

Role of density and resource competition in determining aggressive behaviour in salamanders

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Abstract

Intraspecific aggression is an important aspect of social behaviour that can significantly affect individual survivorship and population dynamics. Aggressiveness may be particularly important in food-deprived habitats, like caves, but few studies have investigated behavioural and ecological determinants of aggressiveness and cannibalism in cave vertebrates. In this study, we evaluated the role of density, competition for food, starvation and rearing history under cave-like conditions in promoting aggression. We performed a behavioural experiment on larvae collected from multiple underground springs and neighbouring streams, and we reared them under two different settings: total darkness and outdoor conditions. Intraspecific attacks were very scarce in hatchlings, and increased after 1 month. Starvation periods and high density clearly increased aggressiveness. Furthermore, aggressiveness strongly increased under competition conditions (presence of other larvae feeding). Aggressiveness increased with age more quickly in larvae from caves than in those from streams. Environmental features typically experienced by cave populations, such as starvation and high density, increase intraspecific attacks and might promote cannibalism. Strong plasticity for aggression may enhance the possibility to successfully colonize and exploit food-deprived environments where no other predators can survive, such as underground environments.

Introduction

Intraspecific aggression is a common phenomenon in vertebrates and invertebrates, being an important aspect of social behaviour. Aggressive interference can significantly affect individual survivorship and population composition (Luke & Bechler, 2010). In nature, interacting conspecifics often differ in size, age, experience and state, and these conditions may affect the outcome of intraspecific interactions (Arnott & Elwood, 2008, 2009). Moreover, for several species, smaller conspecifics may represent an important resource for the large ones in food-deprived environments (Wise, 2006). In this case, aggression can grade into full-scale cannibalism with important ecological consequences (Kishida *et al.*, 2009; Jefferson *et al.*, 2014). Cannibalism is relatively frequent in nature, with complex effects on food webs (Elgar & Crespi, 1992). Cannibalism is usually observed in predator populations (Kudo & Shirai, 2012). In a system in which one or more predators use cannibalism as a trophic resource, the classical predator–prey interactions occur within the same population and trophic level, with consequences for population features (Rudolf, Kamo & Boots, 2010). Aggressiveness and cannibalism

therefore increase the number of ecological parameters that can affect predation rates and predator–prey survival, and the complexity of relationships (Wise, 2006). Aggressive intraspecific interactions potentially grading into cannibalism are thus expected to strongly affect the structure and dynamics of communities (Rudolf *et al.*, 2010; Wissinger *et al.*, 2010).

Several factors can induce aggression such as high population density, limited availability of other prey, the morphology of interacting individuals and the occurrence of other predators (Hammar, 2000; Wissinger *et al.*, 2004), but our knowledge of the behavioural and ecological mechanisms involved in the expression of aggression remains incomplete. Salamanders are an excellent biological model for such studies. Salamander larvae are carnivorous; most species are generalist predators of small invertebrates and can have an important role in determining the structure of freshwater communities (Petranka, 1989). Intraspecific aggressions are often reported, with larger larvae that snap at smaller conspecifics, producing severe injuries, including the loss of part of the tails or entire limbs, that can significantly reduce survivorship (Belden, Wildy & Blaustein, 2000), and these aggressions may

ultimately result in cannibalism (Elgar & Crespi, 1992; Wells, 2007). Cannibalism may provide important trophic advantages to salamander larvae (Denoël, Whiteman & Wissinger, 2006). This is particularly important in oligotrophic or ephemeral environments where prey is scarce and conspecifics represent a rich and easily available food source (Sadeh, Mangel & Blaustein, 2009).

