

# Mirrors inhibit growth and stimulate lateralized response to prey in larvae of the Mexican axolotl *Ambystoma mexicanum*

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## Abstract

Laterality of brain and behavior at the individual and/or group level is a characteristic of all vertebrate classes, including amphibians. It is well recognized that the right-eye/left-hemisphere system is more efficient in discrimination of edible and non-edible items. However, the ontogenesis of this or other lateralized responses has rarely been investigated. Here we present the first evidence of ontogenesis of right-eye/left-hemisphere response to live food in the Mexican axolotl, *Ambystoma mexicanum*. Furthermore, we test a possible influence of mirror images imitating the presence of conspecifics on laterality of the reaction to food, as well as on the growth and development of axolotls; we then compare the results to those known for anuran tadpoles. We placed the newly hatched axolotls into aquaria either with transparent or mirror walls individually (first experiment), or in a group of 25 animals (second experiment). The larvae were fed *Artemia salina* nauplii and regularly scored for the number and direction (either left or right) of their attacks towards the prey. Body length was measured twice a month. The statistically significant lateralized response to food was first detected in only three-week-old larvae and was right-sided, suggesting processing of visual information in the left brain hemisphere. At the same age, the left hemisphere seems to start its specialization in more efficient perception of prey. In both experiments we found a significant difference between the larvae growing in the transparent and mirror aquaria, with a tendency towards a stronger right-sided lateralization in the mirror aquaria. Also, the mirror walls inhibited larval growth, especially at the age of 60–75 days. A similar phenomenon is known in overcrowding of anuran tadpoles, which is discussed in terms of visual brain stimulation.

**Keywords:** Mexican axolotl, *Ambystoma mexicanum*, caudate amphibians, lateralized response to food, response to mirrors, ontogenesis of lateralization, perceptual lateralization, larvae growth, inihemispheric visual stimulation.

## Introduction

Visually guided asymmetric behavioral response to food is a well-established phenomenon characteristic to many, if not all, vertebrate species (Mench and Andrew, 1986; Güntürkün and Kesch, 1987; Alonso, 1998; Vallortigara et al., 1998; Miklosi, Andrew, and Gasparini, 2001; Robins and Rogers, 2004; Robins, Chen, Bea-

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zley, and Dunlop, 2005; Bonati, Csermely, and Romani, 2008; Giljov, Karenina, and Malashichev, 2009; Lippolis, Joss, and Rogers, 2009; Karenina, Giljov, Ivkovich, and Malashichev, 2016). These reactions are considered to be a reflection of asymmetric processing of sensory inputs coming from the right eye into the contralateral left brain hemisphere. The right-eye/left-hemisphere system is characterized by a slower reaction to environmental cues, such as novel stimuli or predators, but performs better in situations when considered response is necessary, e.g., in discrimination between food and non-food items (Rogers and Anson, 1979; Rogers, Vallortigara, and Andrew, 2013).

A number of studies describe lateralization in response to food in anuran amphibians (Robins et al., 1998; Robins and Rogers, 2004, 2006). Immature Iberian ribbed newts, *Pleurodeles waltl*, were also shown to have a strong rightward bias in attacking live prey (Giljov, Karenina, and Malashichev, 2009), which supports the general role of the right-eye/left-hemisphere system for processing information on potential prey in caudate amphibians, too. Most recently, we have shown that a group tendency to attack prey appearing in the right visual hemifield exists also in the Mexican axolotl (Izvekov et al., 2018). Also, axolotls react to their mirror images as to conspecifics and not potential prey, and more readily inspect the images if the mirror is placed to the left of the axolotls, suggesting (given a decussating optic tract) left-eye/right-hemisphere processing of socially significant images (Izvekov et al., 2018). Eye preference takes place both in schooling fish and anuran larvae (which also form schools). Placed in between mirrors, tadpoles of five anuran species (*Bufo bufo*, *Bufo viridis*, *Rana temporaria*, *Pelophylax esculentus* and *Bombina variegata*) preferred to approach the mirror to the left of them and keep themselves near to the mirror (Bisazza, De Santi, Bonso, and Sovrano, 2002). In the majority of previous studies, other vertebrates also preferred to observe conspecifics with their left eye, which indicates right-hemisphere processing. Moreover, the ability of mirror self-recognition by primates seems also to be a function of the right brain hemisphere (Hecht, Mahovetz, Preuss, and Hopkins, 2017). Hence, it seems most correct to assume that observation of the animal's reflection in the mirror is functionally identical to observation of another conspecific (Sovrano, Rainoldi, Bisazza, and Vallortigara, 1999; Sovrano, Bisazza, and Vallortigara, 2001).

