



Fitness effects of shelter provision for captive amphibian tadpoles

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The larvae of many amphibians are highly sensitive to environmental conditions. Tadpoles can adjust their development in response to stressors, but this may come at a cost in terms of fitness. The captive environment may be the source of stressors and may therefore influence the fitness of larval amphibians reared for conservation. We investigated the effects of shelter provision on fitness in captive tadpoles of the red-eyed treefrog (*Agalychnis callidryas*). Larvae maintained with shelter metamorphosed significantly larger and later than animals maintained with either no shelter or with shade only. Behavioural data suggest that the positive effect of shelter provision on fitness may be due to a reduced stress response; animals showed more extreme anti-predator behaviour when housed with no shelter and intermediate responses when housed with shade alone. Our data show that the design of captive enclosures can influence the fitness of captive amphibians.

Key words: amphibian, enrichment, *ex situ* conservation, fitness, husbandry, phenotypic plasticity, shelter, tadpole

INTRODUCTION

Amphibian larvae often develop in highly unpredictable and variable environments. This has driven the evolution of developmental and phenotypic plasticity in tadpoles, which allows phenotypic adaptation to local conditions (Newman, 1992). Threats including predation pressure (Babbitt & Tanner, 1997; 1998; Griffiths et al., 1998; Relyea, 2001; 2004; Kopp et al., 2006) may trigger plastic responses, such as adjusting larval period duration (Glennemeier & Denver, 2002; Denver, 2009). All else being equal tadpoles that metamorphose later, and so spend longer feeding and growing, will transform at a larger size (Newman, 1989; Harris, 1999). Larger metamorphs typically enjoy higher fitness, mainly through effects of size on survivorship and fecundity (Werner, 1986; Bardsley & Beebee, 1998; Semlitsch et al., 1988; Altwegg & Reyer, 2003; Rowe & Beebee, 2003; see Discussion). Developmental responses to threats typically result in reduced size at transformation (Audo et al. 1995; Newman, 1988; 1989; 1992; 1998; Glennemeier & Denver, 2002; Denver, 2009) and so tadpoles pay a fitness cost in order to escape threats and survive until metamorphosis (Newman, 1992).

Many amphibian species are now maintained in captivity, not least as assurance populations and in head starting programmes as part of the *ex situ* response to global amphibian declines (Gascon et al., 2007). Given the sensitivity of tadpoles and their fitness to their environment in the wild, husbandry may directly influence the fitness of amphibians reared in captivity

by activating phenotypic plasticity and its associated fitness costs. Although husbandry practices such as diet (Martins et al., 2013) are known to influence fitness traits in tadpoles, impacts of enclosure design are poorly understood. Environmental complexity and enrichment are poorly studied in and infrequently used for captive amphibians (Burghardt, 2013; Michaels et al., 2014b), and particularly larvae, but may have important implications both for their welfare and conservation (Michaels et al., 2014b; Reading et al., 2013). Shelter is a prominent component of the larval environment under natural conditions and is important in mediating predation pressure (Babbitt & Tanner, 1997; 1998). It has also been shown to benefit both welfare and fitness in post-metamorphic amphibians (reviewed by Michaels et al., 2014a; b). However, refuges are frequently entirely absent from captive enclosures for tadpoles, in order to facilitate maintenance (CM, pers. obs.). The effects of this practice on tadpole fitness are unknown. Here we use the tadpoles of the red-eyed tree frog (*Agalychnis callidryas*) to investigate the effect of shelter provision in captive enclosures on fitness and behaviour of anuran larvae in captivity.

METHODS

Tadpoles were from a clutch produced by one pair of F2 *A. callidryas* at the University of Manchester. We used this species as it is congeneric with several threatened frog species, including *A. lemur*, *A. annae* and *A. moreletii*. Moreover, this species has already been the focus of

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captive husbandry research (e.g., Antwis et al., 2014; Michaels et al., 2014a) and so provides opportunity for a more complete understanding of one model species.

Tadpoles were maintained in aquaria (ExoTerra Faunarium 'Large') measuring 370x220x250 mm for the duration of the study. Water quality and temperature reflected wild conditions (23–24°C, pH 6.5–7.0; HabiData database). Powdered *Spirulina* algae and crushed flaked fish food (Tetramin Tropical Flakes, Tetra), were provided *ad libitum*. Water was filtered using an air-driven sponge filter and 25% water changes were performed every three days to remove nitrogenous waste. Water quality

(pH and ammonia) was monitored every two days using aquarium test strips ('EasyStrips 6-in-1 Aquarium Test Strips' and 'EasyStrips Aquarium Ammonia Test Strips', Tetra). There was no difference in water quality measures between treatments (pH in the 6.5–7.0 band, ammonia undetectable).

