Hatching responses of four newt species to predatory fish chemical cues

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For organisms with complex life cycles, hatching represents a crucial life history switch point that is often associated with high mortality rates due to predation. Not surprisingly, embryos and hatchlings of many species develop predator-induced behavioural and/or morphological responses to reduce the likelihood of mortality. Using laboratory experiments, we examined the effects of constant exposure to predator chemical cues (brown trout, *Salmo trutta*) on the hatching characteristics and hatchling morphology of four newt species (genus *Triturus*). Exposure to predator cues did not affect the timing of hatching, but led to an earlier development stage at hatching and smaller hatchling size. Furthermore, hatchlings exposed to predator chemical cues during embryonic development had shorter and shallower heads, shorter tails, and shallower tail muscles and tail fins relative to their body size than control animals. According to information available from previous studies, these morphological responses are unlikely to enhance the probability of larval survival under elevated predation risk. Hence, these morphological responses might represent non-adaptive developmental costs associated with the presence of predators.

Introduction

Ontogenetic niche shifts during animal histories are critical switch points influencing individual performance, and modifications of the timing or conditions during these changes can have long-lasting effects on an individuals' subsequent development (Werner 1986, Rowe & Ludwig 1991). Theory predicts that pre-reproductive organisms should switch stages in a way that maximizes the ratio between growth and mortality (Werner 1986, Ludwig & Rowe 1990). During these life history transitions, individuals are faced with new predatory and competitive challenges (Werner & Anholt 1993, Skelly 1994). Hatching and metamorphosis are the main life history switch points in organisms with complex life cycles, and morphological and behavioural changes in these life history switch points have been interpreted as risk avoidance mechanisms (Alford 1999). Hatching involves a change from an intracapsular to a free-living stage which is subject to new environmental conditions. As such, this is often a crucial period influencing subsequent survival and development of oviparous species (Warkentin 1995, 1999). Plasticity in hatching responses allows organisms to balance the conflicting demands of pre- and post-

hatching environments. For instance, by altering their hatching characteristics, aquatic organisms could respond to mortality factors such as fungal infections, hypoxia or egg predation, (Petranka *et al.* 1982, Alford 1999, Warkentin *et al.* 2001, Wedekind 2002).

For high-fecundity organisms lacking parental care, predation is typically much more intense on early rather than later developmental stages (Helfman 1986, Richards & Bull 1990), and several studies have found evidence for predator induced modifications in prey behaviour, morphology and development (Sih 1987, Kats & Dill 1998, Lima 1998). In fact, predator-induced plasticity in hatching responses (i.e., timing of hatching, hatchling size or morphology) seems to be widespread in aquatic ectotherms (Sih & Moore 1993, Warkentin 1995, Moore *et al.* 1996, Warkentin 2000, Chivers *et al.* 2001, Mirza *et al.* 2001, Laurila *et al.* 2002, Jones *et al.* 2003). These responses have usually been interpreted as being adaptive, although the functional significance of the induced responses remains unclear in most cases (West-Eberhard 2003).

Amphibians are good models to study predation-induced responses because they exhibit a great variety of life histories that include differences in habitat use (temporal or permanent), feeding regime (carnivorous or herbivorous larvae) and reproductive mode (oviparity or ovoviviparity; Duellman & Trueb 1986). A large number of studies have demonstrated that amphibian larvae are sensitive to direct predator cues (e.g., predator presence), as well as indirect predator cues (e.g., chemical cues; Sih 1987, Lima 1998, Alford 1999). However, most studies have focused on larval development and metamorphosis (Werner 1986, McDiarmid & Altig 1999), whereas effects at time of hatching have been less well studied. Several studies have reported that amphibian hatching is strongly influenced by predators: egg-predators can induce earlier hatching in embryos (Warkentin 1995, 2000, Chivers *et al.* 2001), and larvae-predators influence a delay in egg hatching (Sih & Moore 1993, Moore *et al.* 1996). However, the results of these studies are not always congruent and more information is needed to better understand predator effects on amphibian hatching.

