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Flatworms (*Schmidtea nova*) prey upon embryos of the common frog (*Rana temporaria*) and induce minor developmental acceleration

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Abstract. Amphibians vary in the degree of pre-metamorphic developmental plasticity in response to risk of predation. Changes in hatching time and development rate can increase egg or tadpole survival respectively by shortening the duration of the more vulnerable stages. The intensity of predator induced developmental response and its direction, i.e. delayed, accelerated, or none, varies considerably between amphibian and predator species. We surveyed freshly deposited clutches of the European common frog *Rana temporaria* in a population in Braunschweig, Germany and found that 62% (N = 20) of the clutches contained planarians (*Schmidtea nova*), with an average of 3.94 ± 0.79 and a maximum of 13 planarians per clutch. A laboratory predation experiment confirmed that this planaria preys on *R. temporaria* eggs and early embryos. We further exposed freshly laid egg masses to either free, caged, or no planarians treatments using floating containers within a breeding pond where the two species co-occur. After 10 days exposure, embryos showed developmental stages 14-25 along the Gosner scale with statistically significant positive effects of both predator treatments. The observed effect was rather slight as predator-exposed individuals showed an increase by a single Gosner stage relative to those raised without planarians. The detected trend suggests that direct and indirect cues from flatworms, rarely considered as anuran predators, might induce a developmental response in *R. temporaria* early developmental stages.

Keywords: Anura, aquatic eggs, induced developmental plasticity, planaria, predation risk, prey response, waterborne chemical cues.

Introduction

Numerous animals show adaptive plasticity in their developmental response to risk of predation. This adaptive plasticity often results in changes in the duration of vulnerable developmental stages and a simultaneous-life history change to reduce per capita probability of predator attack. In amphibians, the direction of a developmental response, i.e. delayed, accelerated, or none, varies among: (i) amphibian species (Anderson and Petranka, 2003; Johnson et al., 2003; Anderson and Brown, 2009), (ii) predator species (Schalk et al., 2002; Ireland et al., 2007), and (iii) the specific life-stage exposed to predation (Schalk et al. 2002). For most anuran larvae with aquatic eggs, developmental stages 20-25 typically mark the transition from stationary and often immobile embryos within clutches or nests to mobile, typically free-swimming tadpoles (Gosner, 1960). A number of egg predators have been shown to induce early hatching (Chivers et al., 2001; Saenz et al., 2003) while several hatchling predators have been shown to induce delayed hatching (Sih and Moore, 1993; Schalk et al., 2002). The simultaneous exposure of eggs to egg and hatchling predators induced no change in the timing of hatching (Ireland et al., 2007).

Predator-cued hatching plasticity has been observed thus far in aquatic eggs from four amphibian families: Ranidae, Bufonidae, Hylidae, and Ambystomatidae (Warkentin, 2011). Six species of Ranidae have been tested for predator-induced plasticity in time to hatching and three out of these six have been tested for predator-induced plasticity in hatchling size, stage, and morphology (table 1). Regardless of extensive investigation of predator-cued hatching plasticity and developmental plasticity in the common frog *Rana temporaria*, evidence concerning this species' ability to respond to different predators, and the direction of its developmental response, are inconclusive (table 1). In

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Table 1. Studies of predator-induced effects on hatching and hatchlings stage, size, and morphology in frogs of the family Ranidae with aquatic eggs. None = no effect observed; NA = effect was not tested.

