Embryonic exposure to predator odour modulates visual lateralization in cuttlefish

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Predation pressure acts on the behaviour and morphology of prey species. In fish, the degree of lateralization varies between high- and low-predation populations. While lateralization appears to be widespread in invertebrates, we do not know whether heredity and early experience interact during development as in vertebrates. Here we show, for the first time, that an exposure to predator odour prior to hatching modulates visual lateralization in newly hatched cuttlefish. Only cuttlefish that have been exposed to predator odour display a left-turning bias when tested with blank seawater in a T-shaped apparatus. Exposure to predator odour all the incubation long could appear as an acute predictor of a high-predation surrounding environment. In addition, cuttlefish of all groups display a left-turning preference when tested with predator odour in the apparatus. This suggests the ability of cuttlefish to innately recognize predator odour. To our knowledge, this is the first clear demonstration that lateralization is vulnerable to ecological challenges encountered during embryonic life, and that environmental stimulation of the embryo through the olfactory system could influence the development of subsequent visual lateralization.

1. Introduction

The development and final expression of a phenotype involves dynamic interactions between genes and the environment [1,2]. Understanding the effect of these interactions on development is essential for expanding our knowledge of behaviour and brain function [3]. This phenotypic plasticity plays a key role in survival and fitness of individuals by adapting them to the environmental conditions they live in.

In the last decades, an increasing number of studies in both vertebrates and invertebrates have pointed towards the universality of brain and behavioural lateralization (e.g. in insects, molluscs, fish, birds, primates; for reviews, see [4–6]). Despite the growing amount of evidence of its advantages (e.g. strongly lateralized individuals possess cognitive advantages compared with their less-lateralized counterparts; [7–9]), there is substantial inter-individual variation in both the strength and the direction of behavioural asymmetries. It is therefore important to explore how and to what extent environmental pressures act on the development of lateralization.

In vertebrates, there are some well-known examples indicating that the ontogeny of lateralization depends on the interaction of genes and experience. In poeciliid fish, Brachyraphis epsiciopi, individuals collected in areas of high- and low-predation pressure differ in both the strength and direction of lateralized responses [10]. The strength of lateralization in laboratory-reared offspring is conserved between generations, consistent with it being a heritable character [11–13]. This may provide fitness benefits, both in terms of coordinated group anti-predator responses and in terms of dual information processing [14]. Conversely, when females are collected from a high-predation pressure environment, the direction of lateralization is different between the wild-caught females and their laboratory-reared offspring [11]. According to the authors, it is probable
that experience with predators may influence the way stimuli are processed by individuals. In several species of birds, differential visual stimulation of the two eyes prior to hatching generates an asymmetry in eye use (chicks [15], pigeons [16]; for a review, see [17]). Recently, Andrew et al. [18] showed that this is also the case in zebrafish. It is likely that mechanisms affecting lateralization differ: light acts on visual pathways in birds [19], and on asymmetric distribution of parainpial fibres supplying the habenulae in fish [20]. Predation pressure might act directly on the amount of light the bird embryos are exposed to (i.e. the hen being more or less likely to leave the eggs alone), and therefore on brain and behaviour lateralization [14].

Although early experience is known to play a key role in the development of lateralization in vertebrates, nothing is known about the factors leading to the development of lateralization in invertebrates. This raises the following question: are genetic influences overriding in invertebrates, or does early experience influence the development of lateralization? In the cuttlefish Sepia officinalis (cephalopod mollusc), individuals progressively develop a left-turning bias from 3 to 30 days post-hatching [21]. This behavioural asymmetry results from an eye-use preference [21], and is strikingly correlated with an asymmetry of several brain structures implicated in visual processing (vertical and optic lobes; [22]). The existence of a preferential left eye use to look for escape routes in cuttlefish could appear an evolutionary stable strategy [23,24]. According to these theories, intraspecific behavioural polymorphism may be the result of an interaction between genes and environmental pressures. Cuttlefish embryos develop entirely within the egg, there is no larval or planktonic stage and newly hatched cuttlefish do not benefit from parental care [25]. Sensory and cognitive enrichment positively affects the maturation of memory [26] and defensive strategies [27]. Considering that incubation and rearing conditions are relatively easy to control in the laboratory, and the phenotypic plasticity of cuttlefish, it appears pertinent to explore whether genes and early experience interact to determine the pattern and strength of visual lateralization in this species.

The aim of this study was to test whether the perception of chemical signals of a predator by cuttlefish embryos modulates visual asymmetry in newly hatched cuttlefish. Embryos differed according to the type of olfactory stimulation that they were exposed to prior to hatching: predator odour (seabass), non-predator odour (sea urchins), no odour (blank water). Three days after hatching, lateralization was assessed by testing untrained side-turning preferences in a T-shaped apparatus. As the strength of visual lateralization is not only age-dependent, but also motivation-dependent [21], cuttlefish were either tested with or without predator odour in the testing apparatus. We hypothesize that only cuttlefish incubated in a high-predation pressure environment (i.e. with predator odour) will be visually lateralized 3 days post-hatching, and that lateralization will be strengthened when predator odour is present in the apparatus.