The hypogean (underground) environment is an understudied habitat in which aggression grading into full-scale cannibalism might be frequent (Manenti *et al.*, 2009b), as few trophic resources are available compared with the epigeal (outdoor) habitats (Culver & Pipan, 2009). Some species of salamanders are able to exploit both epigeal and underground water for breeding. For instance, the European fire salamander *Salamandra salamandra* usually breeds in epigeal streams. However, in karstic areas, it often uses underground springs for breeding (Manenti *et al.*, 2009b, 2011; Ianc *et al.*, 2012). Breeding underground may provide advantages, such as higher environmental stability and lower risk of desiccation. However, in underground springs, food availability is severely limited and the density of fire salamander larvae is generally high (Limongi *et al.*, in press); therefore, larvae require more time to complete development (Manenti *et al.*, 2011). Food shortage and the coexistence between large, old larvae and newborns are factors that might favour aggression. Aggression might be therefore an adaptive feature allowing cave colonization. The existence of local adaptations to cave environments has been hypothesized, as cave-born larvae are more able to catch prey in total darkness condition than epigeal larvae (Manenti & Ficetola, 2013). Fire salamander larvae show plasticity for foraging strategy between streams and caves, shifting from a typical sit-and-wait strategy under light conditions, to a widely active one in the darkness, cave-born larvae being particularly plastic in their response (Manenti & Ficetola, 2013; Manenti, Denoël & Ficetola, 2013).

In this study, we evaluated the factors affecting intraspecific aggressive behaviour in fire salamander larvae, considering larvae with different origin (cave/stream) to take into account the potential occurrence of local behavioural adaptations and plasticity. We measured biting attempts as the first stage of aggressions, which in salamanders, ranges from initial biting to full-scale cannibalism. We did not allow cannibalism itself to occur owing to ethical considerations. We conducted two separate experiments to evaluate the role of two factors representing the social environment: density of conspecifics and being in presence of another individual that is catching a prey (resource competition). Impact of resource competition on salamander behaviour might determine major consequences in food-deprived environments like caves, where resources are patchily distributed and prey detection is rare. Furthermore, we evaluated whether conditions that are present in caves (food shortage, lack of light), together with larvae body sizes, may promote intraspecific aggression. For instance, in caves larvae often attain larger size and suffer food shortage: both these features are expected to increase aggression rate, and potentially, the propensity to cannibalism (Reques & Tejedo, 1996).

Materials and methods

Study area

We studied fire salamander populations in Lombardy (north-west Italy) from the catchment basins of the Lambro and Seveso rivers (45°48' N, 9°03' E; altitude range: 340–890 m a.s.l.). The area is covered by broadleaved woodlands and a dense hydrographic network flows from hilly and mountainous reliefs. The populations of fire salamander are ovoviviparous (larviparous) in this area and they generally lay larvae in streams and creeks (Manenti, Ficetola & De Bernardi, 2009a; Ficetola *et al.*, 2011), but female salamanders may also use natural and artificial caves with subterranean springs as breeding sites (Manenti *et al.*, 2009b, 2011). Salamanders select the caves more accessible and with more prey items (nevertheless, caves remain food-deprived environments, if compared with superficial waters) (Manenti *et al.*, 2009b); salamander larvae successfully develop and metamorphose in these environments (Manenti *et al.*, 2011). The study system was approved by the ethics committee of Lombardy Region Authority and authorized complying with the regional law 10/2008, p.n.: F1.2013.0002091.

Experimental settings

We used behavioural experiments to assess the factors promoting aggression in larvae laid in underground and epigeal streams. We collected newborn larvae (one larva per each spring/stream) to be used for tests (hereafter called 'test' larvae) from 13 different underground springs and 12 neighbouring epigeal streams. At the beginning of the experiment, all larvae were at the developmental stage one (Juszczyc & Zakrzewski, 1981) corresponding to newborn larvae and recognized by the dorsal caudal membrane position. We reared larvae for 30 days under two different light conditions: 7 larvae from caves and 6 from streams were raised in total darkness conditions (light intensity constantly <0.01 lux), while 6 from caves and 6 from streams were raised under outdoor-like conditions (photoperiod of 12 h of light, using a laboratory neon lamp, and 12 h of darkness). All the larvae were maintained at an average temperature of 18°C. Thirty days is a temporal window allowing a significant increase in larval body size (Juszczyc & Zakrzewski, 1981). Larvae were individually maintained in 10 × 11-cm plastic boxes (depth: 5 cm), clustered in two blocks per each light treatment. Larvae were fed three times per week with live prey (Chironomid larvae, *Artemia salina* and *Tubifex* sp specimens). Every week we gave the same typology and the same amount of prey to each larva: 20 prey per week during the first 20 days and 30 preys per week during the other 10 days. Total length was similar between cave and stream larvae, both at the beginning [mean ± standard deviation (SD) = 33 mm ± 0.2] and at the end (mean ± SD = 48 mm ± 1.3) of the experiment (*t*-test: both $P > 0.7$).

