

# Density is more important than predation risk for predicting growth and developmental outcomes in tadpoles of spotted tree frog, *Litoria spenceri* (Dubois 1984)

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**Abstract** The diverse benefits of group living include protection against predators through dilution effects and greater group vigilance. However, intraspecific aggregation can decrease developmental rates and survival in prey species. We investigated the impact on tadpole development and behaviour of the interaction between population density and predation risk. Spotted tree frog (*Litoria spenceri*: Hylidae, Dubois 1984) tadpoles were kept at one of three different densities (two tadpoles per litre, five tadpoles per litre or 10 tadpoles per litre) until metamorphosis in the presence or absence of predatory cues. We aimed to determine the influence of population density, predation and the interaction of both factors in determining growth rates in tadpoles. Tadpoles were measured weekly to assess growth and development and filmed to quantify differences in activity and feeding frequency between groups. Generally, tadpoles housed without predators had longer developmental periods when housed with a predator, but there was no effect on tail length or total length. There was no effect of either predation cues or density on percentage of individuals feeding or moving. Although the effects of the presence of predators alone may appear to be less than the effects of the presence of competitors, the prioritisation of competitiveness over predator avoidance may increase vulnerability of tadpoles to the lethal threat of predators. This is particularly important in species such as *L. spenceri*, which is at risk from introduced fish predators.

**Key words:** behaviour, life history, *Litoria spenceri*, morphology, phenotypic plasticity.

## INTRODUCTION

Anuran populations are in severe decline (Wake 1991; Stuart *et al.* 2004) and face many threats, including habitat loss and modification, reducing the area in which they are able to persist (Cushman 2006). In addition, the introduction of predatory fish has had devastating effects on population numbers (Lawler *et al.* 1999; Knapp & Matthews 2000; Gillespie 2001) and creates stress that may negatively affect reproduction and growth (Werner 1986). Synergistic effects are also implicated in amphibian declines (Alton *et al.* 2010) and the interaction of environmental pressures on development, and phylogenetic restraints on morphological plasticity are integral to recruitment and population persistence. Knowledge of development and behaviour in different ecological conditions is required in order to understand the evolution of habitat choice and life-history strategy. The combination of predator effects and other stressors, such as population density, need to be studied to advance our understanding of metamorphosis, growth and timing (Relyea 2007).

The benefits afforded by being in a group can significantly increase lifespan, resource exploitation, mating opportunities, safety, cooperation and predator evasion (Alexander 1974; Clark & Mangel 1986; Baglione *et al.* 2002; Stephens *et al.* 2002; Berec *et al.* 2007). However, group living can also result in an increase in interspecific competition with increasing density of the population. In tadpoles, this is particularly important because chemical competition has strong effects on the growth and development of some individuals (Griffiths *et al.* 1991; Faragher & Jaeger 1998). Tadpoles and frogs are restricted in range occupancy and ability to travel due to their requirement for water, the risk of desiccation and limited body size and are therefore often clumped in distribution (Smith & Green 2005; Semlitsch 2008; Pittman *et al.* 2014). Within these dense groups, rates of activity are often reported to be higher in more dense groups as individuals are obliged to increase their movement in the face of competitive behaviour (Griffiths & Foster 1998) and higher densities allow for more risky behaviour because the danger of predation is diluted (Spieler 2005).

An increased rate of activity permits higher rates of feeding, but may place tadpoles at greater risk from aquatic predators. Under the risk of predation, the

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tadpoles of some species aggregate as a form of defence (Watt *et al.* 1997) and usually display reduced activity (Horat & Semlitsch 1994; Relyea 2001, but see Urban 2007), but less active tadpoles tend to be poorer intraspecific competitors. In two North American hylid frog species (*Pseudacris crucifer* and *P. triseriata*), there is a positive relationship between competitive ability and susceptibility to predation (Skelly 1995). This is reflected in differential survival of species according to their innate behaviour and the change of behaviour of tadpoles exposed to predation risk (Lawler 1989). Individuals exhibit different behaviour according to group size (Relyea 2002). High-density tadpole aggregations are a widely employed antipredator mechanism and tadpoles tend to group together to exploit 'safety in numbers' and exhibit higher movement rates in dense populations, but only when predators are not present (Spieler & Linsenmair 1999; DeVito 2003; Bateman & Fleming 2015); in the presence of a predator, tadpoles in aggregations have been observed to move less (Horat & Semlitsch 1994; Relyea 2001).

In addition to behavioural differences, tadpole morphology and developmental rates are altered by population density and predation risk. Exposure to environmental stressors such as salinity (Chinathamby *et al.* 2006; Squires *et al.* 2008; Kearney *et al.* 2012), UV radiation (Broomhall *et al.* 2000; Blaustein *et al.* 2003) and limited food availability (Leips & Travis 1994) can reduce post-metamorphic immune response (Clulow *et al.* 2015), growth and developmental rates, although later compensatory growth may be possible (Metcalf & Monaghan 2001; Squires *et al.* 2008). Predation can alter competitive outcomes (Morin 1981) and alters growth and the progression of development (differentiation), often resulting in a smaller size at metamorphosis (Benard 2004; Relyea 2007) and earlier metamorphosis (Wilbur & Collins 1973; Relyea 2007), which is suggested to provide an advantage by removing the tadpole from the dangerous aquatic habitat sooner (Wilbur & Collins 1973), which should increase fitness (Smith-Gill 1983; Berrigan & Charnov 1994). Exposure to predators can also result in a shorter body and deeper tail and tail muscle (Relyea & Werner 1999; Lardner 2000; Relyea 2001; Lane & Mahony 2002; Van Buskirk 2002), changes that are thought to increase speed of escape behaviour and act as a physical distraction to the predator (Van Buskirk *et al.* 2003).

Population density has also been shown to alter morphology and development. More interactions with others of the same species (i.e. tadpoles raised in dense groups) tend to cause greater variation in sizes of the tadpoles in the group (Griffiths & Foster 1998) and they take longer to reach metamorphosis and do so at a smaller size (Morand *et al.* 1997).