Importantly, both the viewing of food items and mirror images may influence physiological state and/or development, but with opposite effects. For example, when cows received food which appeared first in their left visual hemifield for a long period of time, they produced more milk and increased their breeding performance (Rizhova and Kokorina, 2005; Rizhova et al., 2006). In a similar experiment with lateral food presen-

tation, rats were healthier and demonstrated better resistance to stress factors (Rizhova and Kokorina, 2005; Rizhova et al., 2006).

These interesting results suggest a strong effect the unilateral activation of the right hemisphere has on animal physiology, including subsequent activation of certain circuits in the neural, endocrine, and immune systems. On the contrary, the effect of multiple mirror images may depress larval growth and development in anuran tadpoles (Rot-Nikcevic, Taylor, and Wassersug, 2006; Gouchie, Roberts, and Wassersug, 2008). Hence, just a presentation of pure visual stimuli may give the same effect as a complex multifactorial situation which usually takes place in overcrowding of tadpoles under natural or laboratory conditions.

Little is known, however, about the ontogenesis of asymmetric behaviors (Güntürkün and Ocklenburg, 2017), when they first appear and whether they change with maturity. The aim of this work was to characterize the ontogenesis of lateralized reaction to food in the Mexican axolotl, *Ambystoma mexicanum*, and possible influence of mirrors both on behavioral lateralization and growth of larvae. We chose axolotl as the experimental object as a widely used laboratory species and relatively solitary carnivorous animal, providing a contrast to most of the available anuran tadpoles, which are filter feeders or vegetarians, readily forming schools. This approach made it easier to trace the ontogenesis of lateralized feeding behavior. At the same time, the axolotls could be less affected by mirror exposure than gregarious anuran larvae. To reach our aim, we employed two types of experimental design. Immediately after hatching, the axolotls were placed (individually or in groups) in two kinds of aquaria (either with transparent glass walls or with all lateral walls made of mirrors), and their reactions to food and body lengths were regularly measured during three months. Here we show that axolotls reveal lateralized reaction to food from the age of three weeks, displaying stronger lateralization when reared in mirror aquaria, and they grow slower when viewing multiple mirror images of conspecifics.

## Materials and Methods

### ANIMAL CULTIVATION

The adult animals (all of the white *dd* strain) were kept in the laboratory under automatic light control (14 h day: 10 h night) and temperature (19–22°C) in the continuous-flow aquaria system. The eggs were obtained from adults after natural spawning initiated by a lower temperature (15–16°C) and shutting off the water flow in the aquaria, according to Khattak et al. (2014). The eggs were incubated in the same aquaria, where the spawning occurred after the parents were removed. In the experi-

ments, we used larvae hatched from eggs belonging to four different spawns obtained from four different pairs of parents. The larvae were fed nauplii of *Artemia salina* daily.

#### EXPERIMENTAL DESIGN

Two experiments were performed in parallel. In the first one, immediately after hatching, the larvae were placed individually in 3 L glass aquaria half-filled with water. Of 20 aquaria, 10 had transparent walls, and the other 10 had mirror walls. The bottoms of all aquaria were transparent, and they were evenly illuminated. To exclude any possible physical gradients (temperature, light, noise, etc.) which could involuntarily influence the results of the experiment, the aquaria with transparent and mirror walls were placed alternately.

