

Article

Simulated predation pressure in *Pelobates cultripes* tadpoles modulates morphology at the metamorphic stage

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Abstract

Studies on the impacts of variation of biotic interactions at key life cycle stages are crucial to understand the interface between ecological and developmental processes. Predators exert a major impact on prey fitness. Although direct consumption entails the greatest effect, predators can affect prey by means of other mechanisms. For instance, injuries inflicted by failed predation attempts can jeopardize prey fitness, even beyond the short-term. In anuran tadpoles, failed predation typically results in partial tail loss, which is known to reduce swimming speed. However, the potential consequences of tadpole partial tail loss after metamorphosis remain understudied. Because tail materials could be important in conforming metamorph body, we assess the effects of tadpole partial tail loss on metamorph body size in Iberian spadefoot toads *Pelobates cultripes*. We clipped 55% tail length of pre-tail-resorption stage anesthetized tadpoles, and compared their body size as metamorphs with anesthetized and non-anesthetized non-tail-clipped controls. Also, we tested whether tail length correlated with metamorph body size of individuals of the control groups. Tail-clipped tadpoles produced smaller metamorphs than both controls (the body size of metamorphs from both controls was similar), which could incur costs in mid-term survival or time to first reproduction. This effect could be particularly important in areas with introduced predators, if autochthonous tadpoles lack defenses against them. Results suggest that materials resorbed from tadpole tail tissues might be reallocated into metamorph body, according to the negative effect of shorter tails in a correlational analysis, and clipped tails in an experimental test, on metamorph body size.

Key words: amphibian, anuran, body condition, body size, failed predation, partial tail loss

The complexity of biotic interactions is a major challenge to understand the impacts of environmental change within and between populations, and experimental approaches are a crucial tool to go beyond. Predation is a capital selective force due to its huge impact on prey fitness (Caro 2005). Future fitness of animals that are preyed on is reduced to zero (Abrams 2000). Moreover, predators can reduce fitness of potential prey by means of different mechanisms besides direct consumption (reviewed in Preisser et al. 2005): behavioral (such as increased refuge use; Martín and López 2000;

Nafus et al. 2017), morphological (Castellanos et al. 2015), or physiological defenses against predators (Zvereva et al. 2017), as well as life-history changes (Chivers et al. 1999), may divert time and resources from other fitness-enhancing activities. For instance, failed attempts of predation may affect conspecific chemical communication, which might reduce mating success (Aragón et al. 2008). Furthermore, injuries resulting from failed predation are common (Reimchen 1988; Sheader 1998), and can compromise prey future fitness by means of wound infection (Archie 2013) or

increased susceptibility to subsequent attacks (Downes and Shine 2001), often driven by impaired mobility (Cooper et al. 2009). Also, animals can respond to failed predation with behavioral changes that increase responsiveness (Crook et al. 2011; Bliss and Cecala 2017) or diminish conspicuousness to predators, to the detriment of other activities that enhance fitness such as feeding (Low et al. 2014).

However, whereas predation is irreversible, animals can eventually recover from injuries caused by failed predation, mainly in body parts that can be lost without killing the individual, such as tails. Indeed, some lizards attract predator strikes toward their autonomous tails with bright colorations (Fresnillo et al. 2015) or conspicuous movements (Cooper 1998). Plus, although their tails are not detachable, some anuran tadpoles exhibit colored tails that are likely to be targeted by predators (Caldwell 1982). Indeed, colored-tail artificial tadpole models are more often attacked in their tails by insect predators than plain-tail models (Van Buskirk et al. 2004). Other tadpoles respond to predator presence with enlarged tails that do not improve swimming performance, but reduce the chances of being attacked in vulnerable head and body (Van Buskirk et al. 2003). In fact, injuries in tails of anuran tadpoles are common in the wild (Blair and Wassersug 2000), and relate positively to predator abundance (Morin 1985). The fact that tadpoles deflect predator attack toward the tail is biologically relevant, because tails are primarily responsible for tadpole locomotion. Indeed, tail-injured tadpoles have slower swimming speed (Ding et al. 2014b). Tail injury therefore increases the chances of future predation in anuran tadpoles (Semlitsch 1990). Hence, the loss of a piece of tail may compromise tadpole short-term survival. However, regeneration of tissues lost restores tadpole locomotor performance (Ding et al. 2014a). Plus, tails are absent in post-metamorph anuran body plan. Therefore, risk raised by directing attacks toward tails could diminish in the mid-term as the damaged structure will be absent in post-metamorphic stages.

