

# Salamanders breeding in subterranean habitats: local adaptations or behavioural plasticity?

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## Keywords

cave; *Salamandra salamandra*; underground; ecology; predation performance; biospeleology.

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## Abstract

The exploitation of novel habitats requires the expression of specific behaviours. This may occur through both behavioural plasticity and local adaptations, but assessing the relative role of these processes is challenging. Animals colonizing underground environments are exposed to strong selective pressure: epigeous species using caves during one or more phases of their life cycles can help to understand mechanisms allowing cave exploitation. The fire salamander (*Salamandra salamandra*) may breed both in cave springs and in epigeous streams. We compared predation performance of larvae from cave and stream populations, and assessed whether local adaptations or behavioural plasticity (or both) improve predation in underground environments. We performed a behavioural experiment about prey detection and capture. We collected larvae from both caves and streams, and reared them under contrasting conditions: underground and outdoor. In the darkness, we tested two measures of predation performance of larvae: time of head turning towards the prey and frequency of prey capturing. We used an information-theoretic approach to assess the relative support of potential mechanisms (adaptations vs. plasticity). Both cave and stream larvae were able to detect and capture prey in the darkness. Larvae born in caves captured prey with higher success than those from streams. Acclimatization to underground conditions did not improve predation performance, suggesting that plasticity plays a minor role. This study indicates that the exploitation of underground environments leads to behavioural local adaptations, allowing an improved predation performance in environments where prey are both scarce and difficult to detect.

## Introduction

During the exploitation of new habitats, populations are exposed to selective pressures that may lead to evolutionary adaptations for multiple traits, such as behaviour, morphology, metabolism and physiological tolerance. Such intraspecific adaptations can evolve quickly, particularly when natural selection is strong, or generation time is fast (Miner *et al.*, 2005; Steinfartz, Weitere & Tautz, 2007). On the other hand, a single genotype can generate multiple phenotypes in response to variation of abiotic and biotic conditions of environments (phenotypic plasticity; Miner *et al.*, 2005; Gomez-Mestre, Touchon & Warkentin, 2006). Phenotypic plasticity allows a quick response to environmental conditions, may favour the colonization of heterogeneous habitats (Hawlena, Hughes & Schmitz, 2011; Peacor *et al.*, 2011; Jourdan-Pineau, David & Crochet, 2012) and can play an important role in adaptation processes (Price *et al.*, 2003). Although plasticity may absorb the effects of environmental variation and reduce the genetic response to selection, thus retarding evolutionary change (Gomez-Mestre & Buchholz, 2006), it may facilitate evolu-

tionary diversification if environmentally induced phenotypic changes allow populations to survive or colonize different or new habitats, and thus develop independently evolving lineages (Gianoli & Valladares, 2011; Lind & Johansson, 2011).

When the new habitat strongly differs from the usual one, the emerging adaptations are expected to be particularly important (Dreiss, Guillaume & Clobert, 2009). Underground environments are extremely interesting to understand the processes allowing the exploitation of novel environments (Jugovic *et al.*, 2011; Riesch, Plath & Schlupp, 2011). Underground environments differ from the epigeous ones for multiple features, such as light scarcity, limited food and stability of microclimate (Culver *et al.*, 2004). Epigeous species attempting the colonization of underground habitats are exposed to strong selective pressures that can induce dramatic phenotypic changes, and may lead to genetic adaptations favouring individual development, survival and fitness (Friedrich *et al.*, 2011; Riesch *et al.*, 2011). These changes can be both morphological, such as in underground populations of the fish *Astyanax fasciatus* that evolved typical troglomorphic features (loss of eyes and pigmentation; Salin *et al.*, 2010), and

behavioural. This is the case of cave populations of the newt *Calotriton asper*, which uses its magnetic compass to move in subterranean environments (Schlegel, Steinfartz & Bulog, 2009) with a mnemotactic feature (Schlegel *et al.*, 2009).