Prey species	Predator species or signal		Effect on hatching timing	Effect on hatchlings or tadpoles	Reference
Rana temporaria	diving beetle (*Dytiscus marginalis*) larvae		none	short body and deeper tail	Laurila et al., 2001
	dragonfly larvae (*Aeshna* spp.)		none	short tail tadpoles	Laurila et al., 2002
	three-spine stickleback (*Gasterosteus aculeatus*)		delay		
	leech (*Haemopis sanguisuga*)		none		
	salamander (*Salamandra salamandra*) larvae	fed with conspecific	none	decrease in weight and shorter body	Saglio and Mandrillon, 2006
		unfed	none	none	
	crayfish (*Astacus leptodactylus*)	fed with conspecific	none	none	
		unfed	none	decrease in weight	
	crushed conspecific tadpole		none	none	
	crushed conspecific eggs		none	increase in weight	Mandrillon and Saglio, 2007
	crushed conspecific tadpole		delay		
	leech (*Haemopis sanguisuga*)	fed with conspecific	accelerate	NA	Capellan and Nicieza, 2010
		unfed with conspecific	none	NA	
	dragonfly larvae (*Aeshna cyanea*)	fed with conspecific	accelerate	NA	
		unfed with conspecific	none	NA	
Lithobates clamitans	leech (*Macrobdella decora*)		delay	large size	Schalk et al., 2002
	leech (*Nephelopsis obscura*)		accelerate	early stage and small size	Ireland et al., 2007
	dragonfly larvae (*Aeschna canadensis*)		delay	late stage and large size	
	dragonfly + leech		none	none	
	crayfish (*Procambarus nigrocinctus*)		accelerate (lower prop. hatched)	NA	Anderson and Brown, 2009
	dragonfly larvae (*Anax junius*)		accelerate	NA	
	dragonfly + crayfish		accelerate (lower prop. hatched)	NA	

Table 1. (Continued.)

Prey species	Predator species or signal		Effect on hatching timing	Effect on hatchlings or tadpoles	Reference
Lithobates sphenocephalus	crayfish (*Procambarus nigrocinctus*)		accelerate	short body	Johnson et al., 2003
	diving beetle (*Cybister* sp.) larvae		none	NA	
	dragonfly larvae (*Anax junius*)				
	crayfish (*Procambarus nigrocinctus*) free		accelerate	NA	Saenz et al., 2003
	crayfish (*Procambarus nigrocinctus*) caged				
Rana arvalis	dragonfly larvae (*Aeschna* spp.)		accelerate	early stage and small size	Laurila et al., 2002
	three-spine stickleback (*Gasterosteus aculeatus*)		accelerate		
	leech (*Haemopis sanguisuga*)		accelerate		
Rana cascadae	leech (*Desserobdella picta*)		accelerate	NA	Chivers et al., 2001
	earthworm (*Lumbricus terrestris*)		none	NA	
	crushed conspecific eggs		none	NA	
Rana sylvatica	dragonfly larvae (*Anax junius*)	fed with conspecific	none	none	Anderson and Petranka, 2003
		unfed with conspecific	none	none	
	crushed conspecific tadpole		none	NA	Touchon et al., 2006
	crushed conspecific eggs		none	NA	
	mosquitofish (*Gambusia affinis*) free		accelerate	NA	Smith and Fortune, 2009
	mosquitofish (*Gambusia affinis*) caged		accelerate	none	
	dragonfly larvae (Libellulidae and Aeshnidae)		none	none	Dibble et al., 2009
	mosquitofish (*Gambusia affinis*)		none	none	
	competitor tadpole (*Lithobates clamitans* and *L. catesbeianus*)		none	none	

one series of studies, embryos of *R. temporaria* delayed hatching in response to the presence of a caged fish (stickleback), but were not affected by cues from leeches (*Haemopis sanguisuga*), dragonfly larvae (*Aeshna* spp.), or diving beetle larvae (*Dytiscus marginalis*) (Laurila et al., 2001, 2002). Additionally, *R. temporaria* delayed hatching when exposed to crushed conspecific tadpoles, but not to crushed conspecific eggs (Mandrillon and Saglio, 2007), and the response of the embryos correlated with the intensity of the predator stimulus (Saglio and Mandrillon, 2006). Conversely, Capellan and Nicieza (2010) reported accelerated hatching in response to cues from crushed conspecific eggs, leeches (*Haemopis sanguisuga*), and dragonfly larvae (*Aeshna cyanea*), but only when these predators had previously been fed with conspecific eggs or tadpoles.

Due to high variation in development rates of siblings, and given the difficulties for registering the exact time of spawning in field-collected clutches, ‘time to hatching’ may prove insufficient as a sole indicator for assessing the effect of predators, or predator cues, on amphibian embryonic development. An alternative approach adopted herein takes into account within-clutch developmental variability by scoring the developmental stage of specimens using the scale of Gosner (1960).