In this study, we exposed embryos of four newt species (genus *Triturus*) to brown trout (*Salmo trutta*) chemical cues to investigate the effect of predator signals on developmental rate and hatching characteristics of newts. Previous studies have reported that newt larvae can modify their behaviour, morphology and development when confronted with non-lethal exposure to dragonflies (Van Buskirk & Schmidt 2000, Schmidt & Van Buskirk 2001) and predatory fish (Orizaola & Braña 2003a). Our experiments were based on non-feeding chemical cues, which have been suggested to be an important source of information in aquatic environments (Kats & Dill 1998). Brown trout, the fish used in the experiments, has been described as an important predator of amphibian larvae (Bradford 1989, Braña *et al.* 1996, Tyler *et al.* 1998, Pilliod & Peterson 2001), but apparently, the trout does not consume amphibian eggs. We tested predator chemical cue effects on hatching success, timing of hatching, developmental rate, and hatchling size and morphology.

Materials and methods

We collected females of four newt species (*Triturus helveticus*, $n = 12$, *T. alpestris*, $n = 13$, *T. boscai*, $n = 44$ and *T. marmoratus*, $n = 50$) from small ponds, cattle-watering tanks and lakes of Asturias, northern Spain, during the reproductive seasons in 1999–2001. Due to space limitation in the laboratory, experiments were conducted in different years (1999 for *T. helveticus*, 2000 for *T. alpestris*, and 2001 for *T. marmoratus* and *T. boscai*). Newts were kept under 12:12 h photoperiod in a constant temperature (17 °C) laboratory room. The females were allowed to lay eggs in cylindrical plastic containers (18 cm in diameter) supplied with 0.5 litres of dechlorinated tap water and leaves of *Glyceria* sp. collected from nearby newt-occupied ponds. We examined the containers every morning for eggs. When eggs were present, we carefully cut off the leaves with wrapped eggs and placed them in 0.3-l plastic bottles, which were then maintained at the same temperature and light conditions. We placed no more than ten eggs in every bottle, using dif-
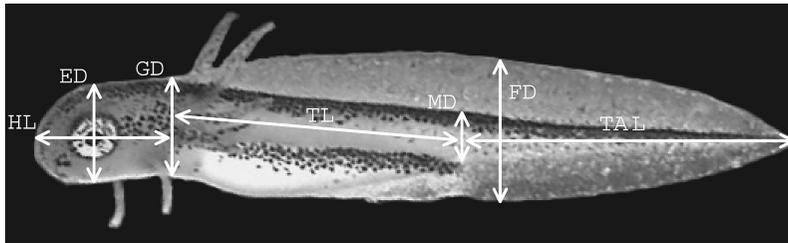
Fig. 1. Lateral view of a marbled newt (*Triturus marmoratus*) hatchling showing the linear measures used in morphological analyses. ED: head depth at eye level; GD: head depth at gill level; HL: head length; TL: trunk length; TAL: tail length; MD: tail muscle depth; FD: maximum tail fin depth.

ferent bottles for 'female' and for 'collection day'. Half of the eggs laid by each female were placed in bottles containing water conditioned with predator cues and the other half in non-conditioned water. We changed predator-conditioned water daily (about 0.25 l per bottle) to maintain a high level of predator cues. Non-conditioned water was changed at the same time to equalise the effect of water management in both treatments. We checked the bottles twice a day to determine the timing of hatching. Hatchlings were removed and immediately examined under a dissecting microscope to determine their developmental stage, which was classified based on a modification of the key proposed by Watson and Russell (2000; *see* Appendix). At the same time, we took a lateral image of every larva with a CCTV Panasonic camera connected to a dissecting microscope. Seven morphological measurements defining the size and shape of newt hatchlings were taken with the image analysis software. The examined traits were: head depth at eye level, head depth at gill level, head length, trunk length, tail length, maximum tail muscle depth, and maximum tail fin depth (Fig. 1). We measured yolk surface and total larval surface of 50 randomly selected larvae of each species to estimate yolk consumption at hatching.