In order to understand the ecological importance of increased density resulting from environmental changes, we determined the relative importance of predation and group density on tadpole development. We investigated the effects of predation risk and population density on development and behaviour using tadpoles of the spotted tree frog (*Litoria spenceri*: Hylidae, Dubois 1984), a streamside anuran that is threatened by introduced fish species (Gillespie 2001), habitat disturbance (Tyler 1997) and chytridiomycosis spotted tree frog are found in low (Scheele *et al.* 2017). The tadpoles of the spotted tree frog are found in low-density groups in slow running streams or connected pools (Gillespie 2002), although they are not known to exhibit schooling behaviour. We predicted that (i) tadpoles exposed to predation risk would metamorphose faster and smaller than control groups, (ii) tadpoles kept at lower densities would grow faster and larger than those at higher densities, (iii) activity levels would increase with increasing group size, and (iv) tadpoles in the presence of fish would move and feed less than the control groups. As protection against predators is the most often cited explanation for animals living in groups, we predicted that the antipredator measures in the tadpoles would be reduced in the higher density group.

## METHODS

### Study species and husbandry

The spotted tree frog is medium-sized (24–52 mm snout-vent length, Tyler & Knight 2011) anuran native to upland south-east Australia (Cogger 2014) and is now restricted to rocky, fast-flowing streams between 300 and 1100 m (Gillespie & Hollis 1996). It breeds in streams, backwaters and connected pools during spring and summer (Hero *et al.* 1995) and is Critically Endangered in the wild (Global assessment, Hero *et al.* 2004), being threatened by habitat disturbance from logging, recreation, cattle (Tyler 1997), predation by introduced trout (Gillespie 2001, 2010) and chytridiomycosis, the latter of which is likely the main reason for its decline (Scheele *et al.* 2017). In the wild, the species has been documented at a mean density of 29 tadpoles per m<sup>2</sup> (range 4–60; maximum stream depth 35 cm; Gillespie 2002). Predator-naïve tadpoles were obtained from the Amphibian Research Centre within a few days of hatching, transported to the laboratory and distributed amongst treatments. All treatment containers were maintained in a temperature-controlled room (21 ± 2°C). Reverse osmosis water with 0.14 g L<sup>-1</sup> aquarium salt was used to maintain tadpoles in large plastic containers, 30 × 25 × 15 cm. The containers were placed on a windowsill, did not receive direct sunlight and were kept on ambient day/night cycle. Once a week, we performed a 75% water change. Tadpoles were fed-frozen endive *ad libitum* and aquarium fish food flakes as a protein supplement once a week to ensure nutrition did not limit growth.

Adult Eastern mosquitofish (*Gambusia holbrooki*) were collected from a pond on the Monash University, Clayton campus (37.908 S, 145.138 E), returned to the laboratory and maintained individually in plastic containers  $10 \times 10 \times 5$  cm, covered with a fine mesh net and partially submerged in the container with the tadpoles. Mosquitofish are a small fish (35–60 mm; Gomon & Bray 2011), widely introduced in Australia and known to prey upon *Litoria* tadpoles (Morgan & Buttemer 1996). Thus, although the predator could not physically interact with the tadpoles, visual and chemical cues were not obstructed. To create a chemical cue for predation, the fish were fed one crushed tadpole a week, after a water change.

### Experimental set-up

Tadpoles were separated into eight plastic containers with 4 L of water and stocked with tadpoles at one of three different densities: four containers of two tadpoles per litre each, two containers of five tadpoles per litre and two containers of 10 tadpoles per litre (9.1, 22.9 and 45.7 individuals  $m^{-2}$ , respectively). Tadpole behaviour was filmed by JVC Everio GZ-E100 camcorders suspended over the containers and sampled for 5 s every 40 mins for approximately 5 h a day for 12 days (between days 26 and 50). The number of individual tadpoles moving and the number of tadpoles feeding were recorded in each 5-s period. Where tadpoles were waving their tails when feeding, they were classified as feeding only. Tadpoles that travelled away from, over, under or through the food source for at least one tail sweep in both directions were classified as moving (and also feeding, if feeding behaviour was also observed).

Tadpoles were measured approximately 3 weeks after hatching and every week thereafter until metamorphosis. Tadpoles were removed from the water and placed on their sides in a moist Petri dish for measurement. Each individual was staged using the developmental chronology of Anstis (2013), and body length and total length were measured with a digital calliper to the nearest 0.01 mm. Tadpoles with a body length greater than 10 mm were weighed with a digital scale to the nearest 0.001 g. Metamorphosis was defined as reaching developmental stage 42 (first emergence of one or both front limbs.) After this point, it is not possible to confine the tadpoles to the water and treatment ceased and the individual was replaced with one from the replicate set-up (see below). Within 24 h of reaching stage 42, we recorded mass, body length and the length of the larval period (days elapsed since the beginning of the experiment) for each individual.

In order to maintain densities in the treatments, an identical ('backup') set-up was maintained from which to source replacement tadpoles that had been reared in the same treatments. This arguably introduces a confounding element, but we consider it similar to a natural situation where immigration into a waterbody is maintaining tadpole density at a given carrying capacity. There are few options for otherwise maintaining tadpole density as individuals metamorphose, and we consider it a reasonable approach. Additionally, for those in the identical set-up lost through natural attrition, tadpoles kept at arbitrary densities (either with or without predators) were kept as 'replacement stock'

to be added to the backup containers as any tadpoles were lost. Tadpoles from the replacement stock container had a small amount of their tail tips removed to identify them as replacement tadpoles when they were placed in the backup containers and they were not measured as part of the developmental record. Filming ceased when all backup tadpoles finished the experiments and replacement stock tadpoles were added to the experimental containers.

### Designation of landmarks

Using the developmental chronology of Anstis (2013), we selected four major morphological landmarks to measure the effects of density and predation risk on tadpole developmental rate (after Chinathamby *et al.* 2006). The landmarks were defined as: (i) appearance of hindlimb bud (Anstis stages 27–28); (ii) differentiation of toes begins (Anstis stages 31–32), (iii) differentiation of toes complete (Anstis stage 37) and (iv) emergence of forelimbs (metamorphosis-Anstis stage 42).