Until now, a variety of freshwater predators, vertebrates (fish, salamanders) and invertebrates (dragonflies, leeches, and beetles) have been tested for inducing plasticity in time to hatching and/or speed of embryonic or larval development in amphibians. During behavioral observations on the European common frog (*Rana temporaria*), we observed a high incidence of planarians (identified as *Schmidtea nova*) on and within freshly laid clutches. *Schmidtea nova* is a free-living freshwater planarian species (family Dugesiidae, order Tricladida, suborder Continenticola, phylum Platyhelminthes). Little is known about the species’ ecology. The only three other species in the genus, *S. mediterranea*, *S. lugubris*, and *S. polychroa*, are known to prey on oligochaetes and gastropods (Harrath et al., 2004). There are only a handful of reports on amphibian eggs or larvae predation by planarians (Malone, 2006). Sih and Moore (1993) have found that cues from a flatworm (*Phagocolus gracilis*) delayed hatching in eggs of streamside salamanders (*Ambystoma barbouri*), but we are not aware of any previous work examining the effect of cues released by flatworms on the development of an anuran species. In this study, we review studies of predator-induced developmental changes in Ranidae with aquatic eggs, document the predatory behavior of planarians in a laboratory experiment and conducted a field experiment to test whether *Schmidtea nova* can induce changes in embryonic development of *Rana temporaria*. We specifically tested if *R. temporaria* can: (i) accelerate embryonic development in the presence of planarians and (ii) vary in this response when exposed to free versus caged planarians.

Materials and methods

Observations and experiments were carried out in 2012 and 2013 in a pond at Kleiwiesen, Braunschweig, Germany (52°32′85″N; 10°58′22″E), and on specimens collected from this pond.

To estimate the prevalence of *R. temporaria* egg predation by *Schmidtea nova*, we collected 32 clutches in the field (10-25 March 2012) and counted the number of eggs (according to Karraker, 2007) and planarians per clutch. To test whether *Schmidtea nova* consume *R. temporaria* eggs, we placed three freshly captured planarians with 10 freshly laid *R. temporaria* eggs (in early embryonic stages) in a petri dish (diameter 90 mm) filled with pond water (ca. 10 mm depth) for 48 hours at ca. 18°C. Planarians and eggs were collected at the same site. The experiment was replicated 10 times, 7 replicates with planarians and 3 replicates with eggs only and no planarian.

We tested for predator induced developmental plasticity of embryos by exposing eggs for 10 days to free and caged planarians using floating mesh containers within their original breeding pond. By this approach we attempted to simulate the relevant ecological conditions during egg development because variation in oxygen levels and temperature are known to affect pre-hatching processes (Mills and Barnhart, 1999). We collected 10 freshly laid (<12 h) *Rana temporaria* clutches and 40 planarian specimens from other *R. temporaria* clutches at the same breeding pond. Planarians were enclosed inside the floating containers on the same day they were captured. We manually divided each clutch evenly into three treatments: no (control, NP), free (FP), and caged planarians (CP), but avoided counting the exact number of eggs to prevent damaging the eggs due to excessive manipulation. Each third of an egg mass was placed inside a floating containers made of a polystyrene ring (22 cm diameter) wrapped in a sleeve made of a nylon stocking (Vojar et al., 2012). Two planarians were allocated to each of the free and caged treatments based on the average density found in egg clutches in the field (see results). Caged planarians were enclosed in a 5 × 5 cm pouch made of nylon stocking (the same material used to build the enclosures) and placed inside the floating container. Containers were randomly distributed in a shallow area at the edge of the breeding pond at a depth of 20-30 cm, with ∼40 cm minimum distance between containers, and a distance of at least 50 m to the nearest natural clutch aggregation. Eggs were allowed to develop undisturbed for 10 days (12-22 April 2013) and then were removed to be analyzed in the laboratory. The developmental stage of each embryo was scored according to Gosner's (1960) staging system, using a Binocular (Zeiss Stemi SR). After counting, individuals were released back into their original breeding pond. In cases when adjacent Gosner stages were difficult to discern the higher of the two possible stages was scored. We did not measure time to hatching because in *R. temporaria*, the jelly envelope surrounding the eggs can starts liquefying rather soon, making it sometimes difficult to distinguish between hatched and unhatched specimens.