Troglophilous species can use underground environments during one or more periods of their life cycles, but do not strictly depend on them. Only some of their life-history stages, or some populations, are indeed facing the selective pressure of underground environments. Understanding the relative role of plasticity and local adaptation for the colonization of new habitats is challenging. For instance, studies on troglomorphic populations of the cave fish *A. fasciatus* [totally blind and depigmented (Romero, 2011)] found that while fishes raised under total darkness did not develop any noticeable eye, fishes from the same population raised in outdoor conditions did, suggesting a prominent role of plasticity in the evolution of troglomorphic animals. Nevertheless, the actual importance of phenotypic plasticity for the adaptation to cave environments remains debated (Poulson, 2011).

The fire salamander, *Salamandra salamandra*, is a highly plastic amphibian that usually breeds in epigeal streams, but in karstic areas often uses underground springs as breeding habitat (Manenti *et al.*, 2009a, 2011; Ianc *et al.*, 2012). The subterranean environment strongly influences the development of larvae which, for instance, require more time to reach metamorphosis than in epigeal streams (Manenti *et al.*, 2009a, 2011). Therefore, the exploitation of underground environments likely requires the development or the elaboration of already existing features, allowing to face the surrounding environment, and to grow and develop in this habitat. In subterranean areas, prey are both rare and difficult to detect and capture; therefore, food scarcity poses major limits to organisms living underground, and may influence predation behaviour (Hervant, Mathieu & Durand, 2000; Plenet, Hervant & Joly, 2000; Schneider, Kay & Fagan, 2010). Cave-dwelling salamanders use specific, active strategies of prey search, combined with mechanic-, chemo- and eventually electro-perception (Schlegel *et al.*, 2009). Analyzing predation performance of salamanders from cave-dwelling populations may provide important insights on the level of adaptation to these environments.

Here, we studied behavioural adaptations of salamander populations breeding in caves, by testing whether they have better predation ability in underground conditions, compared to populations breeding in epigeal streams, and we assessed whether predation performance can be explained by local adaptations or behavioural plasticity. Larvae with different origin (cave vs. epigeal streams) were reared under different conditions. We used a hypothetico-deductive approach, within the information-theoretic statistical framework, to assess the support of three hypotheses on mechanisms allowing salamander larvae to exploit underground environments. H0: the same phenotype may be able to use both environments [generalist phenotype (Jourdan-Pineau *et al.*, 2012)]; H1: Cave-breeding populations have local adaptations improving their predation performance in underground conditions. This hypothesis predicts that larvae from cave-breeding populations have better predation performance in the darkness,

irrespective of the conditions in which they are maintained. H2: Behavioural plasticity improves predation performance in underground conditions. This predicts that larvae grown in underground conditions improve their predation performance in the darkness, irrespective of the population of origin (cave/stream).

## Materials and methods

### Study system

We studied fire salamander populations from the catchment basins of the Lambro, Adda and Seveso rivers in Northwest Italy (see map in Manenti *et al.*, 2011). It is characterized by hilly and mountainous reliefs with broadleaved woodlands and a dense hydrographic network. In this area, the fire salamander is ovoviviparous and it frequently lays larvae in streams and creeks (Ficetola, Padoa-Schioppa & De Bernardi, 2009; Manenti, Ficetola & De Bernardi, 2009b). In the most karstic part of the study area, there are several natural and artificial caves with subterranean springs where fire salamanders regularly give birth to their larvae. The caves chosen are all underground springs exclusively filled by the subterranean aquifer without resurgence of superficial water. Adult salamanders enter in caves, and select them as breeding sites if they have suitable environmental features (accessibility, presence of prey); salamander larvae successfully develop and metamorphose in these environments (see Manenti, 2008; Manenti *et al.*, 2009a, 2011).

### Experiment setting

We performed a behavioural experiment to assess predation performance in larvae laid in underground and epigeal streams. We collected newborn larvae from 20 different underground springs and from 20 neighbouring epigeal streams (one larva per each spring/stream). At the beginning of the experiment, average total length ( $\pm$ SD) was  $34.7 \pm 5.0$  mm; all larvae were at first developmental stage (Zakrzewski, 1987). We sampled the stream closest to each cave with salamander larvae; the average distance between caves and the nearest stream with larvae was around 350 m (mean 349 m, SD 32.45). Larvae were reared for 30 days under two different conditions: 10 larvae from caves and 10 from streams were raised in underground conditions, while 10 from caves and 10 from streams were raised under outdoor conditions. Larvae were fed twice per week with 10 mm live chironomid larvae (one chironomid/salamander). Chironomids were chosen because they are often found in cave streams (R. M., unpubl. data). In preliminary tests, movements of the chironomid larvae were not reduced by the dark conditions for at least half an hour. Salamander larvae were individually maintained in  $10 \times 11$  cm finely punched plastic boxes placed in one pool ( $450 \times 100$  cm, depth 5 cm) of the underground laboratory, and in one tank ( $320 \times 150$  cm, depth 5 cm) in outdoor conditions. Larvae reared in cave were maintained in a laboratory set-up in a 60-m draining gallery built in the 17th century (access