Four newly hatched tadpoles were allocated randomly from the single hatched clutch to each of the three aquaria in four treatment groups; Live plants (L), Artificial plants (A), Bare Lit (B) and Bare Shaded (S). Six tadpoles died close to metamorphosis and were replaced with non-experimental animals in order to maintain equal stocking density. Replacement animals were identified through a combination of photo IDs and their degree of development in comparison to other individuals. L aquaria contained live floating and mid-water aquatic plants (*Limnobium* sp. and *Egeria densa*) and submerged leaves (*Bambusa* sp.). A aquaria contained artificial plastic aquatic plants resembling the species used in L tanks and artificial plastic leaf litter, made from food-grade plastic. Planting density was equal in all aquaria with refuges. B and S tanks contained no shelter. All aquaria were lit with a fluorescent lamp ('Freshwater' T8 linear fluorescent lamp, Arcadia) on a 12:12 photoperiod, but S tanks were partially shaded through the use of opaque aquarium covers.

The duration of larval period and the order in which tadpoles metamorphosed were recorded, using the day on which animals climbed out of the water as the date of metamorphosis. At this point, animals still have remnant tails, but for the purposes of this study this represented the ecological shift between aquatic and terrestrial environments. The SVL of new metamorphs was measured three times using photography against a scale and ImageJ (NIH), with mean values being used for analysis.

On days 14, 15 and 16 of development, tadpoles were disturbed by tapping once on the aquarium with fingertips in a standardised manner. Tadpoles of *A. callidryas* typically swim at an angle near to the surface, but when disturbed rapidly swim away and may leap from the water. The number of leaping tadpoles, as opposed to those not reacting or swimming in other directions, was counted following each disturbance. The assay was conducted at 1100 hours each day and the order in which tanks were disturbed was randomised daily.

SVL and larval duration data were analysed using SPSS v.20 for Windows, $n=42$ for all tests reported. Generalised Linear Models (GLMs) using Wald tests were used to analyse size at metamorphosis (SVL) and larval duration using the following terms; Treatment=the four shelter treatments; Tank=individual tanks, nested within Treatment; Order=the order in which individuals metamorphosed within a tank (which may have influenced output variables through short-term changes in stocking density), with 1 being the first and 4 the last, and simultaneous metamorphoses given equal intermediate values. Terms that were not statistically significant were removed from the model and the analyses repeated (only significant results are reported below; variables not reported were found to be non-