To obtain predator cues, we maintained pairs of large brown trout (17.0 to 30.5 cm fork length, mean ± SE = 24.1 ± 1.1 cm) in a 90-l tank with dechlorinated and aerated tap water. Brown trout is the most common fish species in rivers and streams in the study area (Reyes-Gavilán *et al.* 1995), and is known to be an active predator of newts (Orizaola & Braña 2003a). Trout were food-deprived to prevent fouling of water. A similar 90-l tank filled with dechlorinated and aerated tap water was used to provide unconditioned water. Trout were replaced several times during the experiments, and trouts and newts (both females and larvae) were released at their place of origin after the experiments.

To analyse treatment effects on hatching success we regressed the number of eggs laid against the number of eggs hatched and used the residuals in a two-way ANOVA test, with treatment and species identity as factors. We computed hatching success in both treatments as the mean percentage of hatched eggs for each female. We tested the effects of fish cues on developmental stage at hatching with the Mann-Whitney *U*-test separately for each species. We analysed differences in stages at hatching between species with the Kruskal-Wallis test and *a posteriori* Mann-Whitney test. Two-way ANOVA (water type and species identity as factors) and the post-hoc Scheffé test were used to test for differences in length and yolk size at hatching, as well as time until hatching. Yolk size was estimated as the surface of yolk relative to total hatchling surface (yolk excluded). Spearman rank correlation was used to investigate the relationship between yolk size and developmental stage. To test the effect of predator cues on hatchling morphology, we analysed the residuals from the least squares regressions of the six morphological measures of head and tail on body length (*see* Fig. 1). We used a multivariate analysis of variance (MANOVA) on residuals of head and tail measures for each species to test the null hypothesis that predator cues do not affect hatchling morphology. Univariate ANOVA was subsequently performed on each particular morphological trait. Deviations from normality
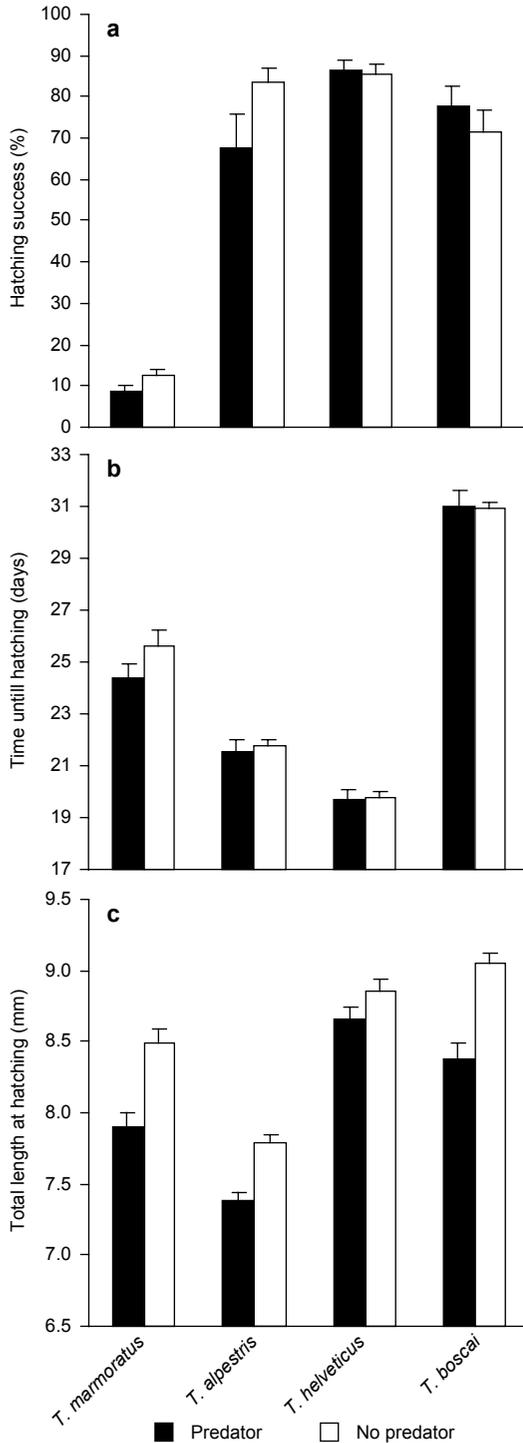
Fig. 2. Effect of trout cues on hatching characteristics in four newt species. — **a**: Hatching success; — **b**: Time between egg laid and larvae hatching; — **c**: Total larvae length at hatching. All results are expressed as mean + SE.