### Statistical analyses

Analysis of this experiment is complicated by nonindependence of tadpoles within containers. The set-up has low container replicates (two containers of two tadpoles per litre with a predator; two containers of two tadpoles per litre without a predator; one container of five tadpoles per litre with a predator; one container of five tadpoles per litre without a predator; one container of 10 tadpoles per litre with a predator; one container of 10 tadpoles per litre without a predator). However, the within-container subsamples are more substantive (per container subsamples: 2 individuals  $\times$  4 L; 5 individuals  $\times$  4 L; 10 individuals  $\times$  4 L). In a set-up like this, there will likely be substantial variation within the independent samples (i.e. containers) that can be parsed out from the container effects if the whole dataset is included in the same model. Such an approach is potentially inflating type II error, as it is likely that some of the treatment effects will be attributed to containers, but it is probably the best approach given the design. At the very least, it should reduce risks of pseudoreplication and obtaining false significances. The number of tadpoles and the number of containers are not balanced, so that a repeated-measures ANOVA would be unreliable. Therefore, a linear mixed-effects model is more sensible for the 'total length', 'tail length', 'body length' and 'mass' measures, as well as the two behavioural measures ('feeding' and 'moving'). Prior to any analysis, feeding and moving needed to be changed into percentages of tadpoles feeding and/or moving; however, percentages are seldom appropriate for linear models. Initial checks of assumptions (diagnostic plots in R) indicated this was the case here (i.e. residuals were not equally distributed and normality of residuals was questionable). Therefore, we applied a standard transformation for assumptions, the arcsine-square-root transformation, which tends to help with both normality of residuals and removes the 'bounding' of 0 and 100 that is secondary problem for percentage data. These data, along with the size measurements, were fitted to linear mixed-effects models using the 'lme' function in 'nlme' in R (although 'lme4' is somewhat mathematically preferable to 'nlme', the results of the 'nlme'

package is considered publishable, and furthermore, our preference is to opt for the mathematically simpler choice at each statistical step, given the dataset. We think that using the simplest valid statistical choice will somewhat address concerns that we might generate significance by implemented models of opaque complexity). The models used ‘container’ as a random effect and ‘sampling day’ + ‘predator’ \* ‘density’. It is possible to investigate interaction terms given the set-up only because once variance attributable to ‘container’ is removed, the leftover variance can be used to examine interactive effects based on the subsamples (although the model still restricts the degrees of freedom to limit type I error rates). We report the model with the interaction term predator:density, and where this term is nonsignificant, we have removed it to examine main effects. Opinions differ on this approach. It is argued for strongly by some authors (e.g. Engqvist 2005), whilst there are also those critical of it (e.g. Parker *et al.* 2016). The underlying rationale for removing the term is that by retaining the interaction term in a model, we are in effect asking the model to take into account a relationship that does not actually exist, and this makes the tests of the main effects unreliable. The counter-argument is that this can be viewed as a way of attempting to inflate the degrees of freedom for the main effects. In either instance, by reporting both models, a reader can decide which model they think is the more reliable. Our interpretation will tend to be of the model with nonsignificant interactions removed, with a caveat that some findings should be viewed as tentative if they were only evident in the reduced model.

Where a significant result for ‘density’ was obtained via a linear mixed effects model, we conducted a multiple comparison with Bonferroni correction (‘glht’ in library ‘multcomp’).

To check the mass at metamorphosis, we used the same approach as above, except that we only used the subset of tadpoles that had reached Landmark 4.

As for the time to reach a given Landmark stage, these data are suitable for survival analysis. Because we are interested in the time to reach Landmarks 1, 2, 3 and 4, a multiple endpoints survival analysis would be ideal, but to our knowledge no such survival analysis with mixed effects has been implemented in R. Therefore, we opted to use a non-parametric Cox’s survival analysis with mixed effects (‘coxme’ in library ‘coxme’). Parametric regression analyses (i.e. those in library ‘survival’) are also inappropriate because they also do not allow for random effects, which is essential given the experimental set-up. Conducting multiple analyses

at every measurement stage is undesirable, as this would inflate type I error, and it is unclear whether *P* value corrections (i.e. Bonferroni) would be effectual in this instance. Measurements of the entire tadpole cohort (and attribution of ‘landmark’) were conducted at days 16, 23, 30, 37, 44, 51 and 65 (allowing for some mortality). Almost all tadpoles had reached Landmark 1 by day 16. Because no measurements before day 16 were taken, a survival analysis, a survival analysis for Landmark 1 is probably not valid. Therefore, Landmark 1 was omitted from the analysis. We conducted separate survival analysis for Landmarks 2, 3 and 4, using ‘sampling day’ as a covariate, and the predictors ‘predator’ and ‘density’ and the interaction term ‘predator:density’. The container was deemed to be a random effect in the model. In the case of the survival analyses, the majority of results indicated a significant interaction; therefore, we have only presented results including the interaction term. Where density was significant, we used ‘glht’ in library ‘multcomp’ to obtain a multiple comparison of density levels using a Dunnett correct. Where the interaction term was significant, we generated a composite term (‘paste’ in core stats) that concatenated ‘predator’ and ‘density’ and checked this term using the same Cox’s mixed effects and multiple comparison approach.

## RESULTS

### Development rates

As a general trend, tadpoles reached Landmarks 2, 3 and 4 more rapidly under low (2/L) compared to medium (5/L) and high (10/L) densities (Tables 1–3; Figs 1, 2). There were no clearly convincing effects of predator presence/absence for time to Landmarks 3 and 4. A significant interaction of density and predator for Landmark 2 seemed to indicate that ‘2/L & no predator’ reached Landmark 2 significantly faster than ‘5/L & predator’ and ‘10L & predator’ but was not different to any other treatment combinations (i.e. the presence of a predator made no difference for tadpoles reaching Landmark 2 when the density was also 2/L; Table 3).