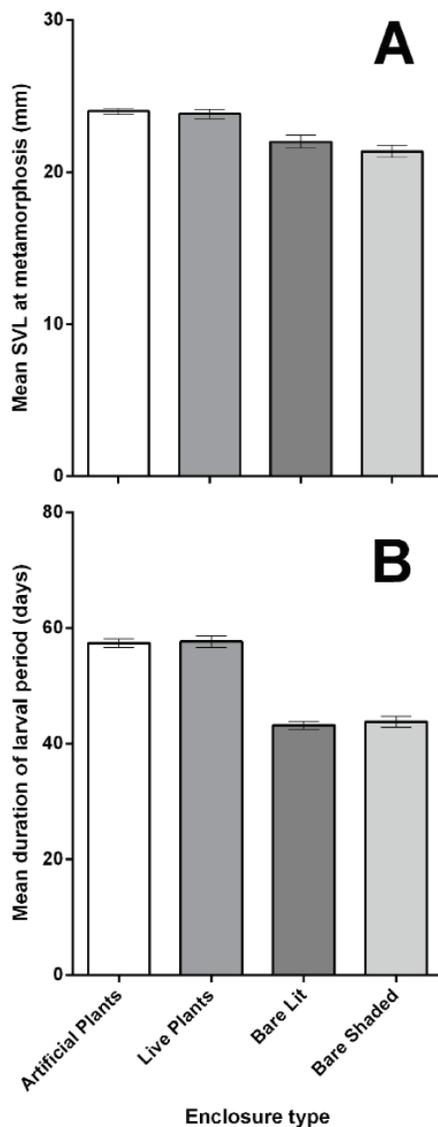


Fig. 1. (A) Mean SVL at metamorphosis of tadpoles in each treatment group ($n=42$). 'Artificial Plants' (A; $n=10$) and 'Live Plants' (L; $n=11$) tadpoles metamorphosed significantly larger than 'Bare Lit' (B; $n=11$) and 'Bare Shaded' (S; $n=10$) tadpoles (Effect of Treatment: Wald Chi-Squared₃₈=45.608, $p<0.001$). (B) Fig. 2. Mean larval period duration in each treatment group. 'A' and 'L' tadpoles metamorphosed significantly later than 'B' and 'S' tadpoles (Effect of Treatment: Wald Chi-Squared₃₈=318.918, $p<0.001$). Error bars represent the SEM.

significant and dropped from final models). However, we report the results of models including and excluding Tank(Treatment) to demonstrate the robustness of this approach to avoiding pseudoreplication.

A regression was run to determine if there was a relationship between larval period and size at metamorphosis. The total number of leaping tadpoles in each treatment group across the three trial days was calculated and data analysed using a Chi-Squared test for differences.

RESULTS

There was no significant effect of Tank(Treatment) on size of tadpoles at metamorphosis (Wald Chi-Squared₃₈=11.449, $p=0.178$). There was a significant effect of treatment on the size of tadpoles at metamorphosis, both including and excluding Tank(Treatment) in the model (Including Tank(Treatment): Wald Chi-Squared=58.040, $p<0.001$; excluding Tank(Treatment): Wald Chi-Squared₃₈=45.608, $p<0.001$; Fig. 1A). Both treatments A and L significantly increase SVL, but were not significantly different from one another (post-hoc LSD test, $p=0.720$). Treatments B and S were not significantly different from one another (post-hoc LSD test, $p=0.219$), but both treatments resulted in significantly smaller metamorph SVL.

There was no significant effect of Tank(Treatment) on larval duration (Wald Chi-Squared₃₈=9.287; $p=0.287$). Treatment had a significant effect on larval duration, both including and excluding Tank(Treatment) in the model (Including Tank(Treatment): Wald Chi-Squared₃₈=392.569, $p<0.001$; Excluding Tank(Treatment): Wald Chi-Squared₃₈=318.918, $p<0.001$; Fig. 1B). A and L tanks increased larval period above the mean, but were not significantly different from one another (post-hoc LSD test, $p=0.747$). B and S tanks decreased larval period, but were not significantly different from one another (post-hoc LSD test, $p=0.610$).

There was a positive correlation between larval duration and size at metamorphosis (SVL) across the whole population (Regression; $r^2=0.717$, $F_{38}=42.312$, $p<0.001$). In order to test whether the correlation between SVL and Duration was due to a direct effect, or due to mediation of one effect by the other, with the ultimate cause being Treatment, the model SVL=Treatment+Duration+Treatment*Duration (Sequential SS) was fitted. This model allows for the variation explained by Treatment before testing for an effect of Duration or the interaction Treatment*Duration; the significance of Duration (Wald Chi-Squared₃₈=2.822, $p=0.093$) and the interaction term disappears (Wald Chi-Squared₃₈=2.048, $p=0.563$), while Treatment remains highly significant (Wald Chi-Squared₃₈=50.896, $p<0.001$). Tank(Treatment) is not significant when fitted in this model (Wald Chi-Squared₃₈=14.783, $p=0.064$). This model therefore suggests that Treatment affects Duration, which in turn mediates SVL.

There was a significant effect of treatment on leaping behaviour ($\chi^2_3=50.827$, $p<0.01$). Figure 2 shows the frequency of leaping behaviour in each treatment.

Tadpoles in A and L tanks leapt less than expected (2 and 1 leaps, respectively). Tadpoles in B tanks leapt the most, with 33 leaping events counted, while an intermediate, but still higher than expected, number of leaping events was recorded from S tank (22 events).

Six tadpoles died and were therefore replaced with non-experimental animals, with the following distribution: A: 2, B: 1, S: 2, L: 1. Given that there was no difference in SVL and duration of larval period between A & L and B & S treatments, it is reasonable to conclude that there was no effect of treatment on mortality; the mean mortality values for treatments with and without shelter were both 1.5.