Twice a week, we fed larvae in a Petri dish by dropping water full of *Artemia* from a pipette just over and in front of the larva. The nauplii of *Artemia* swam randomly around the head of the axolotl larva, being, presumably, evenly distributed in the left and right visual hemifields of the latter. Every time the individual larva was fed, we scored a total of 15 prey strikes directed either to the left or right. The attacks were usually directed at the prey appearing over the head and laterally on either side of the axolotl larva, approximately at the angle of 45° to the main body axis; attacks followed the accentuated movement of the head and the forepart of the body towards the prey, which made it very easy to classify the movement as left- or right-sided. This test was performed 25 times for each of the 20 larvae until they reached the age of three months, yielding a total of 375 responses per individual. By the end of the experiment, the larvae reached a size over 60–70 mm and switched to another type of food. The body length in each larva was measured twice a month during feeding in a Petri dish. The length was measured to the nearest millimeter using graph paper placed under the dish. For the final analysis of larval growth, we used only the data obtained from those larvae that reached the age of three months and for which the number of measurements were minimally enough for statistical evaluation ( $n = 10$ ). When considering behavioral characteristics, the data on larvae that died before the age of two weeks were discarded since they were insufficient for statistical analysis.

The second experiment was made on two groups of larvae ( $n = 25$  each). Immediately after hatching they were placed into two rectangular 12 L glass aquaria half-filled with water. The water in the aquaria was changed daily. As in the first experiment, one of the aquaria was fully transparent, while the other had lateral walls made of mirrors. Both aquaria were evenly illuminated from above. The axolotls were fed *Artemia salina* nauplii daily. Twice a month, the length of each animal was

measured as described above. The experiment lasted for 2.5 months, after which time it was terminated to prevent serious injuries to limbs and gills as well as larval cannibalism that might occur in *Ambystoma* species in crowded colonies (Wells, 2007). By the end of the experiment, only 11 axolotls remained alive in each aquarium, although the exact cause of each death was not known (stress by overcrowding? infections? etc.).