We used R software (R Core Team, 2014) and lme4 package (Bates et al., 2014) to perform a mixed effects model analysis of the effect of planaria presence on embryo development, the response variable. We considered treatment as a fixed effect with three levels (NP, CP, and FP) and to account for inter-clutch variation we included "clutch" as a random effect in the model. Owing to the discrete nature of the response variable (Gosner stages), the data were fitted with a generalized linear mixed effect model with Poisson distribution. No signs of overdispersion were observed after comparing the model residual deviance and residual degrees of freedom. We hypothesized that the presence of planarians (FP & CP treatments) should alter embryonic development, which should be detected as a shift in the distribution of developmental stages with respect to the NP (control) treatment resulting in statistically significant effects of the planarian treatments. We here define embryonic development as also encompassing early free-swimming developmental stages which were attained by some of the embryos over the time of the experiment, and we stress that our setup does not allow to assess at which developmental stage(s) possible shifts in developmental speed might occur. We assessed the statistical significance of FP and CP treatment effects compared to the baseline treatment (NP) using the default Wald tests in lme4 package. The sign of the estimates should indicate whether the effect is a delay (−) or an acceleration (+) in development and a predator-induced cue effect can be hypothesized if not only the FP but also the CP treatment shows a significant effect on egg development.

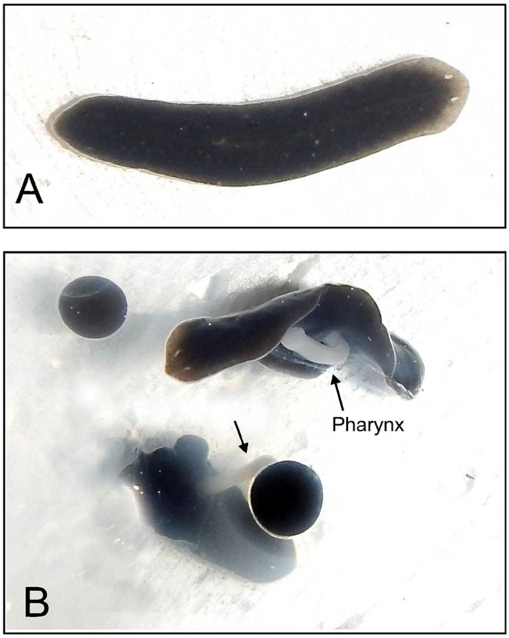

Figure 1. Photographs of *Schmidtea nova* from Kleiwiesen, Germany. (A) Dorsal view; (B) Two planarians feeding on *Rana temporaria* eggs. The lower specimen has positioned itself underneath an egg and has extended its pharynx towards to start feeding on the egg. This figure is published in colour in the online version.

Results

Our predation exposure experiment (fig. 1) verified that *Schmidtea nova* preys on *R. temporaria* eggs and early embryos, causing a decrease in survival rate. On average 95% of the eggs in the seven replicates failed to develop or were completely consumed after 48 hours while all the eggs in the control replicates, survived and eventually developed into tadpoles.

Our field survey of *R. temporaria* clutches revealed that 62% (N = 20) of the clutches at the study site contained planarians with mean ± SE = 3.94 ± 0.79 and a maximum of 13 planarians per clutch. Considering the 20 clutches with planarians, the average number of eggs per planarian was 172. We used this planarian-egg ratio as a guideline for the predator-induced development experiment.

After 10 days of the experiment, a total of 10 942 individuals were scored, with developmental stages varying between stages 14-25

Table 2. Developmental plasticity of *Rana temporaria* eggs exposed to three experimental treatments involving *Schmidtea nova* planarians: NP: no planarians; CP: caged planarians, and FP: free planarians. Descriptive statistics of the embryos after the 10 day experiment are provided as median (minimum-maximum), development stage was measured using the Gosner scale. A summary of a generalized linear mixed effect model relating the three experimental treatments and the observed stages (response variable) while controlling for inter-clutch variation (random effect) are also provided. Confidence interval (95%) range is reported for each fixed effect term with the corresponding test of significance.