was tested with the Shapiro-Wilk test and homogeneity of variance with the Bartlett-Box test.

Results

Hatching success was not influenced by the presence of predator chemical cues (ANOVA: $F_{1,210}$ = 0.034, P = 0.852), but differed among the species ($F_{3,210}$ = 169.35, P < 0.001). *T. marmoratus* experienced higher embryonic mortality than the other species (post-hoc Scheffé test: P < 0.001), and most embryos died after about one week of development. Likewise, time until hatching was significantly different among species ($F_{3,455}$ = 273.44, P < 0.001; post-hoc Scheffé test: *T. boscai* > *T. marmoratus* > *T. alpestris* > *T. helveticus*, in both treatments; Fig. 2b), but no effect of predator chemical cues was detected ($F_{1,455}$ = 1.217, P = 0.271). However, hatchlings of all four newt species hatched at an earlier developmental stage when reared with trout cues as compared with controls (Mann-Whitney U-test: P < 0.01 in all cases; Fig. 3). *T. helveticus* and *T. boscai* hatched at a more developed stage than *T. marmoratus* and *T. alpestris* (Kruskal-Wallis test: H_3 = 400.719, P < 0.01; Mann-Whitney U-test: P < 0.001), and also exhibited a broader range of stages at hatching (Fig. 3). Larvae from predator treatments had more yolk left at hatching than those from control treatments (ANOVA: $F_{1,392}$ = 65.846, P < 0.001). Species identity ($F_{3,392}$ = 46.509, P < 0.001) also affected the proportion of yolk left at hatching, *T. helveticus* and *T. boscai* having more than the other two species (Scheffé test: P < 0.001). The proportion of yolk was a negative function of developmental stage in all four species (Spearman rank correlation: r_s > 0.63, P < 0.001).

Eggs subjected to fish chemical cues produced smaller hatchlings in terms of total length than those from the controls ($F_{1,455}$ = 52.161, P < 0.001; Fig. 2c). Despite having smaller eggs (Orizaola & Braña 2003b) hatchlings of *T. helveticus* and *T. boscai* were larger at hatching than those of the other species ($F_{3,455}$ = 66.262, P < 0.001; Scheffé test: *T. helveticus* = *T. boscai* > *T. marmoratus* > *T. alpestris* in both treatments), which is probably associated with their more advanced developmental stage at hatching (*see* Fig. 3). A

significant species × treatment interaction ($F_{3,455}$ = 2.769, $P = 0.041$) indicated that the differences between treatments were especially pronounced for *T. marmoratus* and *T. boscai*. Body length did not differ between treatments for any of the species (ANOVA: $F_{3,455} = 0.044$, $P = 0.834$), but there were generalised morphological differences between the treatments (MANOVA, Table 1). Univariate ANOVA indicated that hatchlings from the control treatment had, relative to body length, larger and deeper heads, longer tails, and deeper tail muscles and tail fins than those developed in the presence of trout cues (Fig. 4). Most of these differences were significant even after applying the sequential Bonferroni adjustments (Table 1).