2. Material and methods

(a) Animals

Cuttlefish (Sepia officinalis) used in this experiment (N = 193) all came from stranded clutches of eggs gathered in May 2010 on the beach of Agon-Coutainville (France). The eggs were divided into three groups (approx. 80 eggs per group) maintained in strainers floating in tanks supplied with running oxygenated seawater (water temperature = 18 ± 1°C). To control for possible between-clutch variation, we intermixed eggs for each group from at least 10 clutches of eggs.

The three groups of embryos were exposed to a different type of olfactory stimulation prior to hatching. The first group was incubated with a predator fish odour: the strainer containing eggs was floating in the tank (150 × 80 × 35 cm) of a European seabass (Dicentrarchus labrax; predator group). A perforated gate located next to the outflow allowed the strainer to be isolated from direct contact with the fish. The second group was incubated with a non-predator odour: the strainer was floating in a tank (80 × 60 × 40 cm) containing three sea urchins (Paracentrotus lividus; non-predator group). The third group was incubated in a strainer floating in a tank (80 × 60 × 40 cm) with no other animal (control group).

We collected hatchlings at 9:00 from eggs that had hatched during the night (Day 0). Cuttlefish were placed individually in rectangular tanks (4 × 11 × 8 cm) supplied with running oxygenated blank seawater (18 ± 1°C). Cuttlefish were maintained under daylight conditions and fed ad libitum with shrimps of suitable size until behavioural experiments began. In the experiments, we did not include cuttlefish that hatched from eggs exposed for less than 10 days to predator or non-predator odour.

The seabass and the sea urchins were obtained from the Centre de Recherches en Environnement Côtier (C.R.E.C., Luc-sur-Mer, France). They were fed commercial fish pellets (Le Gouessant, France), and red algae (collected on the beach of Luc-sur-Mer, respectively). After completion of behavioural testing, the seaweed and the sea urchins were given back to the C.R.E.C., and the cuttlefish were released at low tide, in pools where prey were abundant (Saint-Aubin-sur-Mer).

(b) T-shaped apparatus

The T-shaped apparatus (fully described in [21,22]) was constructed entirely from white PVC. One arm was used as the start box (5 cm long × 3 cm wide), whereas the other two arms formed the central alley (12 cm long × 4 cm wide). The start box was separated from the central alley by a Plexiglas sliding door. The far end of each goal arm (4 × 3 cm) was covered with a sand layer and darkened by an opaque sliding PVC top. The T-shaped apparatus was placed in a plastic box (35 × 40 × 15 cm) surrounded by opaque plastic walls (50 cm high), and on the right and left sides. The apparatus was illuminated by a 100-W lamp positioned 1 m above the centre of the apparatus.

In each group, cuttlefish were tested in the T-shaped apparatus with 3 cm deep seawater collected from either the seabass tank (‘tested with predator odour’), the sea urchins tank (‘tested with non-predator odour’) or blank seawater (‘tested with blank water’). Seawater was collected in a bucket from the appropriate tank to renew seawater in the apparatus between each trial. Ferrari et al. [28] have shown that a probable degradation over the time of chemical cues in the water can attenuate the risk assessment of predation by prey resulting in a decrease of anti-predator behaviour. Therefore, water inside the bucket was replaced by water from the appropriate tank between each cuttlefish (approx. every 30 min).

(c) Procedure

Cuttlefish were tested 3 days after hatching. Each cuttlefish was placed individually in the start box for 30 s before the Plexiglas sliding door to the central alley was removed. The cuttlefish was allowed to move freely out of the start box and into either arm of the apparatus. In order to avoid a positive reinforcement, as soon as the cuttlefish reached the end of the right or left arm, it

\[ 1 \]
was gently lifted out of water with a small net and placed back into the start box. The arm-choice was recorded for each trial. This procedure was repeated until 10 choices were made. Each cuttlefish was tested in only one testing condition (1 test = 10 choices) either with predator odour, non-predator odour or blank water.

(d) Statistical analysis
All data were analysed using VASSARSTATS software (http://faculty.vassar.edu/lowry/VassarStats.html; Richard Lowry).