**Table 1.** Days taken (mean ± SD (range)) per experimental group of *Litoria spenceri* tadpoles to reach developmental landmarks

	Days to Landmark 1	Days to Landmark 2	Days to Landmark 3	Days to Landmark 4
Low-density control	16.0 ( <i>n</i> = 1) <sup>a</sup>	26.5 ± 8.1 (16–44) <sup>a</sup>	45.8 ± 6.2 (37–51) <sup>a***</sup>	55.1 ± 6.4 (50–65) <sup>a</sup>
Low-density predator	18.8 ± 6.3 (16–30) <sup>a</sup>	26.3 ± 6.9 (16–44) <sup>a</sup>	45 ± 6.3 (37–51) <sup>a***</sup>	57.8 ± 5.4 (54–64) <sup>a</sup>
Medium-density control	23.0 ± 7 (16–30) <sup>a</sup>	25.0 ± 8.0 (16–51) <sup>b***</sup>	48.7 ± 9.6 (37–65) <sup>b**</sup>	57.5 ± 4.6 (52–64) <sup>a</sup>
Medium-density predator	24.5 ± 7.8 (16–44) <sup>a</sup>	47.8 ± 12.7 (30–65) <sup>c***</sup>	49.0 ± 7.7 (44–65) <sup>b**</sup>	58.5 ± 3.4 (54–62) <sup>a</sup>
High-density control	19.9 ± 6.6 (16–44) <sup>a</sup>	35.2 ± 10.1 (23–65) <sup>b***</sup>	55.4 ± 8.3 (44–65) <sup>c**</sup>	60.3 ± 6.4 (53–65) <sup>a</sup>
High-density predator	22.9 ± 8.2 (16–51) <sup>a</sup>	45.4 ± 11.8 (23–51) <sup>c***</sup>	58.0 ± 11.7 (37–65) <sup>c**</sup>	61 ± 5.7 (57–65) <sup>a</sup>

The landmarks were defined as: (1) appearance of hindlimb bud (Anstis stages 27–28), (2) differentiation of toes begins (Anstis stage 31–32), (3) differentiation of toes complete (Anstis stage 37) and (4) emergence of forelimbs (metamorphosis – Anstis stage 42). Different superscript letters denote statistical differences between groups within Landmarks. \**P* ≤ 0.05, \*\**P* ≤ 0.01, \*\*\**P* ≤ 0.001.

**Table 2.** Results of nonparametric Cox's mixed-effects probable hazards models for the numbers of days to reach Landmark 2, Landmark 3 and Landmark 4 (i.e. metamorphosis)

	Coef.	Exp(coef.)	SE(coef.)	<i>z</i>	<i>P</i>
Landmark 2					
Predator (yes)	-0.06	0.94	0.35	-0.17	0.860
Density (05/L)	-0.28	0.76	0.34	-0.81	0.420
Density (10/L)	-1.10	0.33	0.30	-3.64	<0.001*
Predator (yes):density (05/L)	-1.48	0.23	0.48	-3.07	0.002*
Predator (yes):density (10/L)	-0.70	0.50	0.42	-1.66	0.098
Landmark 3					
Predator (yes)	-0.12	0.89	0.38	-0.31	0.760
Density (05/L)	-1.47	0.23	0.45	-3.30	0.001*
Density (10/L)	-1.03	0.36	0.33	-3.08	0.002*
Predator (yes):density (05/L)	-0.35	0.71	0.66	-0.53	0.600
Predator (yes):density (10/L)	-0.32	0.73	0.48	-0.67	0.510
Landmark 4					
Predator (yes)	-0.58	0.56	0.47	-1.24	0.220
Density (05/L)	-1.21	0.30	0.51	-2.38	0.017*
Density (10/L)	-2.75	0.06	0.65	-4.21	<0.001*
Predator (yes):density (05/L)	0.10	1.10	0.80	0.12	0.900
Predator (yes):density (10/L)	0.13	1.14	1.02	0.13	0.900

Note that Landmarks 2 and 3 were only assessed at set intervals (days 16, 23, 30, 37, 44, 51 and 65); however, because metamorphosed frogs could not be contained in the water, the exact day of metamorphosis was recorded for Landmark 4. Note that we have not presented any reduced models because at least one of the models indicated that the interaction term was significant. \**P* < 0.05.

**Table 3.** Where a Cox's mixed-effects probable hazards model returned significance for density (Landmarks 2, 3 and 4), or the interaction of density and predator presence (Landmark 2 only), we applied a multiple comparison test to the model

	Estimate	SE	<i>z</i>	<i>P</i>
Landmark 2				
05/L - 02/L == 0	-1.09	0.40	-2.72	0.012*
10/L - 02/L == 0	-1.51	0.38	-3.96	0.000*
Landmark 2				
Control 05/L - Control 02/L == 0	-0.28	0.34	-0.81	0.856
Control 10/L - Control 02/L == 0	-1.10	0.30	-3.64	0.001*
Predator 02/L - Control 02/L == 0	-0.06	0.35	-0.17	>0.999
Predator 05/L - Control 02/L == 0	-1.82	0.35	-5.15	<0.001*
Predator 10/L - Control 02/L == 0	-1.86	0.31	-5.96	<0.001*
Landmark 3				
05/L - 02/L == 0	-1.63	0.33	-4.93	<0.001*
10/L - 02/L == 0	-1.18	0.24	-4.84	<0.001*
Landmark 4				
05/L - 02/L == 0	-1.17	0.39	-2.99	0.006*
10/L - 02/L == 0	-2.70	0.50	-5.34	<0.001*

Pairwise error rate was controlled using a Dunnett test). The number of pairwise comparisons has been limited to comparisons of all levels with the low-density (2 tadpoles per litre) control (no predator) only. This was a restriction of the testing method and type of model. \**P* < 0.05.