Discussion

The most salient finding of this study was that newt embryonic development was influenced by the presence of trout chemical cues. Larvae from the predator treatments hatched at an earlier developmental stage, but no effect of predator cues was found on the timing of hatching in any of the species used in the experiments. This contradicts the prediction of delayed hatching for amphibian embryos exposed to larval predators (Sih & Moore 1993, Moore *et al.* 1996). A review of the literature on the effects of predators on amphibian hatching reveals that, whereas the predictions of earlier hatching and less developed
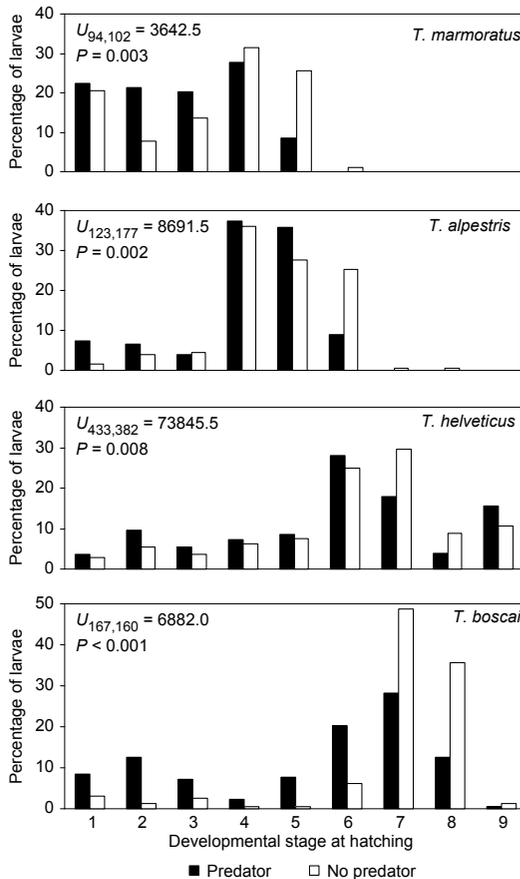
Fig. 3. Developmental stages at hatching for larvae of four newt species reared in presence and absence of trout cues. Values represent percentage of larvae in each stage. *U*, df and *P* values for Mann-Whitney *U*-test are indicated for each species. Developmental stages are described in Appendix 1.

Table 1. Multivariate analysis of variance on the effect of predator environment on six size-free morphological traits in hatchlings of four newt species (MANOVA, Willks's λ) and univariate analyses of variance (ANOVAs, *F* values) of each particular morphological measure. All the analyses were conducted independently for each species. n.s. = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. † Significant after sequential Bonferroni adjustment.