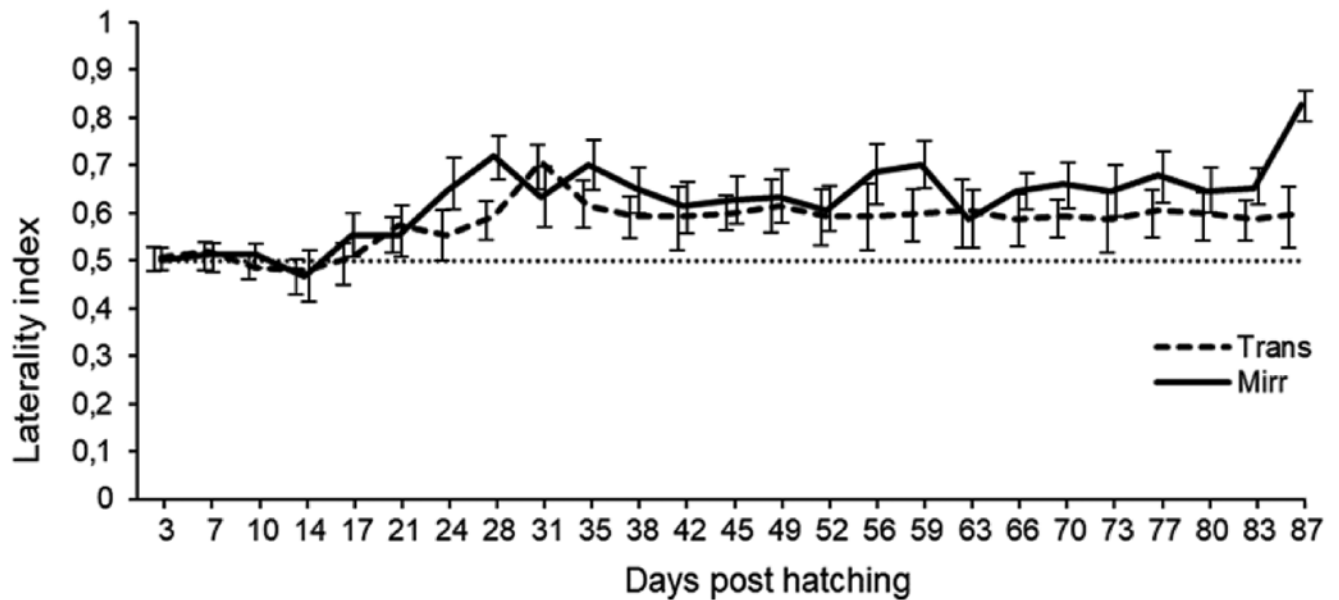
#### STATISTICAL ANALYSES

To quantify behavioural lateralization we used an individual laterality index calculated as the overall percentage of right-sided responses for each specimen throughout the experiment (calculated from a total of 375 responses per larva). The distributions of laterality indices were tested for normality using the Shapiro–Wilk  $W$  test. Group-level lateralization in response to food was scored also as a mean percentage of right-eye use and assessed using the Wilcoxon signed rank test, as these data were not distributed normally. To characterize lateralization at a particular age, we averaged all individual scores for the given age ( $n = 10$ ) and eventually obtained a series of 25 mean values over the test. We analyzed these data using a two-way ANOVA with laterality index as a measurement variable, while the age category and the ‘mirror/transparent walls’ factor were treated as fixed effects. While comparing the general effects of mirror and transparent aquaria on the growth of the larvae, we used factorial ANOVA (with factors Replicate, Type of Wall and Age). Additionally, a non-parametric sign test (Lehmann, 1975) was conducted to compare the larval growth.

## Results

The individual laterality index was above 0.5 in most individuals (eight of ten raised in aquaria with transparent walls and nine of ten in aquaria with mirror walls), showing that most larvae reacted more often to food appearing on their right side. A one-sample Wilcoxon signed-rank test indicated that the bias was statistically significant for the axolotls raised in aquaria with mirror walls ( $z = 2.24$ ,  $p < 0.05$ ) and transparent walls ( $z = 2.55$ ,  $p < 0.05$ ). The same was true for a pooled sample of 20 individuals raised in both types of aquaria ( $z = 3.45$ ,  $p < 0.01$ ).

In both types of aquaria, the larvae displayed no lateralization until approximately 17 days post-hatching (Fig. 1). After that, the right-side lateralization started to manifest itself. It grew for about ten days and then, at the age of 28–87 days, remained stable at a level of  $0.604 \pm 0.032$  ( $M \pm SE$ ) for aquaria with transparent walls and  $0.651 \pm 0.034$  for aquaria with mirror walls. In other words, only in 35–40% of cases the larvae preferred to



**Fig. 1.** Average individual indices ( $M \pm SE$ ) of lateralized response to food in *Ambystoma mexicanum* larvae raised in aquaria with transparent (trans) and mirror (mirr) walls at different age (days).

attack the prey appearing in their left visual hemifield, thus favouring the food items on their right side. The two-way ANOVA (factors Age and Type of Wall) indicated no statistically significant interaction between these factors ( $F = 0.661$ , d. f. = 24,  $p > 0.05$ ). Next, the ANOVA revealed a statistically significant effect of the age of the larvae on their laterality index ( $F = 2.627$ , d. f. = 24,  $p < 0.001$ ). Also, it was found that rightward lateralization was significantly higher in ‘mirror’ animals (two-way ANOVA,  $F = 8.815$ , d. f. = 1,  $p < 0.01$ ). Similarly, the sign test applied within the age range of 21 days and older, when the laterality reaches a steady level, also revealed a significantly stronger bias in animals from the mirror aquaria ( $G_{(1)} = 2.91$ ,  $p < 0.05$ ). Their mean laterality indices were higher in 17 out of 20 target age groups, showing that raising the axolotls in aquaria with mirror walls caused stronger rightward lateralization of their feeding behavior.