	NP	CP	FP
DESCRIPTIVE STATISTICS			
Total number of embryos (clutches assayed)	3866 (10)	3659 (10)	3417 (10)
Developed embryos per clutch	356 (226-600)	402 (211-457)	366 (154-467)
Development stage	21 (14-25)	22 (14-25)	22 (14-25)
MODEL SUMMARY			
Estimates	19.68	1.05	1.02
Low/High CI (95%)	18.59/20.83 (intercept)	1.04/1.06 (coefficient)	1.01/1.03 (coefficient)
Z value	113.35, $p < 0.001$	9.86, $p < 0.001$	3.94, $p < 0.001$

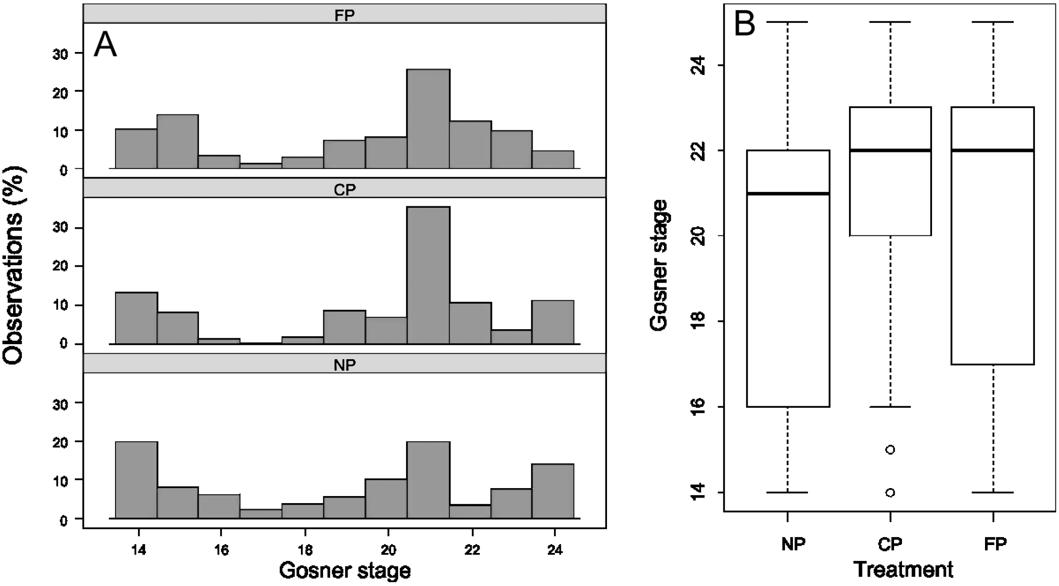

Figure 2. Response of *Rana temporaria* embryos to the presence of planarians. (A) Proportion of embryos observed in each developmental stage (Gosner 1960) after 10 days of the experimental treatments, abbreviated as: NP, no planarians; CP, caged planarians; and FP, free planarians. (B) Median and upper/lower quartiles of the Gosner stages pooled by treatment.

along the Gosner scale (table 2, fig. 2), thus including embryonic as well as early free-swimming tadpole stages. The fitted model indicated a slight positive effect of both predator treatments as eggs raised with free or caged planarians showed developmental stages significantly higher than those raised without planarians. The observed effect was rather slight as the predator-presence estimates were only one Gosner stage higher than the estimate without planarians (table 2). The random effect parameter, representing the variability among clutches not captured by the fixed effects in the model, showed a standard deviation of 1.09 Gosner stages which is comparable to the magnitude of the treatment effects and suggest a high variation among clutches in treatment response.

Discussion

Accelerated development is an adaptive response to the risk of predation by stage-specific egg predators. We here provide the first evidence for the flatworm *Schmidtea nova* preying on eggs of the European common frog, *Rana temporaria*, and found the two species cooccurring under natural conditions. As a relatively small sized and slow-moving predator, *S. nova* should pose a limited threat to tadpoles once they become mobile. Theoretical models of transition in life-histories (Werner, 1986; Rowe and Ludwig, 1991; Day and Row, 2002) predict growth-mortality tradeoffs, time constraints, and developmental thresholds. In the course of an organism ontogeny when development is not yet fixed (Hentschel, 1999), varying the duration of specific stages, e.g. shortening stages that are more susceptible to predation or prolonging stages that are resistant to predation can optimize individual fitness gain. In our study, developmental stages were significantly higher in treatments with caged and free planarians compared to those without planarians implying a slight acceleration of development.