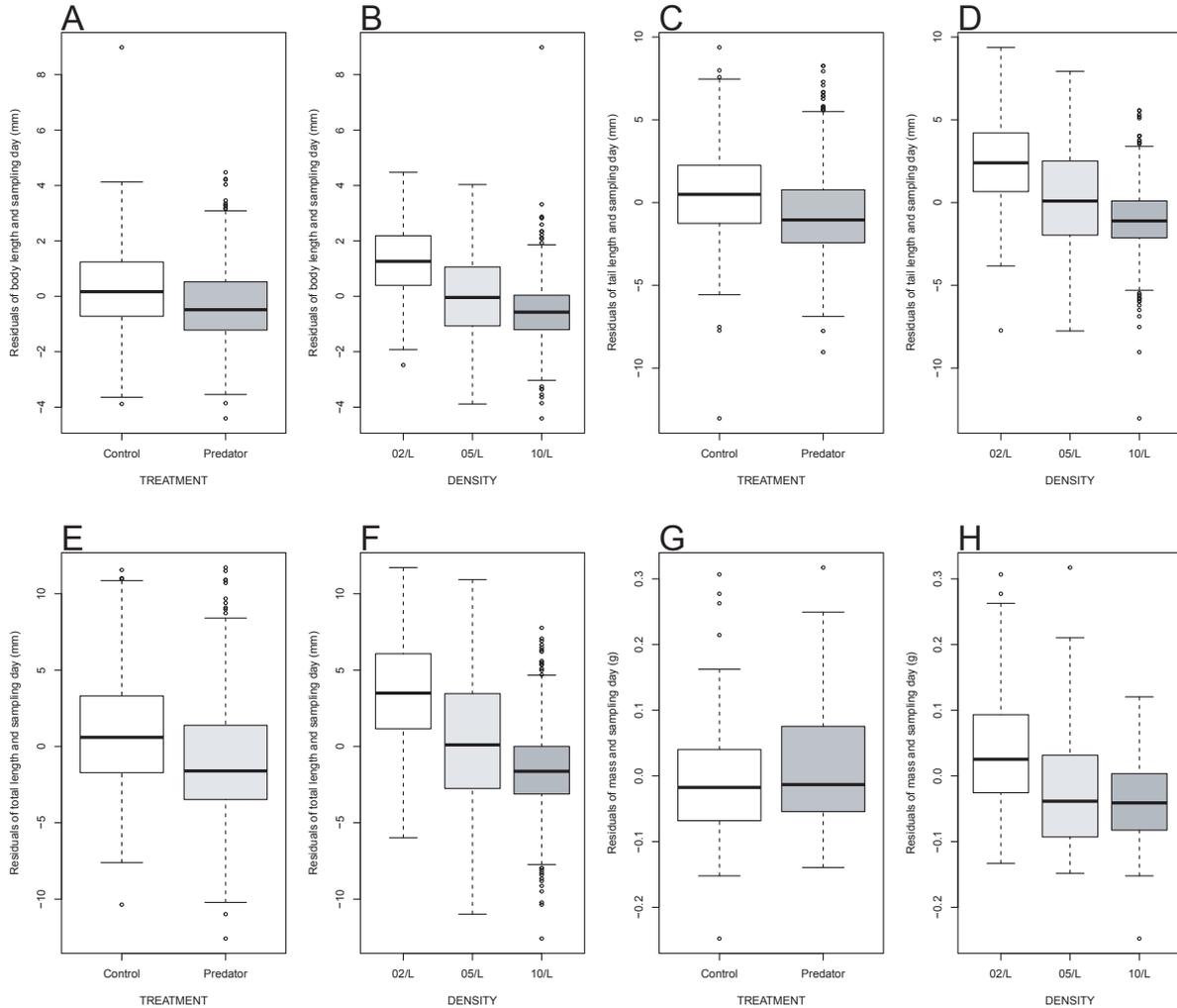
Survival amongst containers varied from 0.5 to 0.6 in low- and medium-density treatments, but was lower in high-density treatments (0.28 in the control and 0.35 in the predator treatments).

**Size**

The interaction term predator:density was never significant for any of the body size or mass measurements. Body length, total length and tail length are all related metrics and are most sensibly considered as a whole. The only effect of predator presence/absence was on body length, where individuals in control (no predator) containers had larger body sizes than individuals in predator containers (Tables 4–6). Density had a more clearly identifiable effect. For body length, 2/L > 5/L > 10/L, whereas for total length and tail length, 2/L > 5/L & 10/L (Table 5). That is, the overall trend appeared to be that of larger individuals in the less densely stocked containers. Mass, however, showed no convincing effects either for all stages of development, or the mass measured only at the point of metamorphosis (Table 5).

**Behaviour**

Neither percentage of individuals feeding, nor percentage of individuals moving was convincingly



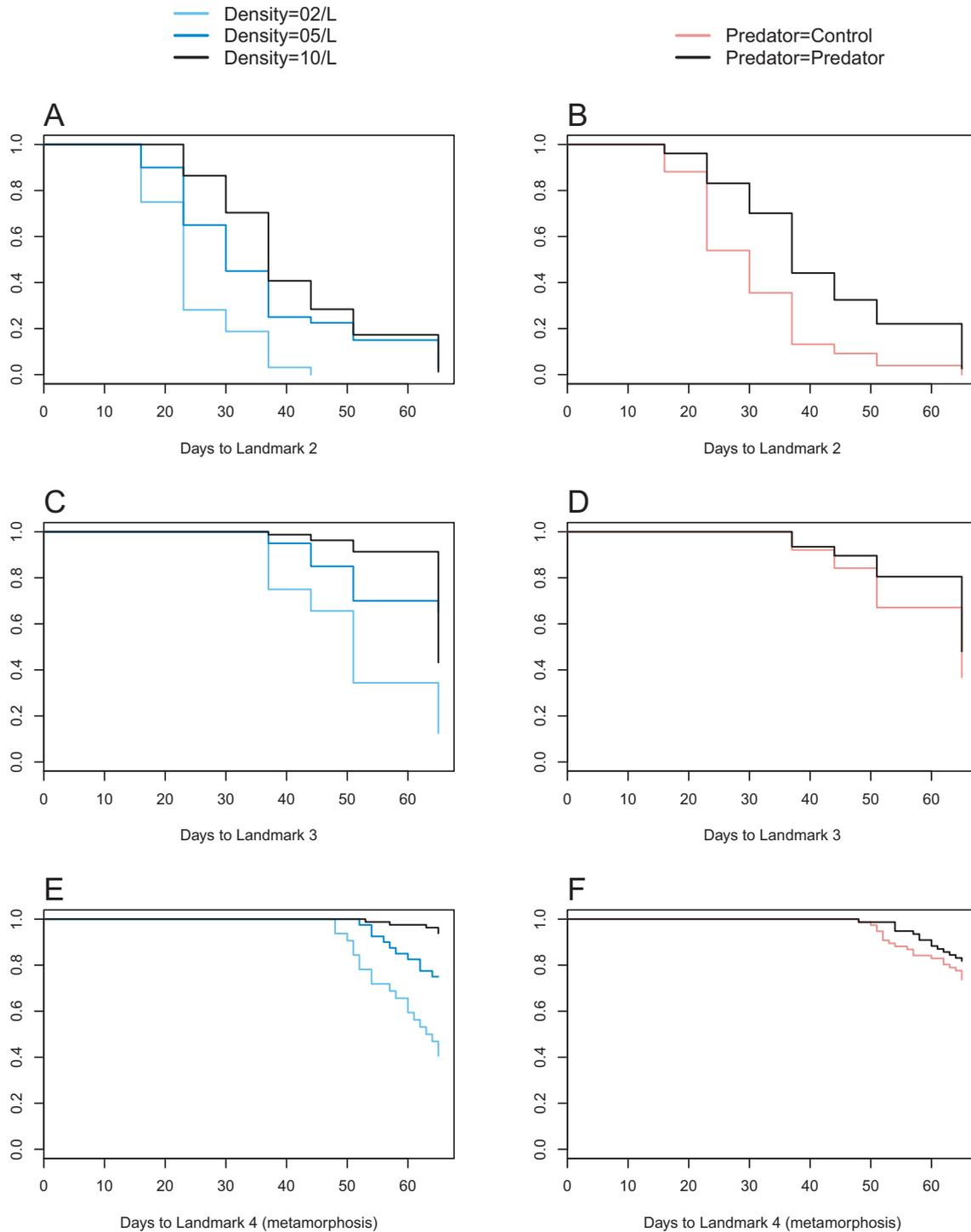
**Fig. 1.** Boxplots showing median, upper and lower quartiles, and ranges for (a) body length for predator and control groups, (b) body length for densities at tadpoles of 2, 5 and 10/L, (c) tail length for predator and control groups, (d) tail length for densities at tadpoles of 2, 5 and 10/L, (e) total length for predator and control groups, (f) total length for densities at tadpoles of 2, 5 and 10/L, (g) mass for all stages for predator and control groups, (h) mass for all stages for densities at tadpoles of 2, 5 and 10/L. Residuals of the values as a function of sampling (ordinary least squares regression) are shown as this is indicative of the actual differences that would be identifiable in a model with sampling day as a covariate. Values above zero are above the mean, and the opposite is true for values below zero. The units on the vertical axes remain the units of the measurement (i.e. either mm, or mg). \*Significant at 0.05. Letters indicate significant groupings derived from multiple comparisons of density groups. All significances based on the reduced linear mixed-effects models (i.e. those with nonsignificant interaction terms removed). Container was included as a random effect to reduce risks of pseudoreplication.