(i) Laterality index
Lateral asymmetries were analysed by calculating for each subject a ‘laterality index’ (LI; [29]) using the formula: 
\[ LI = \frac{(\text{turn to the right} - \text{turn to the left})}{(\text{turn to the right} + \text{turn to the left})} \times 100. \]
LI is a continuous variable ranging from +100 to −100. A positive value indicates a right-turning bias, a negative value indicates a left-turning bias, and the value of 0 indicates no side-turning bias. Departures from random choices (0%) were estimated by one sample t-tests performed on the mean values of the LI for each testing condition in each incubation group. The data followed the assumptions of normality and homoscedasticity allowing us to perform parametric analyses. We tested for the effect of incubation condition (‘predator group’ versus ‘non-predator group’ versus ‘control group’) and testing condition (‘tested with predator odour’ versus ‘tested with non-predator odour’ versus ‘tested with blank water’) as independent factors using a two-way analysis of variance (ANOVA). If the null hypothesis was rejected, we used t-tests for pairwise post hoc comparisons and a values were adjusted according to the sequential Bonferroni method [30].

(ii) Individual side-turning preference
Side-turning preference was determined using a criterion of nine turns to the side. The arm-choice was recorded for each trial. The cuttlefish were classified into ‘left preference’, ‘right preference’ and ‘no preference’ (the remaining cuttlefish). The two-way ANOVA revealed a significant effect of testing condition (\( F = 3.15, p = 0.0452 \)) but no effect of incubation condition (\( F = 0.58, p = 0.5609 \)) and no interaction between incubation condition and testing condition (\( F = 1.64, p = 0.2162 \); figure 1). Pairwise post hoc comparisons revealed that the LI of cuttlefish tested with predator odour, with non-predator odour or with blank water in the apparatus did not differ neither in the control group (t-tests with sequential Bonferroni correction: \( n_{\text{tested with blank water}} = 22; \) tested with predator odour = 22; tested with non-predator odour = 22) nor in the predator group (t-tests with sequential Bonferroni correction: \( n_{\text{tested with blank water}} = 22; \) tested with predator odour = 17; tested with non-predator odour = 17; tested with predator odour/nondpredator odour: \( t_{38} = -2.04, p = 0.0485 \); tested with blank water/nondpredator odour: \( t_{38} = -0.24, p = 0.8116 \), nor in the predator group (t-tests with sequential Bonferroni correction: \( n_{\text{tested with blank water}} = 22; \) tested with predator odour = 22; tested with non-predator odour = 22; tested with predator odour/nondpredator odour: \( t_{45} = 0.45, p = 0.6550 \); tested with blank water/nondpredator odour: \( t_{45} = 0.15, p = 0.8815 \). Figure 1. Laterality index of each incubation group (control (white bars)/non-predator (grey bars)/predator (black bars)) for each testing condition. Error bars indicate s.e.m. One sample t-tests: * \( p < 0.05 \); *** \( p < 0.001 \); two-way ANOVA for independent samples: \# \( p < 0.05 \).

(b) Do hatchlings display a significant side-turning bias in the apparatus?
The right/left choices were not significantly different from random when cuttlefish were tested with blank water or with non-predator odour in the apparatus: in the control group (one sample t-tests: tested with blank water: \( t_{21} = -1.4926, p = 0.1504 \); tested with non-predator odour: \( t_{21} = -1.715, p = 0.1011 \); figure 1) and in the non-predator group (one sample t-tests: tested with blank water: \( t_{21} = -1.565, p = 0.1345 \); tested with non-predator odour: \( t_{16} = -0.96, p = 0.3513 \); figure 1). However, cuttlefish were more likely to turn to the left side when tested with predator odour in the apparatus: in the control group (one sample t-test: \( t_{21} = -6.9663, p < 0.0001 \); figure 1) and in the non-predator group (one sample t-test: \( t_{21} = -4.7422, p = 0.0001 \); figure 1). In the predator group, whatever the testing condition, cuttlefish were more likely to turn to the left side when tested with predator odour in the apparatus: in the control group (one sample t-test: \( t_{21} = -4.29, p = 0.0003 \); tested with non-predator odour: \( t_{21} = -3.9329, p = 0.0008 \); tested with predator odour: \( t_{21} = -2.6667, p = 0.0144 \); figure 1).

(c) Does incubation condition or testing condition alter the number of hatchlings with a significant side-turning preference?
The distribution of cuttlefish in the ‘left preference’, ‘right preference’ and ‘no preference’ classes was not significantly...
4. Discussion

Our study demonstrates a variation in the strength of side-turning bias in newly hatched cuttlefish when tested with or without predator odour (i.e. seabass odour) inside the apparatus. Cuttlefish of the three groups displayed a significant preference for turning to the left when tested with predator odour. Whereas hatchlings that were not exposed to predator odour during embryonic development (control and non-predator groups) showed no side-turning bias when tested with non-predator odour or with blank water, hatchlings that were exposed to predator odour (predator group) preferentially turned to the left in all testing conditions. This variation is linked to a modulation of the strength of the side-turning bias and not to an increase in the number of individuals with a significant left-turning preference.