Embryonic developmental response of *R. temporaria* to planarian waterborne chemical cues is a plausible hypothesis explaining our results regarding caged planarians. Alternatively, the developmental response might also be due to conspecific chemical alarm cues. Embryos of *R. temporaria* have been found to accelerate hatching in response to post-digestion alarm cues released into the water by both leeches and dragonfly nymph that were fed on conspecifics (Capellan and Nicieza, 2010). In our experiments, all the planarian specimens were collected in situ from within *R. temporaria* clutches few hours prior to their inclusion in the experimental containers and probably had been feeding on *R. temporaria* eggs, thus potentially releasing similar post-digestion alarm cues as the predators studied by Capellan and Nicieza (2010). This might explain the lack of difference in developmental stages between the caged and free planaria treatments.

Conducting an enclosure experiment in a natural breeding pond, as performed in this study, entails its pros and cons. It more accurately reproduces natural conditions and thus enhances the ecological relevance of the results. However, it does not preclude the presence of other water-borne predator cues. Our data suggests a slight but significant treatment effect despite the fact that water-borne chemical cues, derived from planarians and other potential predators of *Rana temporaria* eggs were probably present in the pond and potentially could have obscured the treatment effects. However, the high variation observed among clutches of *R. temporaria*, which has also been observed among wood frog (*Rana sylvatica*) clutches (Dibble et al., 2009), points to the existence of an elevated variability in developmental responses to predator presence, an issue that requires further examination. In order to ascertain this source of variation, future studies should include both a larger number of eggs and a larger number of clutches in predator-pressure experiments.

In accordance with threat sensitivity theory (Helfman, 1989), the effect of predator borne cues on prey should be influenced by (1) the intensity of the cue detection range by the prey (Takahara et al., 2012) and (2) the intensity of the prey anti-predator response (Van Buskirk and Arioli, 2002). A few studies on fish report ‘all-or-none’ response to gradients of alarm cues (Ferrari et al., 2010). This type of response is expected in situations where a predator occurs in a wide range of densities and/or is not ideal free distributed within the habitat occupied by the prey. Being an explosive breeder may preclude temporal heterogeneity in predation whereas variability among *R. temporaria* clutches in the intensity of planarian attacks may promote an adaptive embryonic developmental plasticity to reduce mortality risk. Despite having worked in several other populations of *R. temporaria* we have so far not observed planarians preying upon eggs of these frogs elsewhere which suggests a substantial variation among sites in the presence of these predators.

Due to the characteristics of our experiment (natural settings and uncertainty of exact clutch deposition time) it is difficult to derive a precise interpretation of the biological consequences of the slight developmental acceleration observed in the presence of planarians. An accurate assessment of the effect of planarians on the development of *Rana temporaria* embryos would probably require detailed laboratory experiments in order to delimitate which development stages and structures are particularly accelerated which could provide hints on the biological consequences. Alternatively, a carefully-designed gene expression experiment could pinpoint which genes are activated under predator pressure and potentially identify their precise effect on embryonic development.

Our work added flatworms as an additional group of predators inducing changes of embryonic developmental rate in ranid frogs (table 1). Flatworms are a phylum rarely considered as predators of anuran eggs and embryos. *R. temporaria* and related species are early explosive breeders, often depositing their clutches when ponds are still partly covered in ice and snow. At our study site in Germany there are few invertebrate egg predators active at the time of spawning. We find it likely that planarians are among the most relevant predators of *R. temporaria* eggs in this locality. In general, it seems clear that more studies, such as tests of the dosage effect of various planarian densities on early developmental plasticity, tests on planarian diet-related effects, and stage-specific vulnerability to planarian predation, are necessary to fully understand the complex interplay between anuran early-stage development and this predator.

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