provided by the Monte Barro Regional Park); the gallery is used for breeding by salamanders and provides natural cave-like conditions with constant water temperature (around 11.8°C). Outdoor larvae were maintained nearby a stream where salamanders breed, with similar light and temperature conditions (maximum daily illuminance: 789 lux; average water temperature: 12.5°C). Total length was similar between cave and stream larvae, both at the beginning and at the end of the experiment (*t*-test: both  $P > 0.7$ ).

During March–May 2011, for each larva, we recorded two measures of predation performance: (1) time of the first head turning towards the prey: the larva turns the whole head in the same direction of the prey; this measure was used as a surrogate of the time required for prey detection (hereafter: head turning; Uiblein *et al.*, 1992). (2) Predation success: the larva successfully snapped the prey and ate it within the 10 min trial. We did not consider the time required for capturing the prey as a further measure of predation performance because a large number of salamanders did not capture the prey during the 10-min trials (see Results section).

In order to standardize experimental conditions both at the capture moment and after the month of raising, we stopped feeding the larvae 1 week before behavioural experiment. Before behavioural experiment, each larva was placed in a 12 × 10 cm plastic box (depth 5 cm) and let acclimatizing for 24 h.

At each experimental trial, one chironomid larva was positioned in the box, about 10 cm from the salamander. Each trial lasted up to 10 min. For predation success, we performed two experimental sessions (at the collection moment and after 1-month raising) with three trials per larva during each session. The three trials of each session were performed on a single day. For head turning, we performed only one experimental session after the raising, with three trials per larva. All trials were performed in cave conditions under total darkness (illuminance < 0.01 lux). All observations were performed by the same observer using an infrared visor (Eye Clops PRTGP470256, Jakks Pacific, Malibu, CA) that does not cast any visible light for the larvae. We did not perform tests in light conditions because preliminary experiments showed very quick detection and 100% predation rate for both cave and stream larvae. After behavioural trials, we also measured the

total length of larvae (total length). After the experiment larvae were reared for further 2 weeks and then released in the collection localities.

## Statistical analysis

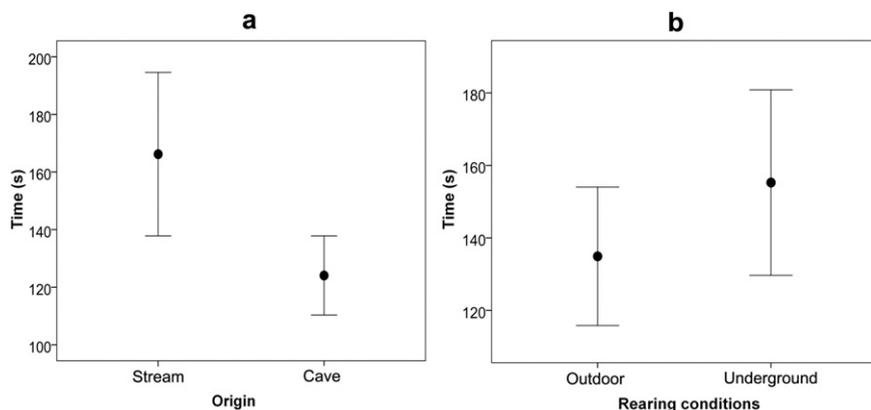
We used generalized linear mixed models (GLMMs) to analyze relationships between the predation performance of larvae and the factors that can affect it. GLMMs allow to analyze dependent variables in which different observations are non-independent; in all our models, we included larva identity as random factor. We built models corresponding to the hypotheses on mechanisms potentially enhancing predation performance in underground environments (generalist phenotype, local adaptation and phenotypic plasticity, see Introduction), and used an information-theoretic approach, based on Akaike's Information Criterion (AIC), to assess their support (Burnham & Anderson, 2002). For head turning, we used a Gaussian error distribution. The model representing to the generalist phenotype hypothesis included the variable 'length' only (no fixed factors); the local adaptation model also included the origin of larvae (cave vs. stream), while the plasticity model also included rearing conditions (cave vs. outdoor) as independent variable (Table 1a).