DISCUSSION

Our results indicate that the captive environment, within the bounds of 'best practice' husbandry, can influence the fitness of amphibian larvae and resulting metamorphs. Tadpoles maintained with physical shelter (A and L treatments) metamorphosed later and at larger sizes, while exhibiting a lower incidence of leaping responses. In nature, the larval environment contributes to size and age at metamorphosis (Werner, 1986; Newman, 1988; 1989; 1992; Denver 1997a; b; 2009; Denver et al., 1998; Rollins-Smith, 1998; 2001; Parris & Cornelius, 2004; Gervasi & Foufopoulos, 2008), as larval development responds

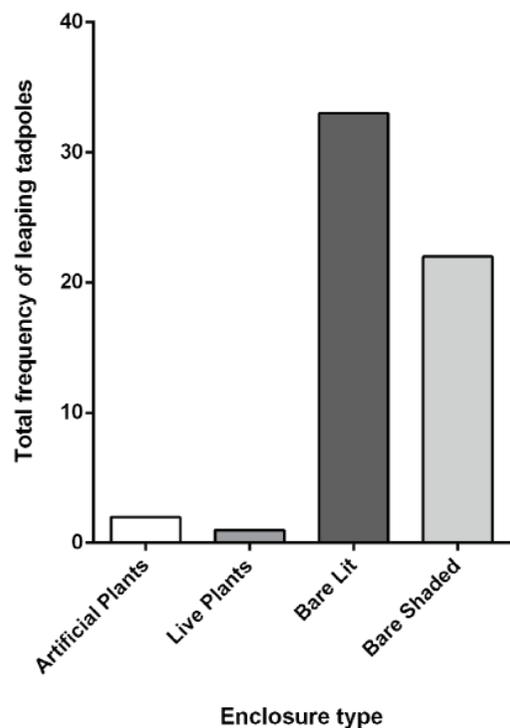


Fig. 2. Mean number of tadpoles exhibiting leaping behaviour in each treatment. 'Bare Lit' (B) tadpoles exhibit significantly more leaping than any other group, 'Artificial Plants' (A) and 'Live Plants' (L) tadpoles exhibit the least amount of leaping, but are not significantly different from one another, while 'Bare Shaded' (S) tadpoles show an intermediate amount of leaping, significantly different from all other groups (Effect of treatment on leaping behaviour: $\chi^2_3=50.827$, $p<0.01$).

plastically to stressors. In hospitable environments, tadpoles will typically extend development, within species constraints, in order to produce larger metamorphs (Newman, 1988; 1989; Denver 1997a; b). Under stressful conditions tadpoles typically extend or reduce larval period duration, but in both cases, smaller metamorphs are produced (Audo et al., 1995; Newman, 1988; 1989; 1998; Glennemeier & Denver, 2002; Denver, 2009). Typically, delayed metamorphosis under stress is only seen under conditions of starvation, where tadpoles must spend longer gathering the resources required for metamorphosis (e.g., Audo et al., 1995). Therefore, accelerated metamorphosis at the cost of size at metamorphosis is more relevant here.

Size at metamorphosis affects survivorship and lifetime fecundity in wild amphibians (Werner, 1986; Bardsley & Beebee, 1998; Semlitsch et al., 1998; Altwegg & Reyer, 2003; Rowe & Beebee, 2003), via post-metamorphic growth rates (e.g., Semlitsch et al., 1988; Altwegg & Reyer, 2003), predation risk (Clarke, 1974; Kusano, 1981; Werner, 1986; Harris, 1999), foraging success (Smith & Petranka, 1987), mobility (Newman, 1989) and perimetamorphic immunocompetency (Rollins-Smith, 1998; 2001; Carey et al., 1999; Gervasi & Foufopoulos, 2008). Size at metamorphosis also predicts adult size (Semlitsch et al., 1988; Altwegg & Reyer, 2003), which partly constrains fecundity and lifetime reproductive output (e.g., Kuramoto, 1978; Semlitsch et al., 1988; Tejado, 1992). In *A. callidryas* specifically, larger adult size improves reproductive fitness in both males (Briggs et al., 2013) and females (Yeager & Gibbons, 2013). For this reason, tadpoles in this study reared under conditions without shelter are likely to be less fit, having accelerated metamorphosis at the cost of growth. This may have implications for conservation projects, mainly those intending to release animals to the wild, where selection pressure are harsh, but also those intending to breed future generations in captivity.