		T. marmoratus		T. alpestris		T. helveticus		T. boscai	
		df	λ	df	λ	df	λ	df	λ
MANOVA									
	Treatment	6,106	0.79***†	6,89	0.71***†	6,114	0.80***†	6,126	0.74***†
ANOVAs									
	Head depth (gill level)	1,111	21.66***†	1,94	14.38***†	1,119	4.29*	1,131	13.24***†
	Head depth (eye level)	1,111	26.82***†	1,94	20.18***†	1,119	9.61**†	1,131	28.82***†
	Head length	1,111	19.14***†	1,94	16.60***†	1,119	8.62**†	1,131	35.51***†
	Tail length	1,111	21.46***†	1,94	16.96***†	1,119	2.97n.s.	1,131	28.43***†
	Muscle depth	1,111	21.85***†	1,94	20.63***†	1,119	10.83**†	1,131	24.75***†
	Maximum tail fin depth	1,111	20.73***†	1,94	9.78**†	1,119	4.17*	1,131	28.71***†
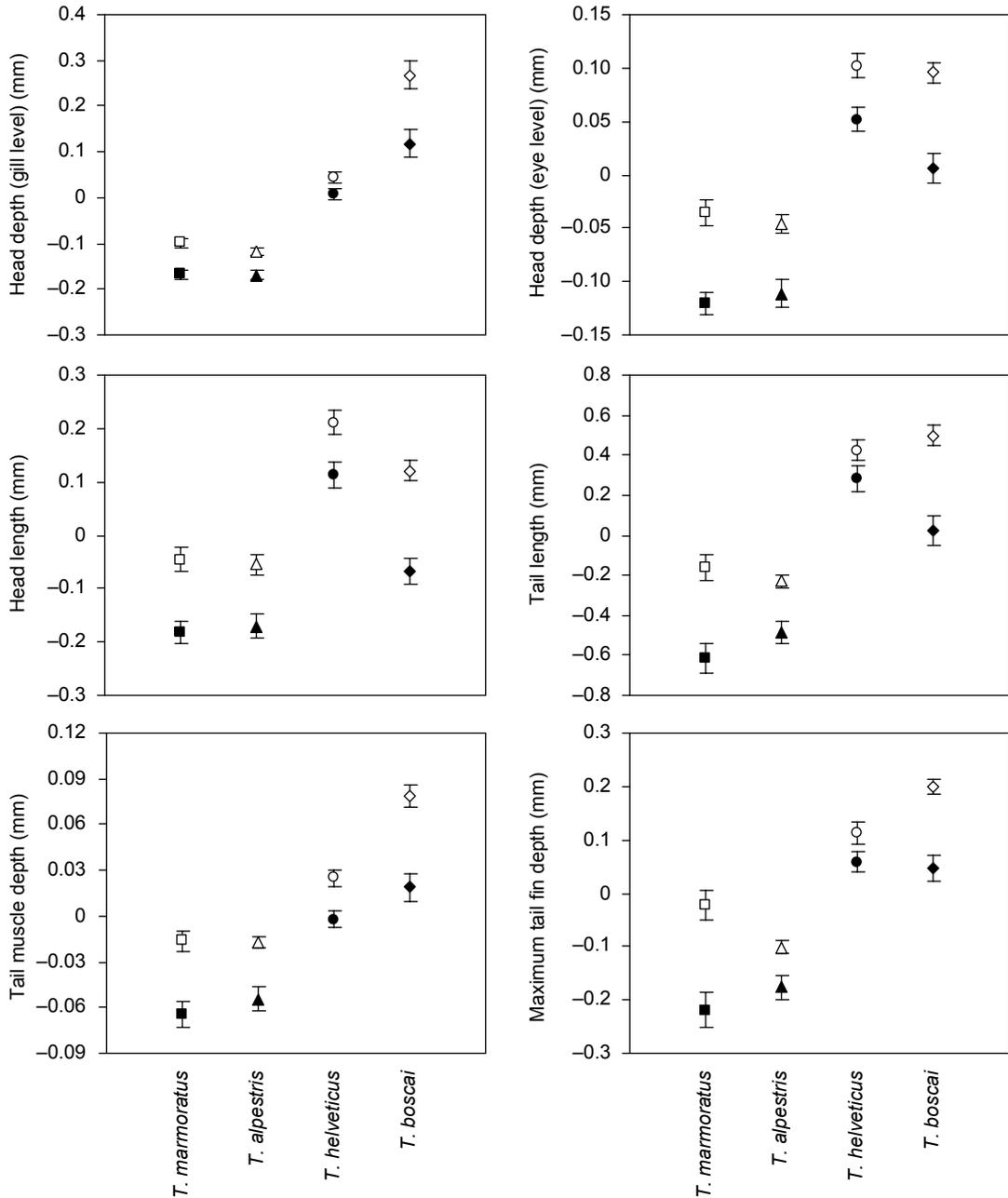
Fig. 4. Relative morphological measurements (mean ± SE; residuals from the least squares regressions on trunk length) for hatchlings of four newt species reared in the presence (solid symbols) and absence (open symbols) of predatory trout cues.

hatchlings in response to egg predators are well supported, those of later hatching and advanced developmental stages in hatchlings exposed to larvae predators were frequently unfulfilled (Table 2). For example, several amphibian species do not show differences either in hatching time or developmental rate when reared with or without larval predator cues (Laurila *et al.* 2001, Laurila *et al.* 2002, Van Buskirk 2002a, Anderson & Petranka 2003, Johnson *et al.* 2003). Indeed, contrary to predictions above, several species hatch earlier, in smaller sizes, and at

Table 2. Summary of the studies reporting predator effects on amphibian hatching time and hatchling size.