Nonetheless, although the effects of tadpole partial tail loss on larval ecology are well studied, its consequences on post-metamorphic stages are poorly understood. In some related vertebrate taxa, such as reptiles (reviewed in Price 2017) and urodeles (Takahashi and Pauley 2010), tails are important storage reservoirs. Tadpoles fast from the emergence of forelimbs until metamorphosis is concluded (Jensen 1967). Therefore, tail tissues could be an important source of materials during metamorphosis, in which case loss of tadpole tail tissues could translate into reduced metamorph body size. However, to our knowledge, this hypothesis has not been tested.

In this work, we assess the effect of partial tail loss in tadpoles of Iberian spadefoot toads (*Pelobates cultripes*) on metamorph body mass, snout-to-vent length (SVL), and body condition. In the wild, these tadpoles often show incomplete tails (Busack and Zug 1976), not only as a consequence of failed native predator attacks, but also due to invasive predators (Nunes et al. 2010) or even cannibal conspecifics (Comas and Escoriza 2015). Following the aforementioned rationales, we predict that partial tail loss in tadpoles will result in reduced metamorph body size.

Materials and Methods

Study species

The Iberian spadefoot is a medium-to-large sized toad distributed in Southern France and wide regions of the Iberian Peninsula (Recuero 2014). These toads dwell in a variety of low- and mid-elevation

habitats, provided that substrate is loose enough for their burrowing habits (Recuero 2014). Reproduction takes place early in the spring in cold areas, or even in the fall and winter in warmer zones. Toads lay eggs in large temporary water masses, mainly ponds and pools, where tadpoles take several months to complete metamorphosis (Lizana et al. 1994; Recuero 2014). Tadpoles are large (up to 120 mm total length in some cases) in relation to other anuran species, yellowish to brownish, and have large tails with massive muscles and deep fins (Recuero 2014). Tadpoles are active day and night, and spend most of their time swimming through the water column feeding on plant and animal organic matter (Recuero 2014). Consequently, they are often attacked by numerous predators, such as diving beetles (e.g., *Dytiscus* sp.), Odonata nymphs (e.g., *Cordulegaster* sp.), urodeles (e.g., *Pleurodeles waltl*), water snakes (e.g., *Natrix maura*), pond turtles (e.g., *Emys orbicularis* and *Mauremys leprosa*), and water birds (e.g., *Ardea cinerea* and *Ciconia nigra*) and mammals (e.g., *Lutra lutra* and *Arvicola sapidus*) (Recuero 2014).

Experimental conditions and design

In March 2017, we collected 112 tadpoles (at 24–26 Gosner stages) with a dip net in a natural temporary pond in Pinares de Cartaya *Pinus pinea* grove (SW Spain: 37°20' N, 7°09' W). Tadpoles were taken to laboratory, and randomly pooled into 2 14-L plastic aquaria placed next to a window that allowed natural light in for adjustment of circadian processes, but avoiding direct sunlight. Tadpoles were fed *ad libitum* with dry cat food, appropriate for their omnivorous diet (37% calories from protein, 29% from fat, and 34% from carbohydrates), and once a day 80% of water volume was replaced with fresh spring water. All tadpoles were set in the same laboratory at room temperature (21°C approximately), and fed the same food, to avoid potential effects of temperature and food quality during larval stage on metamorph body size (Álvarez and Niecieza 2002; Niehaus et al. 2006).