For predation success, we used a binomial error distribution. The generalist phenotype model included experimental session only (immediately after collection, after 1 month of rearing) as fixed factor; the local adaptation model included the origin of larvae (cave vs. stream) and experimental session, while the plasticity hypothesis model included rearing conditions (cave vs. outdoor), session and the interaction between rearing conditions and session (Table 1b). The interaction term was included because tests were performed before and after the month of rearing in the darkness, but the plasticity model predicts an improvement of performance after the rearing. In all models (for both time of head turning and predation success), we also included total length as covariate, to take into account potential differences caused by size. Results of analyses remained identical if total length was not included into models (not shown).

We then calculated the AIC for each model: AIC trades-off explanatory power versus number of predictors; parsimonious

**Table 1** Candidate mixed models representing the hypotheses explaining predation performance of salamander larvae under darkness conditions. *K*, number of parameters in the model; AIC, Akaike's Information Criterion; *w*, AIC weight of the model

(a) Dependent: time of head turning				
Hypothesis	Variables	<i>K</i>	AIC	<i>w</i>
Local adaptation	Origin, length	4	737.02	0.43
Generalist phenotype	Length	3	737.13	0.40
Plasticity	Rearing conditions, length	4	738.83	0.17
(b) Dependent: predation success				
Hypothesis	Variables	<i>K</i>	AIC	<i>w</i>
Local adaptation	Origin, experimental session, length	5	324.58	0.86
Generalist phenotype	Experimental session, length	4	328.54	0.12
Plasticity	Rearing conditions, experimental session, length, interaction between rearing conditions and session	6	331.85	0.02



**Figure 1** Time of first head turning towards the prey in salamander larvae with different origin. (a) Comparison of performance of larvae from stream and cave populations. (b) Comparison of larvae reared under outdoor and underground conditions. Error bars are SE of the mean.

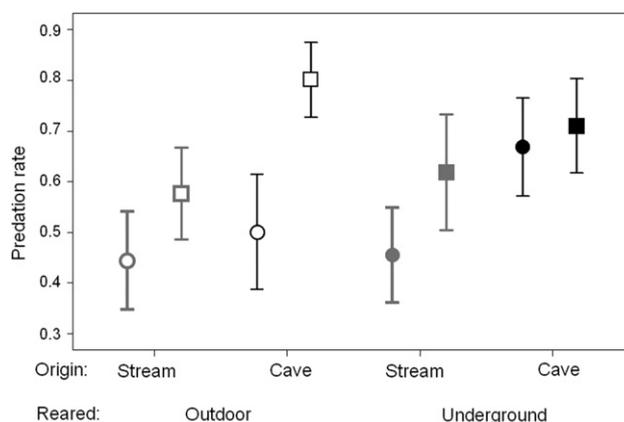
models explaining more variation have the lowest AIC values and are considered to be the ‘best models’. Conclusions were identical if the AIC corrected for small sample size (AICc) was used instead than AIC. For each candidate model, we calculated the Akaike’s weight  $w$  (AIC weight), representing the probability of the different models given the data (Lukacs *et al.*, 2007). Prior to performing the analyses, time of first head turning was square root transformed, and total length of larvae was log transformed to improve normality. We built models with maximum likelihood, and performed analyses using the package LME4 under the R environment (<http://www.r-project.org>).

## Results

Both stream and cave larvae were able to detect and capture prey under darkness conditions. They showed an active foraging behaviour by wandering through the experimental boxes with slow movements, looking for prey. Once they detected the prey, larvae stopped movements for a few seconds and started to slowly move closer to the prey before quickly snapping it. Only in very few cases snaps were unsuccessful. Snapping distances were very short (2 cm or less). We did not detect obvious qualitative differences in foraging behaviours between cave and stream larvae, nor between larvae reared under different conditions.