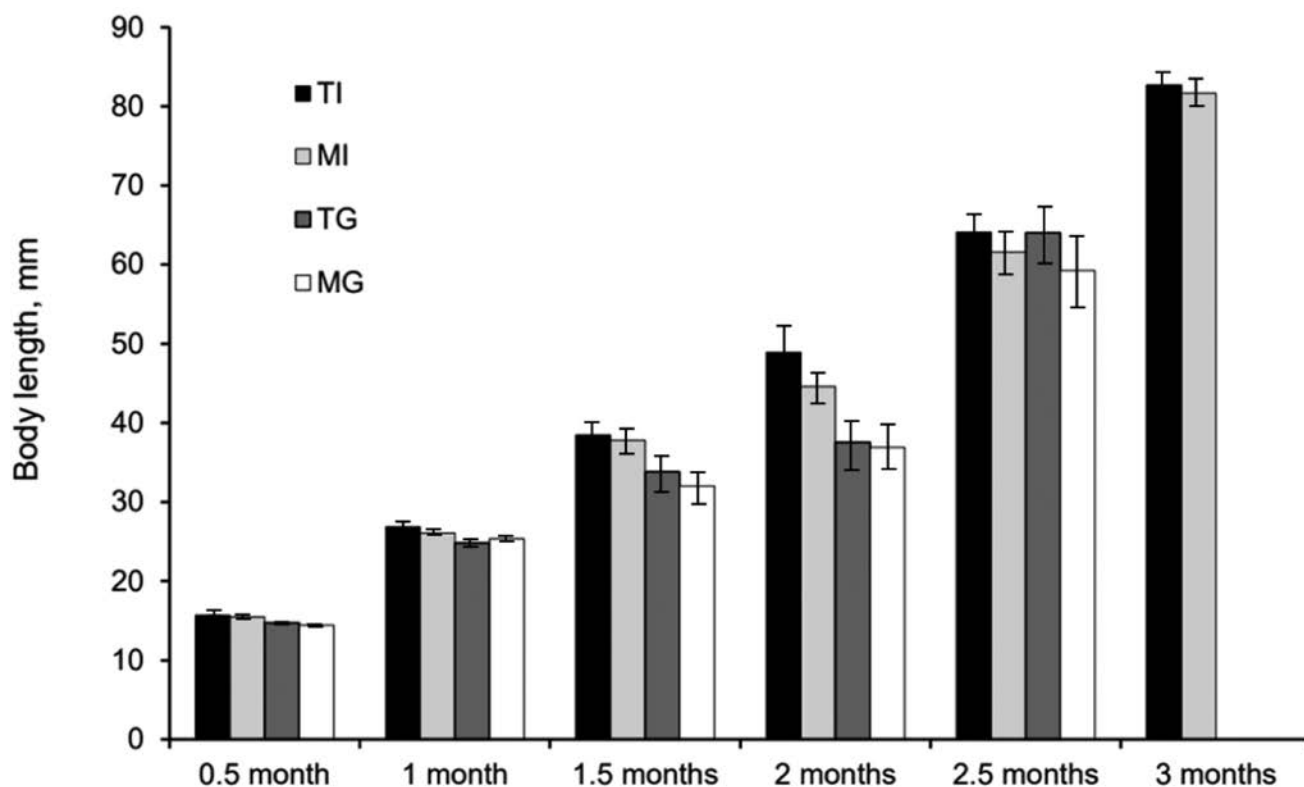
The body lengths of the axolotls kept in aquaria with transparent and mirror walls are given in Fig. 2. The effect exerted by mirrors on the growth rate was analysed using a factorial ANOVA with factors Replicate (df = 1), Type of Wall (df = 1), and Age (df = 4). This analysis, performed on the first five ages and ten individuals from each replicate, yielded no significant three-way interaction ( $F = 0.367$ , d. f. = 4,  $p > 0.05$ ). The factorial ANOVA indicated a  $p < 0.1$  trend for Type of Wall ( $F = 3.241$ , df = 1). Meanwhile, as it is clear from Fig. 2, in nearly all age groups, the animals grew faster in the transparent aquaria than in those with mirror walls. This bias was observed in all six ages for individually housed larvae, and in four out of five ages for the group-housed ones,