Tadpoles were reared under those conditions until they reached Gosner stage 42, when hindlimbs are well developed and forelimbs appear (Gosner 1960). We conducted procedures at 42 Gosner stage because it immediately precedes tail resorption (Gosner 1960), and so any potential effect of energy investment in tail regeneration on body size was thus dodged (Chen et al. 2014). At that moment, tadpoles were measured SVL and tail length with a digital caliper (model ECO T304B.W-1230) to the nearest 0.01 mm, as well as body mass with a scale (model CDS-100, precision 0.01 g), after gently removing excess water with a disposable napkin (1 per individual). Immediately after, tadpoles randomly underwent 1 of 3 treatments. Tadpoles assigned to the experimental group ($n = 35$ individuals) were anesthetized in a clove oil solution (450 mg/L of water; Mitchell et al. 2009) until they did not straighten or move, even as a response to gentle touching stimuli, but before they lost respiratory reflect (from 5 min for smaller tadpoles to 9 min for larger tadpoles, approximately). Then, they were clipped 55% tail length with a sterile scalpel. This was the maximum tail length percentage that could be clipped with no subsequent death of individuals in a preliminary test. The wound was immediately disinfected and stanching with veterinary tissue adhesive (3 M Vetbond 1469), which prevented bleeding. Besides, we defined 2 control groups. In 1 of them ($n = 39$ individuals), tadpoles were anesthetized in clove oil in the same circumstances as the experimental group, but their tails were not clipped. In the second control group ($n = 38$ individuals), tadpoles were not anesthetized nor had their tails clipped.

We immediately transferred tadpoles to individual 0.5-L aquaria until metamorphosis. Water was daily replaced with fresh spring water. After assignment to 1 treatment, tadpoles were no longer fed to avoid confounding effects of differential food intake on metamorph body mass. In fact, tadpoles generally fast after 42 Gosner stage until metamorphosis is completed, due to mouthparts atrophy (Gosner 1960) and digestive tract restructuring (Jenssen 1967). When tadpoles were in Gosner stages 44–45 and tail resorption was advanced (Gosner 1960), they were transferred to 0.5-l individual tilted aquaria to facilitate metamorphs abandon water at Gosner stage 46. Metamorphs were then measured SVL with a mm-marked ruler, and body mass with a scale (model CDS-100, precision 0.01 g). Individuals were then released at their capture site as soon as possible.

Statistical analyses

Because data met the criteria of homoscedasticity (Levene's Test: $F < 0.538$; $P > 0.586$ in all cases) and residual normality (Kolmogorov-Smirnov $d < 0.061$; $P > 0.050$ in all cases), we conducted parametric analyses (Quinn and Keough 2002). First, we checked with a 1-way Analysis of Variance (ANOVA) that initial tadpole SVL, tail length, body mass, and body condition (calculated as the regression residuals of tadpole body mass on total length) before the experimental procedure did not differ among groups. We then tested (excluding individuals that had been tail-clipped) the relationships between tail length, controlled for tadpole body condition, on metamorph body condition with a multiple regression. Finally, we conducted a 1-way Analysis of Covariance (ANCOVA) to test the effect of treatment on metamorph body condition, including tadpole body condition as a covariate. Then, we conducted a Tukey test to assess pairwise differences among the 3 groups. For the calculation of body condition of both larvae and metamorphs, body mass and SVL were not log-transformed because at this stage of the life cycle the allometric relationship is not apparent yet (Figures S1 and S2 in the Supplementary Material, respectively). All analyses were also conducted for metamorph SVL and body mass separately (controlling for tadpole SVL and body mass, respectively).

Results

Tadpoles

Because tadpoles were randomly assigned to 1 of the 3 treatments, there were no differences in tadpole SVL ($F_{2, 109} = 1.139$; $P = 0.324$; Table 1), tail length ($F_{2, 109} = 1.426$; $P = 0.245$; Table 1), body mass ($F_{2, 109} = 0.603$; $P = 0.549$; Table 1), and body condition ($F_{2, 109} = 0.328$; $P = 0.721$; Table 1) among treatments. Tadpole tails ranged from 61.11 to 69.49% total length.

Metamorphs

Multiple regression with metamorph body condition as the dependent variable, and tadpole tail length and body condition as

independent variables was significant ($F_{2, 74} = 26.436$; $R^2 = 0.417$ $P < 0.001$); according to partial correlations, both tail length ($F_{1, 74} = 22.235$; $\beta = 0.426$; $P < 0.001$; Figure 1) and tadpole body condition ($F_{1, 74} = 39.788$; $\beta = 0.570$; $P < 0.001$) had significant, positive relationships with metamorph body condition.