For head turning, the local adaptation model showed the lowest AIC value (Table 1a). Nevertheless, the general phenotype model had very similar AIC; therefore, the support of the local adaptation model was weak. According to the best AIC model, head turning was slightly faster in larvae from cave populations (Fig. 1), but differences were not significant ( $F_{1,35} = 2.041$ ,  $P = 0.162$ ). The effect of total length was very weak and non significant ( $F_{1,35} = 0.440$ ,  $P = 0.511$ ). The generalist phenotype model had a support similar to the local adaptation model, while the plasticity model showed very limited support, on the basis of AIC (Table 1a), and larvae reared in underground conditions did not show fastest head turning (Fig. 1).

For predation success, the local adaptation model showed the lowest AIC value and was considered to be the best model



**Figure 2** Predation success of larvae with different origin, before the acclimatization period (circles) and after the acclimatization period (squares). Grey: larvae from streams; black: larvae from caves; open symbols: larvae reared outdoor; filled symbols: larvae reared underground. Error bars are SE of the mean. Note that no significant differences among treatments are found before the beginning of the experiment ( $\chi^2_1 = 0.11$ ,  $P = 0.74$ ).

(Table 1b). According to this model, predation success in the first experiment was highest in larvae from cave populations ( $\chi^2_1 = 5.968$ ,  $P = 0.015$ ; Fig. 2) and during the second experimental session ( $\chi^2_1 = 5.132$ ,  $P = 0.023$ ; Fig. 2), while the effect of total length was not significant ( $\chi^2_1 = 0.566$ ,  $P = 0.452$ ). The average predation success was 67% in cave larvae, and 52% in stream larvae; cave larvae showed a consistently higher predation success, compared to stream larvae, during both the first and the second experimental session (Fig. 2). The generalist phenotype model and the plasticity model showed higher AIC values and limited weight (Table 1b). The plasticity model showed the lowest weight (Table 1b): rearing larvae in underground conditions did not improve their predation success in the darkness more than maintaining them in outdoor conditions (Fig. 1). According to this model, predation success increased in the second experimental session ( $\chi^2_1 = 4.760$ ,  $P = 0.02$ ) but was not affected by rearing

conditions ( $\chi^2_1 = 0.609$ ,  $P = 0.44$ ) nor by the interaction between rearing conditions and session ( $\chi^2_1 = 0.09$ ,  $P = 0.76$ ).

## Discussion

Larvae from both caves and streams were able to detect and capture prey in underground conditions. Epigeous larvae of the fire salamander are active during both day and night, with a preference for night time when the drift of preys is highest (Oberrisser & Waringer, 2011). The species locates preys mainly by visual detection, and epigeous larvae can rely on night vision (Himstedt, 1971) nevertheless, chemical and mechanical cues can help during night predation (Ferrer & Zimmer, 2007). The ability of predation in night likely plays an important role in allowing salamanders to use caves as breeding sites, and the ability of fire salamander larvae to prey and grow in underground conditions confirms the opportunism of the species (Joly, 1968, Ficetola *et al.*, 2009).

The relationship between head turning and the origin of larvae was weak (Table 1). It is possible that the observation error for this measure is high because larvae can randomly turn head in the same direction of the prey during the exploration of the experiment box, and these movements might have been misinterpreted as detection of the prey. Actually, in several occasions the capture of the prey did not occur immediately after the apparent detection, and in multiple cases larvae never captured the prey even though they turned the head towards them. On the other hand, successful predation was always preceded by head turning. This suggests that such standard measure of prey detection helps to assess predation (Uiblein *et al.*, 1992) but should be corroborated by additional parameters. Actually, we observed a positive relationship between the time of first head turning and the overall time required to capture the preys (Pearson's correlation  $r = 0.506$ ,  $P < 0.001$ ). Finally, for this measure, sample size was smaller than for the analysis of predation success, limiting statistical power.

