdom or is just a coincidence.

In the present experiments, the embryos showed habituation to a repeated stimulus at stage 30 but not at stage 25. In the latter, the neural pathway allowing the behavioral response might not yet be coupled to the storage areas of the brain. Such a neural mediation hypothesis was suggested in the habituation of cardiac response to chemosensory stimulation in rat embryos (Smotherman and Robinson, 1992). In coleoid cephalopods, the brain structures involved in the formation of visual memory are the optic lobes and the vertical lobe complex. In hatchling cuttlefish, the vertical lobe and its connection to the rest of the brain are very immature compared with that in adults (Dickel et al., 1998). The optic lobes, which are involved in visual processing in cuttlefish, have also been shown to be involved in habituation in octopus (Wells, 1978). The early developmental stage of the optic lobes in the cuttlefish embryo might explain the low visual habituation capabilities in stage 25 embryos, even though the visual system has started to function. The brain structures involved in visual learning are subjected to considerable development not only during embryonic development but also after hatching (Dickel et al., 1998; Dickel et al., 2001; Dickel et al., 2006). The findings of this study clearly demonstrate that the visual system is sufficiently well developed to support visual habituation in a stage 30 embryo. As habituation can be tested in an individual in multiple sensory modalities and with different independent measures of behavioral responses, it may be uniquely suited for investigating the functional development of the nervous system before hatching (Smotherman and Robinson, 1992). Habituation allows animals to focus selectively on relevant stimuli and may serve as a prerequisite for other forms of learning (Rankin et al., 2009). In cuttlefish, it is known that some post-hatching behaviors are plastic and dependent on individual embryonic sensory experience (Darmaillacq et al., 2008; Guibé et al., 2010). It is therefore important for embryos to sort out relevant stimuli as they cannot escape from their eggs. These results reveal many possibilities for exploring embryonic sensory perception and learning development in cuttlefish. Taken together, this evidence

suggests that acquisition of environmental information in cuttlefish embryos is essential for survival.

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