altogether in 10 out of 11 comparisons. So the sign test, unlike the factorial ANOVA, yielded a significant difference in the growth rates of these two groups of larvae ( $G_{(1)} = 2.41$ ,  $p < 0.05$ ). However, in the case of group-raised axolotls, the effect of growth inhibition by mirror images seems to be manifested somewhat weaker than in individual growth.

## Discussion

Our data support the previous study reporting that Mexican axolotls prefer to attack prey seen in their right hemifield (Izvekov et al., 2018). Moreover, we have shown this effect both at the individual and group levels. The rightward tendency to react to food was previously found in representatives of all major clades of vertebrates, such as teleost fish (Miklosi and Andrew, 1999), caudate (Giljov, Karenina, and Malashichev, 2009) and anuran amphibians (Vallortigara et al., 1998), squamate reptiles (Robins, Chen, Beazley, and Dunlop, 2005), and birds (Rogers and Anson, 1979; Güntürkün and Kesch, 1987), suggesting that this is a universal feature of all vertebrates. All these data correspond to the general pattern of functional inter-hemispheric asymmetry which is likely typical for all vertebrates (Rogers, 2002) and some invertebrates (Schnell, Hanlon, Benkada, and Jozet-Alves, 2016).

We found that right-sided lateralization in response to food stimuli first appears in axolotls only in the fourth week post-hatching; hence, it is not present from birth, but rather develops with maturation of certain larval brain areas and/or with training. In chicks (Rogers, 1990)



**Fig. 2.** Mean body length ( $M \pm SE$ , mm) of *Ambystoma mexicanum* larvae raised under different conditions (TI — transparent, individual; MI — mirror, individual; TG — transparent, group; MG — mirror, group). Size of animals was measured twice a month up to the age of 2.5–3 months (for each age class,  $n = 10$  for animals from individual aquaria, and  $n = 11$  for those kept in groups).

and fish (Budaev and Andrew, 2009) some (though not all) types of lateralized behaviors can be induced by early light stimulation. This influence presumably takes place through light stimulation of photosensitive pineal and parapineal organs in the epithalamus, the structural and functional asymmetry of which seems to be linked to lateralized behaviors (Güntürkün and Ocklenburg, 2017). To what extent this might be applied to axolotls and whether the maturation of the lateralized response to food is light-dependent in this species is not known. Interestingly, the tadpoles of *Xenopus laevis*, when viewing their mirror images, exhibited a significant eye preference only at certain developmental stages (Gouchie, Roberts, and Wassersug, 2008), while other tadpole species in a similar mirror test demonstrated a stable leftward bias at all stages examined (Bisazza, De Santi, Bonso, and Sovrano, 2002). It is probable that in different anuran species, the degree of such visual lateralization may vary, or the degree of different motor or visual asymmetries just in *X. laevis* is so low that group-level lateralization is not detectable at all or at some stages (Wassersug, Naitoh, and Yamashita, 1999; Kostylev and Malashichev, 2007; Giljov, Karenina, and Malashichev, 2009). In contrast to *Xenopus* and another studied representative of Urodela, *Pleurodeles waltii* (Giljov, Karenina, and Malashichev, 2009), axolotls displayed a stronger

response to food appearing in their right visual hemifield both at the majority of developmental stages (except for the earliest ones; this study), and after sexual maturity (Izvekov et al., 2018). Hence, the results of our work clearly demonstrate for the first time the development of a lateralized behavior in an amphibian species. More importantly, the development of the asymmetric response to food was found to be stimulated in the presence of mirrors.