Twelve additional newborn larvae (average length: 33 mm) to be used as target (hereafter named 'target' larvae) were collected from two additional epigeal streams, 3 km and >25 km from the nearest sampling site of test larvae,

respectively. Thus we were able to have the same length and developmental stage of target larvae at the beginning and end of the 30-day test period, because of variation in altitude of collection streams and the timing of female deposition. Due to the short duration of the trials (less than 30 min including acclimatization), none of the larvae were physically injured by intraspecific interactions. After the experiment, larvae were reared for 2 additional weeks and then released in their collection localities.

Behavioural trials

For each larva, we recorded the number of snaps attempts to a target larva as a measure of aggressive behaviour. As at the collection the starvation status of larvae was unknown, we fed them for 3 consecutive days. Before each experimental trial, we measured the total length of each larva as a proxy of body size. For each larva, the density and competition tests were performed both at the capture and after the month of rearing. We performed two different tests to assess the role of density and competition for food in determining aggressive behaviour.

Test 1: density

The effect of larval density on aggressiveness was assessed recording the number of snaps performed by the tested larvae in two conditions: with one and four target larvae. Each larva was tested under two food starving conditions: satiated (fed 18 h before the test) and starved (not fed for 3 days). One prey per day can be considered as high food availability conditions for fire salamander larvae (Krause, Steinfartz & Caspers, 2011). In each period (at capture/after rearing) we performed three trials for each larva for each density and food shortage condition. In each trial, we counted the number of snaps given by each test larva to the target larvae in a 35 × 25-cm tank for 5 min. Trials started after 2 min of acclimatization, in which the test larva was alone in the arena. All observations were performed by the same observer in light conditions. The order of and status of test and target larvae was always randomly changed.

Test 2: competition for food resources

The effect of competition on aggressiveness was assessed by putting the test larva with a target larva eating a prey. Just before the start of each trial, a target larva was fed with a live *Chironomus* sp. larva in the opposite side of the tank from the test larva. In all the trials the target larva immediately bit the *Chironomus*. The trials started immediately after the bite by the target larvae; both visual and chemical cues from the prey and the conspecific were detectable by test larvae. Trial periods, number and larvae conditions were the same as in density test. Also for the competition test, each larva was tested under both satiated and starved conditions.

Statistical analysis

We performed two separate analyses for the density and competition experiments. Overall, for each test we considered four

variables that could affect aggressiveness: larvae origin (cave/stream), rearing treatment (before rearing, reared under darkness, reared under light), satiety (starved/sate) and size (total length, in mm). We used generalized linear mixed models (GLMMs) to analyse relationships between the aggressive behaviour of larvae and the factors that can affect it. GLMMs allow analyses of dependent variables in which different observations are non-independent; in all our models, we included larva identity as a random factor, as each larva was measured multiple times. We also included trial number as an additional fixed factor, to account for habituation to experimental settings. For the two experiments, the number of biting attempts was considered as the dependent variable. As independent variables we included the following: density or competition status (depending on the experiment), starvation status, origin and light treatment as independent factors, and body size of larvae as covariate. Light treatment was coded as a three-level factor: 0, before the treatment; 1, after the darkness treatment; and 2, after the light treatment. We then used planned orthogonal contrasts to test the differences between light treatments. Orthogonal contrasts allow to test the difference among treatment groups, without inflating the type I error (Crawley, 2007). Specifically, through contrasts we evaluated: (a) whether the number of bites varies between the pre- and the post-treatment tests; (b) whether, after 1 month of raising, the number of bites varies between the light and the darkness treatments. We also tested the interaction between origin and light treatment, as in presence of local adaptations the response to the two light regimes may be different between cave and stream larvae (Manenti *et al.*, 2013); only significant interactions were included in the final model. The dependent variable was a count; therefore, we used a Poisson error distribution. The residual deviance of models was similar to the residual degrees of freedom, suggesting that our models were not affected by overdispersion (for all the models, variance inflation factor <1.5) (Crawley, 2007; Richards, 2008). We used a likelihood ratio test to assess significance of variables; we created models using the lme4 package in R (Bates & Maechler, 2010). We used violin plots to show the relationships between aggressiveness and experimental conditions; violin plots are combinations of kernel density plots and box plots (Hintze & Nelson, 1998) that show the probability density of the data at different values.