Treatment had no effect on metamorph SVL (1-way ANCOVA: $F_{2, 108} = 0.837$; $P = 0.436$; Figure 2A), whereas tadpole SVL (included as a covariable) showed a positive relationship with metamorph SVL ($F_{1, 108} = 252.593$; $\beta = 0.843$; $P < 0.001$).

However, treatment had a significant effect on metamorph body mass (1-way ANCOVA: $F_{2, 108} = 14.839$; $P < 0.001$ Figure 2B), and tadpole body mass (included as a covariable) showed a positive relationship with metamorph body mass ($F_{1, 108} = 989.095$; $\beta = 0.950$; $P < 0.001$). Fisher post-hoc tests showed that body mass of metamorphs from the experimental group differed from both anesthetized (Fisher test, $P = 0.004$; Figure 2B) and non-anesthetized controls (NACs) (Fisher test, $P = 0.014$; Figure 2B), whereas both controls did not differ between them (Fisher test, $P = 0.667$; Figure 2B).

Treatment had a significant effect on metamorph body condition (1-way ANCOVA: $F_{2, 108} = 4.366$; $P = 0.015$; Figure 2C); tadpole body condition, included as a covariate, was positively related to metamorph body condition ($F_{1, 108} = 28.460$; $\beta = 0.440$ $P < 0.001$). Fisher post-hoc tests showed that differences between the experimental group and both anesthetized (Fisher test: $P = 0.049$; Figure 2C) and NACs ($P = 0.001$; Figure 2C) were significant, whereas differences between both controls were non-significant

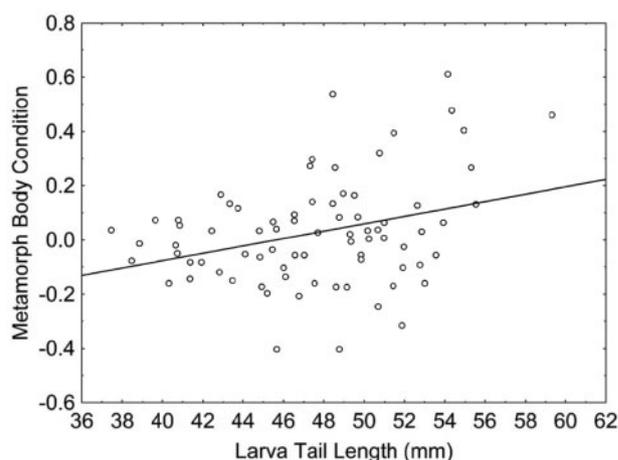


Figure 1. Correlation between larva tail length and metamorph body conditions in the individuals that were not tail-clipped. Sample size was 80 individuals.

Table 1. Mean \pm standard errors of SVL, tail length, body mass, and body condition of tadpoles assigned to NAC, anesthetized control, and experimental group, at Gosner Stage 42

Variable	NAC ($n = 38$)	Anesthetized control ($n = 39$)	Experimental group ($n = 35$)
SVL (mm)	24.671 \pm 0.332	25.093 \pm 0.328	25.389 \pm 0.346
Tail length (mm)	46.859 \pm 0.696	48.261 \pm 0.687	48.350 \pm 0.725
Body mass (g)	2.993 \pm 0.119	3.145 \pm 0.117	3.162 \pm 0.124
Body condition	0.036 \pm 0.059	-0.007 \pm 0.058	-0.031 \pm 0.061

Sample sizes are indicated.