affected by any of the predator or density treatments (Tables 5, 7).

**DISCUSSION**

As predicted, tadpoles reared in the low-density group grew faster than those at higher densities. There was also evidence that tadpoles in the lower density groups grew longer. Tadpoles kept with predation cues grew somewhat longer, but not faster. This is consistent with a previous suggestion that

density-dependent competition regulates development of *L. spenceri* (Gillespie 2002). Furthermore, interference competition is the likely basis for the difference in size and growth rates, as it is often more important than exploitative competition under stress (Steinwascher 1978) such as predation or high population density. This means that the density would be more important than predation risk as density had a larger influence on the size of the metamorphs than predation in our study, suggesting that it is the more significant stressor. Tadpoles in the medium- and high-density groups took longer to reach Landmark 2



**Fig. 2.** Survival analyses plots for the tadpoles at densities 2, 5 and 10 L and predator/control treatments. (a) Days to Landmark 2 for density treatments (differentiation of toes begins (Anstis stages 31–32)), (b) days to Landmark 2 for predator treatments. (c) Days to Landmark 3 for density treatments (differentiation of toes complete (Anstis stage 37)), (d) days to Landmark 3 for predator treatments, (E) days to Landmark 4 for density treatments (emergence of forelimbs (metamorphosis – Anstis stage 42)), (e) days to Landmark 4 for predator treatments. Days to landmarks were significant for all density treatments, but no clear main effect of predator treatment was observed. A significant interaction of density and predator for Landmark 2 seemed to indicate that ‘2/L & no predator’ reached Landmark 2 significantly faster than ‘5/L & predator’ and ‘10L & predator’ but was not different to any other treatment combinations (i.e. the presence of a predator made no difference for tadpoles reaching Landmark 2 when the density was also 2/L).

**Table 4.** Mean morphological measurements ( $\pm$ SD (range)) of *Litoria spenceri* metamorphs per experimental group

	Body length at Landmark 1 (mm)	Body length at Landmark 2 (mm)	Body length at Landmark 3 (mm)	Body length at Landmark 4 (mm)	Metamorph mass (g)
Low-density control	6.21 ( $n = 1$ ) <sup>a</sup>	8.60 $\pm$ 0.74 (7.44– 9.97) <sup>a</sup>	12.13 $\pm$ 1.05 (10.62–13.49) <sup>a*</sup>	12.58 $\pm$ 0.83 (10.98–13.47) <sup>a*</sup>	0.367 $\pm$ 0.08 (0.22–0.50) <sup>a*</sup>
Low-density predator	7.32 $\pm$ 0.67 (6.57– 8.36) <sup>a</sup>	8.43 $\pm$ 0.57 (7.42– 9.43) <sup>a</sup>	11.30 $\pm$ 1.19 (9.6– 13.55) <sup>b*</sup>	12.30 $\pm$ 0.51 (11.61–13.16) <sup>a*</sup>	0.326 $\pm$ 0.04 (0.275–0.404) <sup>a*</sup>
Medium- density control	7.08 $\pm$ 0.43 (6.39– 7.44) <sup>a</sup>	8.61 $\pm$ 0.51 (7.66– 9.61) <sup>a</sup>	10.28 $\pm$ 0.67 (9.76 –11.53) <sup>b*</sup>	11.48 $\pm$ 0.86 (10.68–12.74) <sup>b*</sup>	0.271 $\pm$ 0.05 (0.23–0.35) <sup>b*</sup>
Medium- density predator	6.59 $\pm$ 0.62 (5.65– 8.26) <sup>a</sup>	8.62 $\pm$ 0.61 (7.7– 9.23) <sup>a</sup>	12.06 $\pm$ 1.09 (10.5 –13.38) <sup>a*</sup>	12.34 $\pm$ 1.04 (11.32–13.79) <sup>b*</sup>	0.351 $\pm$ 0.05 (0.3 –0.41) <sup>b*</sup>
High-density control	6.76 $\pm$ 0.63 (5.40– 8.12) <sup>a</sup>	8.35 $\pm$ 0.63 (7.16– 10.41) <sup>a</sup>	10.90 $\pm$ 0.72 (10.09–11.97) <sup>a*</sup>	11.67 $\pm$ 0.90 (10.63–12.25) <sup>c*</sup>	0.272 $\pm$ 0.05 (0.21–0.32) <sup>c*</sup>
High-density predator	6.66 $\pm$ 0.77 (4.88– 8.09) <sup>a</sup>	8.26 $\pm$ 0.70 (6.58– 9.37) <sup>a</sup>	10.71 $\pm$ 0.73 (9.71 –11.43) <sup>b*</sup>	11.36 $\pm$ 1.15 (10.55–12.17) <sup>c*</sup>	0.249 $\pm$ 0.06 (0.202–0.303) <sup>c*</sup>