Results

At least one biting attempt was observed in 214 of the total 852 5-min trials performed; the number of snaps ranged between one and five per trial.

Density experiment

Density played a major role in determining the number of snaps performed by test larvae, as the number of snaps clearly increased at high densities of 'target' larvae (Table 1, Fig. 1a). Furthermore, the number of snaps was increased by starvation (Table 1; Fig. 1b), while we did not detect any effect of body size of larvae (Table 1). We also observed a significant

Table 1 Results of the density experiment

	Effect size	SE	χ^2	d.f.	P
Density	0.508	0.048	144.7	1	<0.0001
Starvation	0.544	0.114	23.8	1	<0.0001
Body size	1.737	1.330	1.8	1	0.180
Treatment			11.7	2	0.003
Origin	-0.062	0.326	2.4	1	0.124
Origin × treatment			7.4	2	0.025
Trial number			3.1	2	0.208

Effect of experimental conditions on the number of snap attempts of larval salamanders on conspecifics. d.f., degrees of freedom; SE, standard error.

effect of light treatment. Biting attempts significantly increased after the 30-day rearing treatment in both conditions (contrasts: $\chi^2_1 = 6.92$, $P = 0.009$). Furthermore, during the second test period, biting attempts were significantly higher in larvae reared under darkness than in those reared under natural daylight conditions ($\chi^2_1 = 6.93$, $P = 0.008$) (Fig. 1c). A significant interaction between origin and treatment indicated that change in biting rate was different between cave and stream larvae (Table 1). Specifically, during the second test period, cave larvae increased their biting rate more than did stream larvae (orthogonal contrast: $\chi^2_1 = 4.68$, $P = 0.031$; Fig. 2).

Competition experiment

The presence of another larva catching a prey significantly increased the aggressiveness of test larvae (Table 2, Fig. 3a). Furthermore, the number of snaps significantly increased in starved larvae and in larger larvae, while we did not detect a significant effect of origin (Table 2). We also observed a significant effect of light treatment. Biting attempts increased during the second period, even though the increase was marginally non-significant (contrasts: $\chi^2_1 = 3.14$, $P = 0.077$). Furthermore, in the second test period biting attempts were highest in larvae reared under darkness than in those reared under natural daylight conditions ($\chi^2_1 = 7.12$, $P = 0.008$). For this analysis, the interaction between treatment and origin was not significant ($\chi^2_2 = 4.97$, $P = 0.083$); therefore, it was not included into the model. Nevertheless, the results remained identical if the interaction was included in the model (not shown).

Discussion

Larvae from both caves and streams displayed aggressions, and the frequency of aggressive behaviours was influenced by multiple parameters. Density, the presence of food competitors and food shortage clearly increased aggressions. Furthermore, aggressiveness levels were higher for larvae reared under cave-like conditions. Our results help understanding the pressures occurring on salamander populations breeding in small bodies of water where food is scarce and conspecifics density

high, and highlight the complexity of behavioural interactions of salamander larvae.

Density and food competition were among the factors showing the strongest influence on aggression attempts. The number of snaps performed by focal larvae was higher at the greater density (Fig. 1a). In caves, breeding pools are generally small, with particularly high densities. The high density of salamanders observed in these environments can have major effects, by slowing larval growth and reducing survival (Limongi *et al.*, in press). Aggression may allow individuals to gain food resources, but might also represent a first step of cannibalistic behaviours. The positive relationship between density and aggressiveness is well known for several species of Urodels (Wells, 2007), and an increase of the cannibalistic behaviour of fire salamander larvae at high densities was observed by Reques & Tejedo (1996), which showed a strong relationship between the number of aggressive interactions and density. The results of the density experiment might be affected by the fact that in the high-density treatment, more potential targets were available. Nevertheless, our experimental settings matched the conditions observed in some natural populations where the density of larvae is particularly high (Limongi *et al.*, in press), suggesting that aggressive interactions may be particularly frequent there. Additional studies are required to test whether the increase in biting attempts is truly determined by the density of conspecific, or is a by-product of the high number of potential targets. In one of our experiments, we did not detect an effect of body size on aggression rate (Table 1), with results different from Reques & Tejedo (1996). However, the number of individual larvae tested was limited, and this might have reduced the power of this test.