The stronger side-turning bias observed in cuttlefish tested with predator odour, would suggest that cuttlefish are able to recognize predator odour as a chemical evidence of the potential presence of a predator nearby. Several studies have shown that a number of prey species are able to recognize a predator ‘innately’ and display anti-predator responses towards them (in birds [31], amphibians [32] and fishes [33]). Lukowiak et al. [34] showed that the odour of an unknown predator induces behavioural (vigilance behaviour), as well as neuronal (decreased activity of neurons controlling respiration) changes in laboratory-reared *Lymnaea*. Cuttlefish usually avoid open and lit areas where they cannot bury themselves [35]. When placed in our T-shaped apparatus, cuttlefish are primarily concerned with seeking shelters. Sand and shadow provided on both sides of the apparatus would thus appear as potential hiding places. The preference to turn to the left observed in cuttlefish tested with predator odour could well be the result of a greater motivation to hide. Indeed, side biases are not necessarily fixed in an individual; lateralization has been described in the literature as being dependant on motivation. In fish, females display an enhanced lateralization when they need to detour past an obstacle to approach males if they have been isolated from males for two months [36]. Such alteration of the strength of lateralization was also observed in fish when the motivation to shoal is increased (i.e. shortly after capture in a net [37]).

Cuttlefish exposed to predator odour during embryonic development are more likely to turn to the left regardless of testing conditions. Exposure of salamander eggs to predator odour induces more efficient anti-predatory strategies in post-hatching *lirrae* (reduced activity and preference for vegetated habitats [38]). In fish, lateralized behaviour patterns fluctuate between populations of the same species exposed to differing levels of predation pressure [10]. In poeciliid fish, females from high-predation areas display stronger side-turning biases, when faced with a novel object than those from low-predation areas [11]. Surprisingly, in our experiment, cuttlefish exposed to predator odour as embryos are more likely to turn to the left even when tested with blank water.

<table>
<thead>
<tr>
<th>Tested with predator odour</th>
<th>Left preference</th>
<th>Right preference</th>
<th>no preference</th>
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<tr>
<td>Control group</td>
<td>6</td>
<td>16</td>
<td>1</td>
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<tr>
<td>Non-predator group</td>
<td>14</td>
<td>17</td>
<td>0</td>
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<tr>
<td>Predator group</td>
<td>17</td>
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Table 1. Cuttlefish distribution according to incubation groups and testing conditions. Fisher’s exact probability test: NS = not significant, \( p > 0.05 \).
with blank water or non-predator odour. The exposure to predator odour throughout the incubation period could perhaps appear as an acute predictor of a high-predation environment. Cuttlefish could thus behave as if a threat was potentially around even without chemical evidence of a predator nearby. In rats, males exposed to an early social stress (e.g. taken away from their mother several minutes each day for the first 20 days of life) display lateralized behaviour (for a review [5]). It has been suggested that higher levels of stimulation in early life prepare an individual (e.g. by being more lateralized) for life in an environment demanding high cognitive ability [5]. In birds and fish, lateralized individuals show improved ability to forage while remaining vigilant for predators (in birds [9] and in fish [39]). A strengthened cerebral asymmetry is likely to provide fitness benefits to these cuttlefish in terms of dual information processing. This may improve survival of hatchlings in a high-predation environment.

Embryonic exposure to a predator, or at least its odour, would be able to induce an increase in the strength of visual lateralization by modulating the development of cerebral lateralization. Chivers et al. [40] have shown that exposure of young fish to predators induced morphological changes (height and width). It has been demonstrated in chicks that sensory experience (by placing the embryo reversed in the egg) or cancelled (by incubating eggs in the dark; for a review, see [5]). The side biases observed in cuttlefish which were exposed or not exposed to stressful stimuli as embryos could result from a variance in the neurological architecture underlying behavioural asymmetries. The side-turning biases observed in the predator group may be a reflection of both the neurobiological (i.e. brain lateralization) and the psychological (i.e. the way stimuli are perceived and processed by individuals) impacts of predator exposure during embryonic development.

To our knowledge, this is the first clear demonstration that lateralization is vulnerable to ecological challenges encountered during embryonic life, and that environmental stimulation of the embryo through the olfactory system can influence the development of subsequent visual lateralization. Given the advantages of being lateralized described both in vertebrates and invertebrates [7–9], the question arises as to why lateralization has not become fixed in the population. One possibility is that there may be compensatory advantages linked with the lack of asymmetry, such as more balanced awareness [41]. The effects of an environmental factor on the development offer a source of individual variation, potentially allowing selection for each individual of the more advantageous trade-off between being lateralized or not. Research in cuttlefish provides a relevant invertebrate model system for investigating the development of brain and behavioural asymmetries.

The experiments complied with French animal testing laws.

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References