One important stressor in the larval environment is perceived predation risk. This is known to affect larval period duration, size at metamorphosis and tadpole behaviour in amphibians (e.g., Babbitt & Tanner, 1997; 1998; Griffiths et al., 1998; Kopp et al., 2006). This response, and similar responses to other threats, are mediated by adreno-corticosteroid or 'stress' hormones, produced by the Hypothalamus-Pituitary-Adrenal (HPA) axis (Denver, 1997b; Rollins-Smith, 1998; Hossie et al., 2010). The tadpoles in treatment B showed higher incidences of leaping behaviour than those animals maintained in treatments A and L. This is consistent with increased perceived predation risk in tadpoles that had no shelter to retreat to when disturbed; indeed, all tadpoles typically swam to the bottom of the tank initially, with A and L animals remaining hidden while B (and to a lesser extent, S) animals subsequently displayed leaping behaviour. These animals also exhibited shorter larval period duration and smaller size at metamorphosis. Together, these data support a possible role of adreno-corticosteroids in mediating increased perceived predation risk and the effects that we detected on growth and larval period duration. The energetic cost,

in terms of energy expenditure and foraging time lost, of leaping behaviour in response to both experimental and, presumably, routine disturbance may also have contributed to reduced size at metamorphosis by limiting resources available for growth. It is important to note that the behaviour described in this study is in response to mechanical disturbance and not to chemical cues, which may elicit different behavioural responses (e.g., Hossier et al., 2010) as many species tailor anti-predator behaviour to the type of threat present (Relyea, 2001; 2004).

The intermediate behavioural response in S aquaria implies that tadpoles of *A. callidryas* may identify suitable shelter on the basis of both shade and the presence of physical objects. Although it was difficult to test the presence of physical objects without shade (transparent 'plants' still create a degree of shadow), it is clear from our behavioural data that shade alone is not entirely sufficient as a refuge. Our data also show no differences in any variable measured between tadpoles in A and L treatments. This may be the result of the trophic niche of *A. callidryas* tadpoles, which largely feed on suspended food particles or easily-grazed periphyton (Gonzalez et al., 2011), and are largely incapable of feeding on tougher organic material, such as vegetation. It is possible that in herbivorous species, the provision of live, rather than artificial, plants may have a nutritional impact on tadpole development. In enclosures without filtration, live plants may also help to maintain stable, healthy water chemistry through the uptake of heavy metals, ammonia and nitrates and through the harbouring of nitrifying bacteria (Walstad, 2003).

There are no other studies, to the authors' knowledge, that directly investigate the impact of ecologically-relevant shelter provision on fitness and behaviour in captive amphibian larvae. Other studies do not allow the separation of the effect of shelter itself from the presence of predators (Babbitt & Tanner, 1997, 1998) or use shelter that is far removed from the type experienced in natural environments (Calich & Wassersug, 2012). In post-metamorphic amphibians, enrichment through shelter provision has been shown to have beneficial effects on both welfare and fitness (reviewed by Burghardt et al., 2013 and by Michaels et al., 2014b). Michaels et al. (2014a) showed that *A. callidryas* froglets provided with shelter grow faster, achieve better body condition and carry better symbiotic bacterial communities. The same authors also showed that animals actively sought out shelter and found evidence for a stress response in its absence (Michaels et al., 2014a). Our data suggest that the importance of shelter is similar in the larvae of this species and that basic behavioural drives may be maintained across metamorphosis.

In summary, our data therefore suggest that the design of the captive environment may be important in determining the fitness of captive amphibian larvae. Although our sample size is relatively small, the shared genetic background, and therefore reduced variation, of study animals improves the robustness of these results. Using siblings, however, does not allow for the detection of clutch effects, which are common in amphibians (Travis

et al., 1985; Newman, 1988; Allran & Karasov, 2001). These results are therefore, strictly speaking, limited to the clutch used in this study. However, our results illustrate that such effects of the captive environment can occur in amphibian larvae and therefore should be considered in tadpole enclosure design, particularly in conservation initiatives that must aim to maximise the fitness of animals in order to optimise the chances of success.

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All methods used in this study were non-invasive and did not require licensing under UK Law. Treatment groups fall within the normal range of 'best practice' used to maintain tadpoles in captivity. This study was approved by the University of Manchester Ethics Committee.

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