Type of predator	Species	Predator type	Hatching time and developmental stage	Hatchling size	Source
a) Egg predator	*Agalychnis callidryas*	Snake	Earlier, less developed	Smaller	Warkentin 1995
	Agalychnis callidryas	Wasp	Earlier, less developed	Smaller	Warkentin 2000
	Hyla regilla	Leech	Earlier, less developed	Smaller/No effect	Chivers et al. 2001
	Rana cascadae	Leech	Earlier, less developed	Smaller	Chivers et al. 2001
	Rana arvalis	Leech	Earlier, less developed	No effect in overall size	Laurila et al. 2002
	Rana temporaria	Leech	No effect	No effect	Laurila et al. 2002
b) Egg-larvae predator	*Rana sphenocephala*	Crayfish	Earlier	Smaller	Johnson et al. 2003
	Rana sphenocephala	Crayfish	Earlier		Saenz et al. 2003
	Physalaemus sp.	Dipteran	Earlier, less developed		Menin & Giaretta 2003
	Rana sphenocephala	Dytiscid	Earlier/No effect	Smaller/No effect	Johnson et al. 2003
	Rana clamitans	Leech	Later, same dev. stage	Larger	Schalk et al. 2002
	Rana temporaria	Dytiscid	No effect	No effect in overall size	Laurila et al. 2001
	Triturus alpestris	Dytiscid	No effect		Schmidt B.R. (unpub. data)
c) Larvae predator	*Ambystoma barbouri*	Flatworm	Later, advanced dev. stage	Larger	Sih & Moore 1993
	Ambystoma barbouri	Fish	Later, advanced dev. stage	Larger	Moore et al. 1996
	Rana temporaria	Fish	Later, advanced dev. stage	No effect	Laurila et al. 2002
	Rana arvalis	Dragonfly	Earlier, less developed	Smaller	Laurila et al. 2002
	Rana arvalis	Fish	Earlier, less developed	Smaller	Laurila et al. 2002
	Rana temporaria	Dragonfly	No effect	No effect	Laurila et al. 2002
	Rana temporaria	Dragonfly	No effect	No effect	Van Buskirk 2002
	Rana sphenocephala	Dragonfly	No effect	No effect	Johnson et al. 2003
	Rana sylvatica	Dragonfly	No effect	No effect	Anderson & Petranka 2003
	Ambystoma maculatum	Dragonfly	No effect	No effect	Anderson & Petranka 2003
	Triturus alpestris	*T. alpestris*	No effect		Schmidt B.R. (unpub. data.)
	Triturus marmoratus	Fish	Same time, less developed	Smaller	Orizaola & Braña (this study)
	Triturus alpestris	Fish	Same time, less developed	Smaller	Orizaola & Braña (this study)
	Triturus helveticus	Fish	Same time, less developed	Smaller	Orizaola & Braña (this study)
	Triturus boscai	Fish	Same time, less developed	Smaller	Orizaola & Braña (this study)

earlier developmental stage when presented with predator stimuli (Laurila *et al.* 2002).

These findings, together with those obtained in the present study, show that embryos of even closely related amphibian species display great variation in hatching characteristics when exposed to larvae predators. This variability could indicate that other factors associated with experimental designs, such as the predator species involved, the timing of exposure and/or the intensity of predator chemical, tactical or visual cues could strongly influence amphibian responses to predator presence. The fact that the newt species used in this study have longer embryonic periods than the other amphibians in Table 2 could be one explanation for their deviating responses. Newts hatch on average after more than 20 days at 17 °C (Fig. 1b), whereas in the other amphibian species incubation lasted between several hours (*H. regilla* 10–150 h, *R. cascadae* 60–75 h, Chivers *et al.* 2001; *R. sylvatica* 38–43 h., Anderson & Petranka 2003) and a few days (*Rana sphenocephala* 2–4 days, Johnson *et al.* 2003; *A. callidryas* 4–10 days, Warkentin 1995; *R. temporaria* 6–8 days, *R. arvalis* 7–10 days, Laurila *et al.* 2002; *A. maculatum* 12–13 days, Anderson & Petranka 2003). Hence, if the effects of predator exposure were cumulative and time-dependent, they could be manifested differentially in species with comparatively slow development. Furthermore, since other environmental factors (e.g., temperature, desiccation, UV-B exposure) can also affect amphibian development, organisms could develop developmental strategies that could interact with predation in determining hatching responses.