Starvation and living for a month in total darkness significantly increased aggressive attacks. Hungry animals value food resources more than would satiated animals (Arnott & Elwood, 2008). On the one hand, this may increase aggressive attacks in presence of competitors for food resources (Fig. 3b); on the other hand, when trophic resources are scarce, the value of other larvae as potential food source would increase (Sadeh *et al.*, 2009). In cave environments, long periods of starvation are particularly frequent (Schneider, Christman & Fagan, 2011), and the high larval density can exacerbate the lack of prey (Limongi *et al.*, in press). Overall, these observations suggest that, in the wild, aggressive interactions may be particularly frequent in cave populations, possibly increasing the occurrence of cannibalism. Similar patterns occur in ambystomatid mountain lake populations, where several cohorts of larvae coexist (Wissinger *et al.*, 2010). In caves, larval development may take even more than 9 months, thus cohorts born during different seasons often overlap (Manenti *et al.*, 2011), and newborns likely constitute a food source for older larvae (Manenti *et al.*, 2009b). For those old larvae, high aggressiveness may allow to feed on newborn larvae and gain the necessary energy to reach metamorphosis. Although smell is a strong cue for prey detection in salamanders (Hervant, Mathieu & Durand, 2001), the higher aggressiveness of larvae after living 1 month under total darkness might arise because visual detection remains

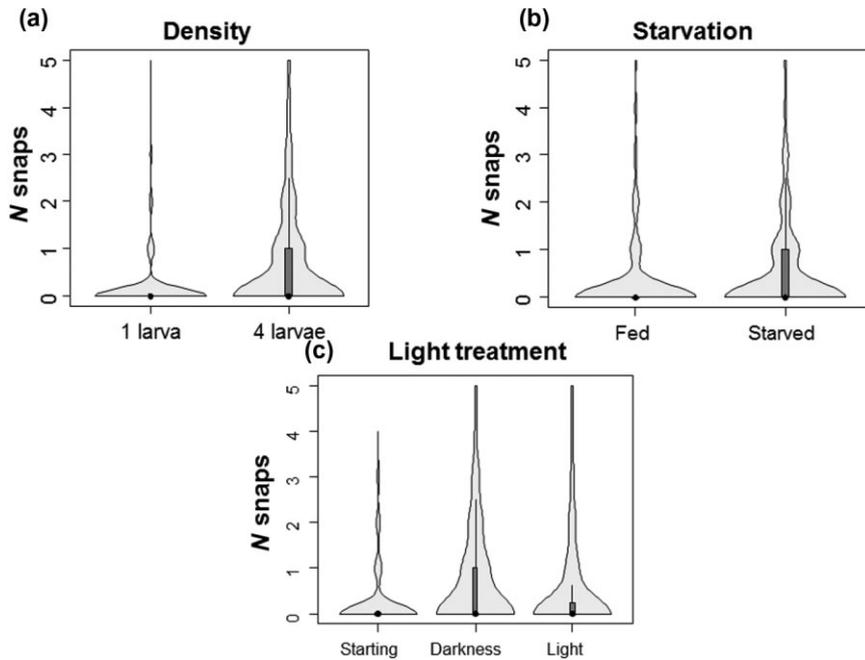


Figure 1 Violin plot showing the relationships between aggressiveness (number of snaps towards conspecifics) of fire salamander *Salamandra salamandra* larvae recorded during density tests, considering different experimental conditions: (a) density; (b) starvation; (c) light treatment. Violin plots are combinations of kernel density plots and box plots; black dots represent median values.