Different superscript letters denote statistical differences between groups within Landmarks. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ .

if they were exposed to predation risk, as seen similarly in previous experiments (Travis & Trexler 1986) where individuals grew slower and smaller if exposed to predation and one other stressor. The low-density groups in our experiments grew faster when exposed to predators, which is predicted by life-history theory to increase fitness by escaping the risks of the aquatic predators by earlier metamorphosis (Wilbur & Collins 1973). Density was more important than the risk of predation in a study on another Hylid frog, *Scinax nasicus* (Gomez & Kehrer 2013). Variation in developmental rates (days to reach a given landmark) was mostly greater in predation groups than control groups at the earlier landmarks, but decreased or reversed by Landmarks 3 and 4. This presumably resulted because the impacts of long-term stress lessened, through acclimation and reallocation of resources to morphological responses instead of behavioural reactions, as the need to feed to survive and develop becomes imperative (Lima & Dill 1990). Predation risk was also found not to impact on feeding behaviour in a recent meta-analysis (Davenport *et al.* 2014) and may vary between populations and/or habitats (Urban 2007).

As predicted, tadpoles kept with predators were shorter in body length than control groups, but high-density groups were longer at Landmark 3 (Tables 4–6) than tadpoles kept at lower densities. Throughout most of the experiment, the low-density control group had the longest mean body size and the high-density predator group had the shortest. The responses of tadpoles to predators usually consist of a deeper tail (Relyea 2001; Lane & Mahony 2002; Van Buskirk 2002), which we did not measure, but may have been more useful in determining responses.

Tadpoles in predator treatments did not reach metamorphosis earlier than control groups, but they were smaller (at high and low densities, tadpoles exposed to predators were shorter and weighed less, but at medium density, the opposite was true). Longer larval periods often result in more prolonged exposure to introduced predators and therefore lower recruitment which likely results from the sublethal stress imposed by active avoidance of predators. This is corroborated by the reduced growth rates often seen in stressed animals (Van Buskirk & Yurewicz 1998; Relyea & Auld 2004; Kearney *et al.* 2014) and in our experiment specifically where stress interferes with metabolic processes (Glennemeier & Denver 2002) and behavioural differences, such as decreased foraging (Crespi & Denver 2004), the latter not seemingly applicable in this study. Tadpoles under physiological stress may not have the resources to metamorphose, even if it would be advantageous to do so (Loman 2002). Furthermore, although a shorter time to metamorphosis is often documented in tadpoles at risk of predation, our results were similar to others that also used caged predators (Relyea 2007). Stress hormones have a direct effect on the rate of development and pattern of growth in tadpoles in the presence of predators (e.g. Middlemis Maher *et al.* 2013), resulting in an earlier metamorphosis at a smaller body size (Denver 1997). Further studies investigating the relationship between environmental factors, hormones and behaviour are warranted (Rose 2005) especially at different population densities.

The smaller mean size of metamorphs in the high-density treatment groups is probably due to a combination of the effects of predation stress and the competitive interaction with other tadpoles. Differential

**Table 5.** Linear mixed-effects model results with one covariate (sampling day), two factors (density and predator presence/absence) and a random effect encompassing container to limit risks of pseudoreplication

Predictors	numDF	denDF	F-value	P
Body length				
(Intercept)	1	1009	6187.98	<0.001*
SAMPLING.DAY	1	1009	795.40	<0.001*
Predator	1	2	6.79	0.121
Density	2	2	22.89	0.042*
Predator:Density	2	2	0.21	0.824
Reduced model				
(Intercept)	1	1009	11494.29	<0.001*
SAMPLING.DAY	1	1009	805.21	<0.001*
Predator	1	4	13.06	0.023*
Density	2	4	42.72	0.002*
Total length				
(Intercept)	1	1009	3822.93	<0.001*
SAMPLING.DAY	1	1009	1008.69	<0.001*
Predator	1	2	4.53	0.167
Density	2	2	16.58	0.057
Predator:Density	2	2	0.63	0.615
Reduced model				
(Intercept)	1	1009	4795.07	<0.001*
SAMPLING.DAY	1	1009	1011.00	<0.001*
Predator	1	4	5.77	0.074
Density	2	4	20.86	0.008*
Tail length				
(Intercept)	1	1009	2681.06	<0.001*
SAMPLING.DAY	1	1009	863.96	<0.001*
Predator	1	2	3.34	0.209
Density	2	2	12.78	0.073
Predator:Density	2	2	0.79	0.558
Reduced model				
(Intercept)	1	1009	3029.86	<0.001*
SAMPLING.DAY	1	1009	864.90	<0.001*
Predator	1	4	3.81	0.123
Density	2	4	14.46	0.015*
Mass (all stages)				
(Intercept)	1	219	462.65	<0.001*
SAMPLING.DAY	1	219	0.50	0.482
Predator	1	2	0.15	0.735
Density	2	2	2.29	0.304
Predator:Density	2	2	0.13	0.888
Reduced model				
(Intercept)	1	219	829.75	<0.001*
SAMPLING.DAY	1	219	0.63	0.427
Predator	1	4	0.28	0.628
Density	2	4	4.10	0.108
Mass (metamorph)				
(Intercept)	1	25	264.01	<0.001*
SAMPLING.DAY	1	25	0.42	0.521
Predator	1	2	0.01	0.925
Density	2	2	1.97	0.337
Predator:Density	2	2	0.81	0.552
Reduced model				
(Intercept)	1	25	277.03	<0.001*
SAMPLING.DAY	1	25	0.41	0.527
Predator	1	4	0.01	0.919
Density	2	4	2.06	0.243
Percentage moving				
(Intercept)	1	792	1122.38	<0.001*