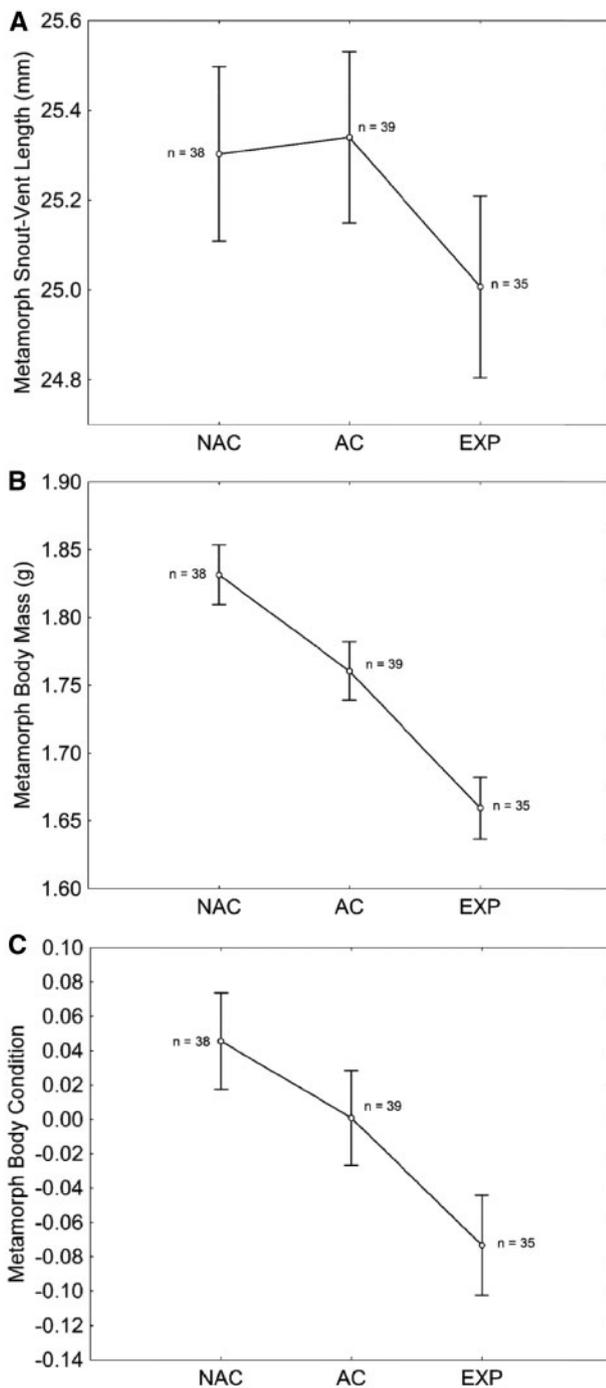


Figure 2. Mean \pm Standard errors of SVL (A), body mass (B), and body condition (C) of metamorphs from tadpoles assigned to NAC group, anesthetized control group (AC), and experimental group (EXP), controlled for tadpole SVL, body mass, and body condition, respectively. Sample sizes are indicated.

($P = 0.163$; Figure 2C). Therefore, we can discard an effect of handling and anesthesia.

Discussion

Body mass at metamorphosis is directly related to survival in anurans (Cabrera-Guzmán et al. 2013), probably mirroring the positive

effect of metamorph lipid reserves on survival (Scott et al. 2007). Intraspecific competition may diminish size at metamorphosis (Travis 1984), and this effect not only can be mediated by density, but also softened by kinship (Saidapur and Girish 2001). Also, changes in predation risk can incur costs in terms of body mass at metamorphic stage (Benard and Fordyce 2003; Richter-Boix et al. 2006). Indirectly, exposition to predator chemical cues during larval stages may trigger body mass loss during metamorphosis (Van Buskirk and Saxer 2001, but see Niecieza 2000), whereas it may have no effect during the egg stage (Capellán and Niecieza 2007). Tadpoles that suffer partial tail loss show reduced swimming speed (Ding et al. 2014a, 2014b), and growth (Wilbur and Semlitsch 1990), which may increase predation risk (Figiel and Semlitsch 1991). Our finding that partial tail loss also results in reduced metamorph body size reports, to our knowledge, the first evidence of a direct across-stage negative effect of predators on anuran body size. Furthermore, according to our results, reduced body size as a consequence of partial tail loss during larval stage could compromise survival in early post-metamorph stages (Altwegg and Reyer 2003; Cabrera-Guzmán et al. 2013). Even in the long term, individuals that metamorphose at greater size can be larger also as adults, and reproduce earlier (Smith 1987). This is particularly important in animals with short lives or high mortality rates such as *P. cultripipes* toads (Leclair et al. 2005), which can have low chances of surviving long enough to reproduce multiple times. Therefore, negative consequences on fitness of partial tail loss in anuran tadpoles are not exclusive of larval phases, but can extend to post-metamorph stages. Nevertheless, our experimental design does not account for genetic distance, so a more complex design controlling for kinship and the potential effects of different discrete populations would have gained in inference but was beyond logistical possibilities because it would incur in differences of developmental and captivity times among ponds.