On the other hand, our models clearly identified the hypothesis best explaining predation success: Success was better in larvae laid in caves, supporting the local adaptation hypothesis (Table 1, Fig. 2). Furthermore, after 1 month of raising all the larvae, from both caves and streams, improved their predation performance. The improved performance after 1 month is probably related to their older age: older larvae may have better performance because of multiple reasons, including more experience with these prey, enhanced sensorial systems or locomotion, higher food requirements. Conversely, predation performance was not affected by raising conditions, as spending 1 month in underground conditions did not increase predation ability. Raising larvae in a single source may in principle increase the likelihood to detect significant treatment effects. However, we did not detect any treatment effect, suggesting that this issue did not pose major problems to the outcome of our study. Our results do not support the plasticity hypothesis, and suggests that previous experience of predation in the darkness does not improve performance.

The highest predation performance was observed in larvae laid in caves, both before and after the acclimatization

(Fig. 2), in agreement with the predictions of the local adaptation hypothesis. In some karstic localities, populations depend on the availability of underground breeding sites (Bressi & Dolce, 1999). Multiple features of cave environments strongly affect larval development and metamorphosis timing of salamanders (Clergue-Gazeau, 1975; Manenti *et al.*, 2011). This exposes larvae to strong selective pressures that are very different from those in outdoor wetlands. For instance, invertebrates are extremely abundant in streams where salamanders usually breed (Manenti *et al.*, 2009b), while underground prey are rare, and the travelling distance between prey items may be large (Uiblein *et al.*, 1992; Culver *et al.*, 2004). Even though salamanders select for breeding those caves with the richest macrobenthos, the abundance of prey in breeding caves remains much lower than in streams (Manenti *et al.*, 2009a, 2011). Moreover, because of lack of light, prey are more difficult to detect, and predators must rely on chemical or mechanical cues (Uiblein *et al.*, 1992). Individuals with the highest predation performance are thus favoured by selection and, if some population depends on underground water for breeding success, selective forces may have led to the evolution of larval traits enhancing predation performance in these challenging environments. Amphibians often show fine scale evolutionary adaptations, particularly in response to the features of breeding sites. For instance, in larvae of frogs, adaptations to wetland features (e.g. temperature differences between typologies of ponds) can evolve across very limited distances (Skelly, 2004, Ficetola & De Bernardi 2006, Richter-Boix *et al.*, 2010). Divergence among populations breeding in different environments can be maintained in the presence of gene flow (Richter-Boix *et al.*, 2011), even though gene flow often limits local adaptations (Storfer, 1999). In the fire salamander, studies have demonstrated that adaptation to contrasting breeding environments (ponds vs. streams) result in differences for development parameters and for the capability to exploit food resources (Weitere *et al.*, 2004; Steinfartz *et al.*, 2007). Behavioural adaptations for feeding strategies can have powerful impact on larval parameters, and therefore play a key role in the evolution of local adaptations (Urban, 2007; Lind & Johansson, 2011).

In the darkness, most of larvae used an active widely foraging mode. When prey are abundant and mobile, predators often adopt a sit-and-wait foraging behaviour (Uiblein *et al.*, 1992). On the contrary, wide foraging strategies are frequently adopted when predator should look for invisible, sedentary or rare prey (Uiblein *et al.*, 1992; Uiblein, Engelke & Parzefall, 1995). A widely foraging mode is described for some cave dwelling urodeles such as the olm, *Proteus anguinus* and the cave populations of the newt *Calotriton asper* (Uiblein *et al.*, 1992). Despite the ability of salamanders to exploit subterranean springs for breeding, larvae are likely to suffer high energetic costs when foraging underground because of the active foraging behaviour. Prey scarcity and darkness determine longer search times and efforts, thus the net energetic gain per prey is limited. The food intake, and the limited energetic gain per prey probably 'act together to' determine the slow larval development in caves, which may require more than 8 months (Manenti *et al.*, 2009a).

Additional strategies, such as cannibalism (Eitam, Blaustein & Mangel, 2005), may be adopted and need to be investigated in future studies.

The study of underground environments may provide powerful insights on the mechanisms allowing the colonization and exploitation of new habitats. Epigeous species can try to use caves during one or multiple life-history stages, but the peculiar features of underground environments (e.g. lack of light, scarcity of trophic resources, microclimatic conditions) are challenging and expose animals to steep and novel selective pressures (Fenolio, Graening & Stout, 2005; Schneider *et al.*, 2010). Our study shows that local adaptations for behavioural parameters can occur over fine spatial scales, as a consequence of the use of underground water for breeding; behavioural adaptations can be extremely important for the exploitation of these unique environments.

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