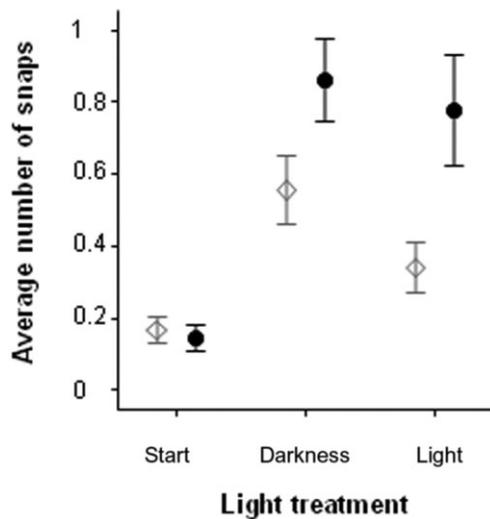


Figure 2 Interactive effect of light treatment and origin on the mean number of snaps performed by larval salamanders on conspecifics. Open diamonds: larvae from streams; closed circles: larvae from caves. Error bars represent standard errors of the mean.

important in fire salamanders for both prey capture (Himstedt, 1971) and intraspecific interactions (Reques & Tejedo, 1996). It is possible that, after 1 month of darkness, the possibility of visually detecting (in light test conditions) the movement of small conspecifics, that might constitute suitable prey, may stimulate aggressiveness. Furthermore, cave larvae increased their aggressive behaviour more than stream larvae, displaying a higher plasticity. A high behavioural plasticity of

Table 2 Results of the competition experiment

	Effect size	SE	χ^2	d.f.	P
Competition	1.777	0.140	224.4	1	<0.0001
Starvation	0.735	0.105	50.7	1	<0.0001
Body size	2.484	1.230	4.1	1	0.042
Treatment			9.1	2	0.010
Origin	0.003	0.244	0.0	1	0.999
Trial number			15.6	2	0.0004

Effect of experimental conditions on the number of snap attempts of larval salamanders on conspecifics. d.f., degrees of freedom; SE, standard error.

fire salamander larvae originated from cave populations has also been observed for foraging behaviour (Manenti *et al.*, 2013). Salamander larvae are able to perform shifts of foraging strategy, from a wide foraging (i.e. food-active searching – the opposite strategy of sit-and-wait foraging) displayed under darkness conditions to the typical sit-and-wait strategy performed in light conditions. Our study reveals the occurrence of both plasticity and local adaptation, and there is an interaction, as cave-born larvae show higher plasticity. Plastic traits may be favoured by selection in population facing cave colonization where at least some individuals rely on underground water for breeding success. Cave larvae show stronger shifts of foraging strategies, if compared with the stream larvae (Manenti *et al.*, 2013), and such behavioural plasticity may favour colonization and exploitation of novel, challenging environments such as caves (Crispo, 2008; Gray *et al.*, 2012). Our study confirms the particularly high plasticity of cave populations of *S. salamandra*. When colonizing new environments, the most plastic individuals can better survive under