On the other hand, the negative short- and mid-term effects of partial tail loss on tadpole locomotion shown in previous studies, and on metamorph body size found in our study, could both be transient. A previous encounter with a predator may predispose tadpoles to survive subsequent predator attacks (Álvarez and Niecieza 2006), which could counteract the reduced locomotor performance due to partial tail loss. Also, tail regeneration can restore tadpole swimming speed (Ding et al. 2014b). Moreover, although Altwegg and Reyer (2003) detected long-term effects of reduced body mass at metamorphosis on fitness components of 2 frog species, Boone (2005) found that compensation in body mass by means of increased feeding rates after metamorphosis may reverse lower metamorph body mass in some cases. Thus, the strategy of many tadpoles that divert predator attacks toward their tails, even developing deeper tail fins (Schalk 2016) or more colorful tails (Touchon and Warkentin 2008) to attract predator attention, avoids potential fatal strikes on head and body, and entails negative effects that, nonetheless, can be reversed in the mid-term. Therefore, not only the costs of partial tail loss outweigh those of predation, but they can also be attenuated.

In spite of this, tadpoles may lack effective defenses against introduced predators, which can thus increase not only tadpole mortality, but also tail injury prevalence and severity (Shulse and Semlitsch 2014). In the area studied, introduced red swamp crayfish *Procambarus clarkii* pose a severe threat to native amphibian populations, including *P. cultripipes* (Cruz et al. 2006; Vázquez et al. 2017). Indeed, frequency of tail-injured *P. cultripipes* tadpoles is greater in ponds where *P. clarkii* is present (Nunes et al. 2010).

However, although naïve *P. cultripes* tadpoles do not display any defensive response to chemical cues of *P. clarkii* crayfish, they do after predator cues are presented along with conspecific alarm cues (Polo-Cavia and Gomez-Mestre 2014). Similarly, sympatric *Pelophylax perezi* frog tadpoles do not immediately develop inducible defenses against *P. clarkii* (Gomez-Mestre and Diaz-Paniagua 2011), whereas strong selection by this introduced predator triggers rapid evolutionary changes that improve survivorship (Nunes et al. 2014). Nonetheless, learned or acquired defenses observed in several anuran tadpoles (Nunes et al. 2013) seem unable to compensate high predation rates, according to the devastating effects of *P. clarkii* invasions on amphibian populations reported in several regions (e.g., Cruz and Rebelo 2005; Cruz et al. 2008; Romano et al. 2016).

Tail resorption is modulated by thyroid hormone that induces muscle cell death and suicide mechanisms (Nakajima and Yaoita 2003), in synergy with corticosterone (Kulkarni and Buchholz 2012), as a part of an immunological rejection response of tadpole triggering apoptosis of its own tail cells (Mahapatra and Mahapatra 2012; Nakai et al. 2017). Our results suggest that tadpole tail tissues resorbed may contribute meaningfully to metamorph tissue anabolism, as shorter tails in a correlational analysis, and clipped tails in an experimental test, resulted in smaller metamorphs. According to these findings, materials resulting from tail resorption could be reallocated into metamorph tissues. The underlying histochemical mechanisms deserve further research.

In conclusion, tail-clipped tadpoles became smaller metamorphs, which could incur costs in mid-term survival or time to first reproduction. This effect could be particularly important in areas with introduced predators, if autochthonous tadpoles lack defenses against them. Results suggest that tadpole tail materials resorbed might be reallocated into metamorph body, according to the negative effect of shorter tails in a correlational analysis, and clipped tails in an experimental test, on metamorph body size. The histochemical mechanisms responsible for materials reallocation should receive further attention.

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Authors' contributions

F.J.Z.-C. and P.A. conceived and designed the experiment. F.J.Z.-C. performed the experiment. F.J.Z.-C., S.C.-M., and P.A. analyzed the data. F.J.Z.-C. wrote the manuscript, with substantial input from S.C.-M. and P.A.

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