While effects on embryonic development were clear, hatching success was relatively high in all the species — except in *T. marmoratus* — and was not affected by predator cues. Low hatching success exhibited by *T. marmoratus* could be partially related to the arrested development syndrome that kills on average half of the eggs in the species of the *T. cristatus* complex, including *T. marmoratus* (Macgregor & Horner 1980). This explanation is supported by the fact that a high fraction of mortality accumulated during the first few days of incubation.

Morphological responses to predator cues can influence predator escape success and

many studies have focused on predator-induced morphology in anuran tadpoles from this perspective (e.g. Lardner 1998, Relyea & Werner 2000, Relyea 2001a, 2001b, Van Buskirk 2002a, 2002b, Fitzpatrick *et al.* 2003, Relyea & Hoverman 2003, Teplitsky *et al.* 2003). In our study, no predator effect was detected on hatchling body length, but the larvae that hatched in the predator environment had shorter and shallower heads, shorter tails, and shallower tail muscles and tail fins than those that developed in the absence of predator cues. Although only a few studies have tested the effects of predator-induced morphological modifications on prey performance, they indicate that amphibian larvae with long and deep tails relative to body size have higher survival probability than those with opposite characteristics (McCollum & Van Buskirk 1996, Van Buskirk *et al.* 1997, Van Buskirk & Schmidt 2000, Van Buskirk *et al.* 2003). On the basis of the results of these previous studies, the small size and morphological characteristics exhibited newt hatchlings reared with predator cues in our study are unlikely to increase their survival under predation risk. The smaller relative size of all the considered traits of the larvae hatched in the predator treatment could be a simple consequence of delayed development of embryos exposed to predator chemical cues. This interpretation is supported by the fact that earlier developmental stages exhibit less developed distal parts (Harrison 1969, Epperlein & Junginger 1982). As such, the higher proportion of yolk surface detected in the predator treatment hatchlings could be also a reflection of the delayed development.

Many studies indicate that newt embryos are well protected during their development because eggs are wrapped in plant leaves, which reduces the risk of predation (Miaud 1993, 1994, Orizaola & Braña 2003b). The same cannot be said about larvae, as newt larvae are frequently preyed upon by aquatic predators, such as dragonflies (Van Buskirk & Schmidt 2000) and fish (Orizaola & Braña 2003a). Accordingly, hatching at an early developmental stage and without any special morphological antipredator defenses could be considered non-adaptive, possibly reflecting predator-induced stress. Several previous studies have reported that chronic exposure to preda-

tor cues could alter growth and development of prey through changes in their metabolic rate and stress hormone levels (Chabot *et al*. 1996, Boonstra *et al*. 1998, Hik *et al*. 2001). Long-term consequences of developmental plasticity at hatching have been rarely studied, but hatchling size and developmental stage at hatching are known to be correlated with growth rate, size at metamorphosis and length of the larval period of several anuran species (e.g., Kaplan 1992, Semlitsch & Schmiedehausen 1994). Hence, future studies investigating the responses of amphibian embryos to a wider range of predators, as well as studies in the adaptive significance of the responses, should be of interest.

Acknowledgements

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Appendix. Correspondence between the developmental stages at hatching used in the present study and those described by Watson and Russell (2000) for *Ambystoma macrodactylum krausei*.

Stage	Watson & Russell (2000)
1	1 and 2
2	3 and 4 if eye are emerged in less than 50%
3	3 and 4 if eye are emerged in more than 50% but less than 100%
4	3 and 4 if eye are completely emerged with or without iridophores
5	5 and 6
6	7
7	8 if forelimb had three fingers
8	8 and 9 if forelimb had three fingers and the fourth emerging as a swelling
9	9 if forelimb had four fingers