Indeed, the tendency for axolotls to strike at prey appearing in their right visual hemifield was steadily stronger in mirror aquaria at the vast majority of age groups tested. The nature of this mirror-induced increase in the lateralized response to food, however, is unclear. It is known that a long-term unilateral presentation of food can, in principle, affect many physiological parameters in rats and cows. If the food first appears on the left side for a long time, the animals feel and perform better, cows give more milk and larger litters, and become healthier (Rizhova and Kokorina, 2005; Rizhova et al., 2006). This phenomenon was interpreted as a result of unilateral visual stimulation of the right brain hemisphere with subsequent effect through the hypothalamo-pituitary-adrenal system. In the case of our experiments, the food was not presented asymmetrically, but was evenly distributed in the water (freely swimming *Artemia* larvae),

and thus could not influence directly via positive feedback stimulation of the right hemisphere. Moreover, the preference of axolotls to ingest food seen in their right visual hemifield and decussation of optic fibers further do not support such an explanation.

The answer may be found only if one considers the results of the experiments altogether. We found that in the mirror aquaria, the axolotls grew slower compared to those in the transparent ones, and had a smaller average size by the end of the experiments. The material of the walls was the only varying factor (see Materials and Methods), suggesting that mirrors might somehow inhibit larval growth. As we have recently assumed elsewhere (Izvekov et al., 2018), the mirror images are perceived by the axolotls not as moving prey or dangerous objects, but as socially significant visual stimuli, i.e., conspecifics, which attract animal's attention, evoking neither attack nor escape. Therefore, a mirror visually multiplying a number of conspecifics creates an illusion of their dense group. This, in turn, can inhibit the growth and development of axolotls, as it has been previously shown in some anuran larvae (Rot-Nikcevic, Taylor, and Wassersug, 2006). Further on, the mirror images can potentially stimulate feeding responses. On the one hand, since the feeding acts are mostly driven by the left hemisphere, thus being directed to the right, an elevated level of readiness to feed may be at least partly responsible for the increase in asymmetry observed in our experiments. A more intensive search for prey and higher competition between the larvae could cause the observed decrease in their growth as a side effect.

Interestingly, in vertebrate and invertebrate species, environmental visual cues may also influence the development of lateralization. A most striking example is the development of left-turning bias in cuttlefish. In this cephalopod mollusk, newly hatched individuals have only individual preferences to turn in empty T-maze and a slight but not significant tendency to turn left, and this bias does not change with age. However, when shelters are available at both ends of the T-maze, they demonstrate a clear and significant increase in turning behavior between days 3 and 60 (Joset-Alves et al., 2012). This suggests an increasing role in motor lateralization of the left side of the brain stimulated by shelters as potential places to hide (visual input is processed by the same side from which it is perceived in cuttlefish (O'Brien, Mezrai, Darmaillacq, and Dickel, 2016)). A phenomenon even more similar to that found in axolotl is known for anuran larvae (Rot-Nikcevic, Taylor, and Wassersug, 2006; Gouchie, Roberts, and Wassersug, 2008). Here, the visual cues (either mirror images or models of conspecifics) elicited a strong inhibiting effect on larval growth. In contrast to most salamander species, the anuran larvae often dwell in temporary waters, form large schools or are forced to live in overcrowded conditions. Previous research has revealed the suppressive action of mirrors through visual stimulation

of the larval brain, which may partially explain the inhibiting effects of overcrowding on the growth and development of anuran larvae (Gouchie, Roberts, and Wassersug, 2008). Our finding of this effect in more solitary and predatory axolotls indicates that it is not necessarily caused by severe overcrowding, but can be quite common in other animal species in less stressful conditions, and potentially can have a strong effect on natural populations.

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