**Table 5 Continued**

Predictors	numDF	denDF	F-value	P
SAMPLING.DAY	1	792	4.03	0.045
PREDATOR	1	4	4.29	0.107
DENSITY	1	4	0.43	0.550
PREDATOR:	1	4	0.03	0.876
DENSITY				
Reduced model				
(Intercept)	1	792	1397.11	<0.001*
SAMPLING.DAY	1	792	4.11	0.043
PREDATOR	1	5	5.28	0.070
DENSITY	1	5	0.53	0.501
Percentage Feeding				
(Intercept)	1	792	337.00	<0.001*
SAMPLING.DAY	1	792	9.46	0.002*
PREDATOR	1	4	4.89	0.092
DENSITY	1	4	0.08	0.797
PREDATOR:	1	4	0.42	0.552
DENSITY				
Reduced model				
(Intercept)	1	792	384.17	<0.001*
SAMPLING.DAY	1	792	9.53	0.002*
PREDATOR	1	5	5.55	0.065
DENSITY	1	5	0.09	0.781

The models including the interaction term PREDATOR:DENSITY and the reduced models with the nonsignificant interaction term removed are both presented. \**P* < 0.05.

developmental rates result from the chemical inhibition of smaller tadpoles by larger individuals and the metamorphosis at a smaller size facilitates escape from unfavourable conditions for growth. The differential growth and inhibition by other individuals create different effects on individual tadpoles, expressed as some exceptionally large and some smaller individuals. The consequences of intraspecific competition

**Table 6.** Pairwise comparisons of density levels where the linear mixed-effects model results indicated significance

	Estimate	SE	z	P
Body length				
05/L – 02/L == 0	-1.24	0.21	-5.86	<0.001*
10/L – 02/L == 0	-1.84	0.21	-8.95	<0.001*
10/L – 05/L == 0	-0.60	0.22	-2.67	0.021*
Total length				
05/L – 02/L == 0	-3.42	0.87	-3.94	<0.001*
10/L – 02/L == 0	-5.33	0.86	-6.21	<0.001*
10/L – 05/L == 0	-1.91	0.97	-1.97	0.119
Tail length				
05/L – 02/L == 0	-2.18	0.68	-3.20	0.004*
10/L – 02/L == 0	-3.49	0.67	-5.19	<0.001*
10/L – 05/L == 0	-1.32	0.76	-1.72	0.196

We have taken significance at the reduced model level as the guide for presented which pairwise comparisons to present. \**P* < 0.05.

**Table 7.** Mean percentage of individual *Litoria spenceri* tadpoles that fed or moved during each 5-s video clip

	Mean ( $\pm$ SD) % of tadpoles moving	Mean ( $\pm$ SD) % of tadpoles feeding
High-density control	26.8 $\pm$ 12.7	19.1 $\pm$ 8.7
Medium-density control	23.2 $\pm$ 11.1	19.1 $\pm$ 12.5
Low-density control	30.6 $\pm$ 18.3	25.7 $\pm$ 19.3
High-density predator	21.1 $\pm$ 11.9	14.2 $\pm$ 7.5
Medium-density predator	21.42 $\pm$ 211.4	15.3 $\pm$ 10.1
Low-density predator	25.2 $\pm$ 18.6	18.03 $\pm$ 18.4

may differ between individuals (Griffiths & Foster 1998). At high densities, some tadpoles grow larger and faster at the expense of the others, through chemical interference competition whereby larger tadpoles suppress the growth of their smaller conspecifics in order to dominate available resources (Wilbur 1977; Steinwascher 1978). Thus, the apparent advantage of being a participant in the medium-density groups is likely a reflection of the skewed representation of maximum growth caused by the experiment ending after the larger tadpoles had metamorphosed, but the small tadpoles had not. As a result of the faster growth of a few individuals, the results documented here, particularly for the slower-growing high-density groups, represent only the earlier stages of development. It is possible that some of the smallest tadpoles in our experiment may never have metamorphosed at those densities, or would have metamorphosed at a larger size as they would have spent more time growing (Wilbur & Collins 1973). The number of tadpoles that metamorphose and the size of the metamorphs are both negatively correlated with population density (Brockelman 1969; Wilbur & Collins 1973), but a denser population is not a guarantee of disadvantage (Brockelman 1969; Travis & Trexler 1986), as access to nutrient-rich faeces increases with more individuals (Steinwascher 1978). This may explain the increased size at metamorphosis of the medium-density group that had a high enough density to benefit, but in low enough numbers to avoid some of the negative consequences of high densities, such as depleted oxygen levels and direct competition for food. There is also a suggestion that limited (i.e. not necessarily large groups) social exposure to conspecifics increases size at metamorphosis, even without a risk of predation (Wells 2010). Furthermore, at very high densities

metamorphosis occurs earlier (and therefore usually at a smaller size) as larval conditions are unfavourable and the terrestrial environment may be more conducive to growth (Wilbur & Collins 1973).

Neither density and predation risk, nor the interaction affected movement and feeding behaviour, suggesting that the growth rates and size were not a factor of amount of food consumed or energy used. A similar study in wood frogs (Ranidae: *Lithobates sylvaticus*) found that the tadpoles were less active in the presence of predators, but that group size made no difference to tadpole activity (Awan & Smith 2007). In another study, African clawed frogs (Pipidae: *Xenopus laevis*) also did not alter their behaviour according to group size (Golden *et al.* 2000). As these species are all found in relatively dense groups in the wild, it is possible that the densities we used in our experiment were not high enough to elicit a typical behavioural response. Alternatively, there is evidence to suggest that in some systems, amphibian larvae are more active as other selective pressures result in the atypical response, for example in high predation-risk environments with gape-limited predators (Urban 2007).

## CONCLUSION

We have identified population density as a more important factor affecting growth and development than predation risk. Although the effects of density on movement and feeding did not meet our expectations, they were the main predictors of growth and development in the tadpoles, along with the interaction between density and predation risk. Both density and predation risk were significant factors affecting growth, suggesting that the acute effects of short-term stress, such as predation risk or crowding, are more detectable than the longer-term developmental effects. Although the effects of the presence of predators alone may appear to be less than the effects of the presence of conspecific competitors, the prioritisation of competitiveness over predator avoidance may increase vulnerability of tadpoles to the lethal threat of predators. This is particularly important in species such as *L. spenceri*, which is at risk from introduced fish predators. Furthermore, other anthropogenic environmental changes that influence densities can put more pressure on populations.

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