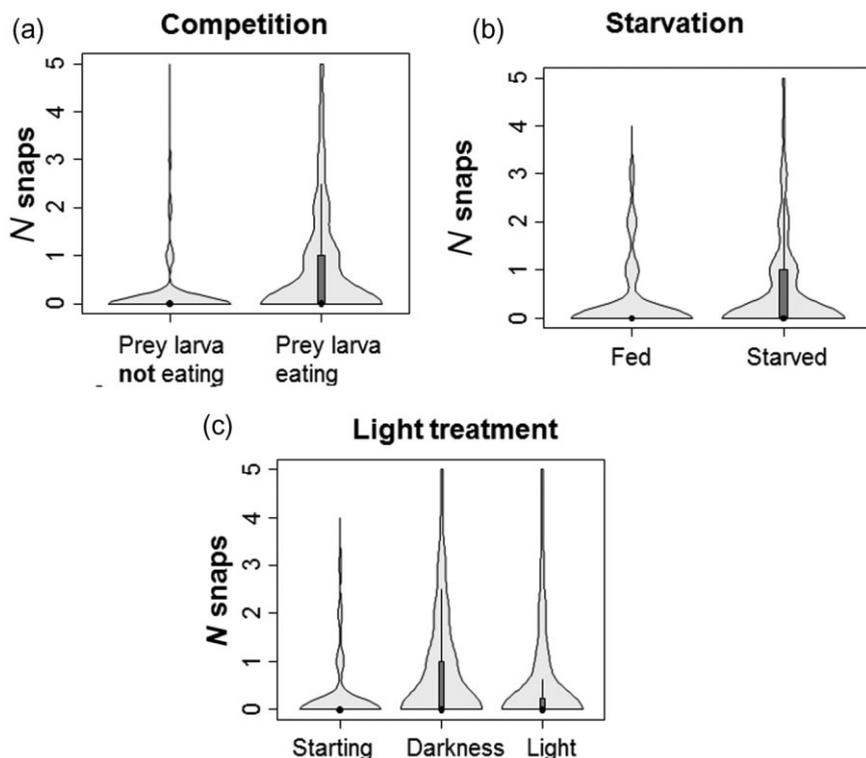


Figure 3 Violin plots showing the relationships between aggressiveness (number of snaps towards conspecifics) of fire salamander *Salamandra salamandra* larvae recorded during competition tests, considering different experimental conditions: (a) density; (b) starvation; (c) light treatment. Violin plots are combinations of kernel density plots and box plots; black dots represent median values.

selective pressures, and plasticity is expected to be selected for in populations experiencing the first stages of adaptation. This scenario may determine higher plasticity in the populations adapting to novel environments, initiating or increasing differentiation among ecotypes (Crispo, 2008; Torres-Dowdall *et al.*, 2012).

Resource competition is a major cause of aggressive behaviours; the typologies of aggression for resources can vary noticeably from species to species, ranging from noncontact interactions to deadly attacks. In competitive interactions, the aggression effort increases with the value of the resource (Arnott & Elwood, 2008). Intraspecific aggressions were stimulated by another larva seizing a prey, and this behaviour was particularly frequent in starved larvae (Fig. 3). Actually, in this experiment the occurrence of competition for food was the factor more important in determining aggressiveness, as it explained much more variation than starvation, body size or treatment (Table 2). Such a strong response to the behaviour of conspecifics catching a prey is a new aspect for studies on amphibian ethology, and can be very important in populations with high larvae density. First, if a larva is attracted towards another that has caught a prey, it increases its possibility to find another prey (e.g. if prey have a clustered distribution) or to catch it if it escapes to the first larva. Second, the attacks may induce the first larva to release the prey. The significant positive relationship between aggressiveness and larvae size might occur because large larvae can have a higher probability of successfully harming the conspecific, causing the prey release, and perhaps a lower probability of being

aggressed (Reques & Tejedo, 1996; Arnott & Elwood, 2009). In general, the higher the value an individual gives to a potential resource, the higher the stimulus to compete for it (Arnott & Elwood, 2008), and the rare prey occurring in food-deprived environments may acquire particularly high value, determining frequent contests. Our study confirms that much research is still needed to fully explore the high complexity of amphibian behaviours.

Environmental variation may strongly influence aggression in salamander larvae. For instance, in the Israeli salamander *Salamandra infraimmaculata*, cannibalism allows larvae to successfully develop in temporary pools, as in these environments cannibals may successfully complete development before wetland drying (Segev & Blaustein, 2007). Similarly, in salamanders of the genera *Hynobius* and *Ambystoma*, individuals showing cannibalistic behaviour have significantly more probability to survive and reach metamorphosis in bodies of water with poor trophic conditions or high conspecific densities (Wildy *et al.*, 2001). Despite a few studies performing behavioural comparisons of epigeal and hypogean populations of the same species of salamander (Uiblein *et al.*, 1992), to our knowledge there are no analyses on their aggressiveness and cannibalism; therefore, comparisons are difficult. Aggressive attacks were also frequent in stream larvae, and this can be explained considering that conditions of scarce food availability can also be experienced in epigeal habitats, especially if larvae density is high and predators are rare (or absent; Kishida *et al.*, 2009). Nevertheless, aggressiveness clearly increases under conditions mimicking those occurring in

caves, and this may enhance the possibility that at least a few larvae successfully complete the development in such a food-deprived environment. The analysis of aggression may allow better understanding on how these animals can successfully breed in aquatic environments with extreme conditions, where no other vertebrates survive. Under these conditions, aggressions may increase the probability to capture the rare available prey, but may also increase the frequency of cannibalistic behaviour (Segev & Blaustein, 2007). Nevertheless, detailed analysis of actual diet, for instance using stable isotopes, or traditional stomach-flushing (Denoël *et al.*, 2006), is required to assess the actual relevance of cannibalism in our study system.

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