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Enriched Diets and the Growth, Development and Survival of *Litoria moorei* (Anura) Tadpoles Reared in Captivity at Low Density

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Abstract Increasing numbers of amphibian species require conservation breeding programs for their survival. A major challenge is the efficient rearing of tadpoles, many of which require complex habitats and specialised diets. Aquatic tadpoles of the West Australian frog *Litoria moorei* were kept at low density (1 tadpole per 1.95 litres water) in aquaria at 25°C. Fed on a staple diet of boiled lettuce and leaf litter, group of diets were supplemented with either control, Wardley® Premium Spirulina Discs, Sera™ GVG-mix tropical fish food, or a combination of Wardley® Premium Spirulina discs and Sera™ GVG-mix fish food. There was a relatively high loss (i.e., found dead, euthanized due to scoliosis, or not found) of tadpoles fed with the lettuce/leaf litter alone, but this was increased significantly when supplemented with Wardley® Premium Spirulina discs, either alone or with Sera™ GVG-mix fish food , and Sera™ GVG fish food alone. However, the survived tadpoles fed on the three supplements were all heavier after three weeks and at metamorphosis than those fed with lettuce/leaf litter alone, and reached metamorphosis quicker. It is concluded that any benefit of the food supplements in terms of increasing the rate of growth and development of the tadpoles is outweighed by greater mortality. There is now a need for the efficient rearing of tadpoles, many from novel species that need complex habitats. Further studies of diet are required due to the current conservation crisis of amphibians.

Keywords *Litoria moorei*, commercial food, protein, tadpole

1. Introduction

Scientifically-based methods for the rearing of tadpoles of threatened and endangered species will help ameliorate the current conservation crisis in amphibians (Marantelli, 1999; Mendelson *et al.*, 2006). Diet is an obvious part of husbandry requiring optimisation even though it is often unclear what tadpoles actually eat in the wild (Altig *et al.*, 2007). Many species have been shown to select foods that promote the most rapid growth (Kupferberg, 1997) and an increased protein content in the diet is often associated with an increased rate of growth and development (Carmona-Osalde *et al.*, 1996; Doughty and Roberts, 2003). Although too rapid a growth rate can result in an impaired physical performance of metamorphs (Alvarez and Nicieza, 2002; Arendt, 2003). Furthermore, skeletal malformations have been seen in some culture systems (Browne *et al.*, 2003; Martinez *et al.*, 1992).

The quality of artificial diet is important for the growth, development and survival of tadpoles (Boonyaratpalin, 2001; Carmona-Osalde *et al.*, 1996; Martinez *et al.*, 1993; Martinez *et al.*, 2004; Martinez *et al.*, 1993), but the main studies on aquatic diets have involved fish aquaculture where the main goal is to maximise the yield of food rather than genetic diversity and reproduction. There have only been a limited number of studies on anuran diet mainly for the commercial production of frogs as food, with these supported by the studies in conservation breeding programmes, museums and zoos. The growth rate of fish larvae is clearly dependant upon diet as illustrated in the case of moi (*Chitala chitala,* Hamilton) (Sarkar *et al.*, 2006). The protein requirements for maxi-

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mal growth varies between fish species, such that a dietary component of 37% protein appears to give optimum growth in the black catfish (*Rhamdia quelen*) (Salhi *et al.*, 2004) but 50% gives maximal growth in the southern catfish (*Siluris meridionalis* Chen) (Fu and Cao, 2006). Growing too fast, however, can often lead to health issues (Metcalfe and Monaghan, 2003). Dietary deficiencies, particularly in vitamins and minerals, have been implicated in causing skeletal abnormalities in larval fish (Lall and Lewis-McCrea, 2007). However, hatchery-reared Senegal sole (*Solea senegalensis*) can have a high incidence of skeletal malformations (Gavaia *et al.*, 2002), possibly caused by dietary deficiencies.

An early attempt to raise *Litoria moorei* tadpoles in captivity under controlled conditions showed a 30% loss due to death and a high incidence of scoliosis (Gaikhorst *et al.*, 2008), and the scoliosis was also shown with the highly dense rearing of Green and Golden bell frog (*L. aurea*) tadpoles (Browne *et al.*, 2003). Found in the southwestern part of Western Australia (Copland, 1957), *L. moorei* is a relatively common species (Tyler *et al.*, 1994) and it serves as a good model to develop adult husbandry and tadpole rearing techniques for species with a similar lifecycle. It is a member of the *L. aurea* species-group of hylid frogs (Tyler and Davies, 1978) and, although populations of *L. moorei* appear stable, one species in this genus is already extinct and two are threatened (Mahoney, 1999). The present study has therefore used *L. moorei* as an analogue species to investigate systematically the effect of protein-rich dietary supplements upon the growth and development of tadpoles in captivity.

2. Materials and Methods

2.1 Animals Male and female *L. moorei* were collected from the grounds of Perth Zoo 31°51′33.60″S and 115°51′21.63″E, and housed in dry enclosures with a gravel substrate, small PVC hides, foliage for cover and a water bowl. The room was kept at a constant temperature of 18°C. The frogs were fed with 3–4 large crickets or woody cockroaches (*Panesthia laevicollis*) dusted with calcium carbonate precipitate twice a week. Once the females were gravid, breeding pairs were placed in glass aquaria filled with water to a depth of 15 cm (Gaikhorst *et al.*, 2008) and inspected daily for the occurrence of amplexus and the presence of eggs. Upon oviposition, the adults were removed and tadpoles kept in the breeding tank for one week to allow them to become mobile, and then they were transferred to the aquaria described below. Tadpoles were kept in aquaria at an initial density of approximately 50 tadpoles per tank (1 tadpole per 1.95 L). Any tadpoles that became sick to the extent that they could not swim or eat were euthanized by immersing in an aqueous solution of benzocaine (Ace Chemical Co, Camden Park, South Australia) at a concentration of 0.5 mg/ml.

2.2 Overall experimental design Four tadpole aquaria were set up in a room at the Perth Zoo Native Species Breeding Programme, and these were used for housing tadpoles that were fed four different diets. A replicate set of aquaria were set up in an adjacent room. Eggs were found in the breeding tank of the first breeding pair on 15th August 2007 and tadpoles selected at random were transferred to the diet aquaria one week later. Upon completion of the first set of tadpoles 13 weeks after oviposition, the diet tanks were emptied, cleaned and set up again. A second breeding pair was then put together and eggs were found on 28th November 2007. Tadpoles were again transferred to the two replicate sets of diet tanks one week later and the study closed 13 weeks after oviposition. The data from the two replicate sets of tanks for each of the two breeding pairs were combined for summary, but analysed as detailed below using mathematical modelling to avoid the problem of pseudoreplication (Millar and Anderson, 2004).

2.3 Tadpole aquaria The aquaria had glass lids and an enclosed biological filtration system, as previously described (Gaikhorst *et al.*, 2008), and a compartment containing the tadpoles held 97.5 L water. The aquaria were kept in a room held at a constant temperature of 21°C, but the water temperature was held at 25°C by 300 watt aquarium heaters. Daily maximum and minimum temperatures were recorded for all the aquaria. A 4 cm layer of eucalyptus leaf litter was placed on the bottom of the tanks to supply detritus. The leaves were collected from trees within the grounds of the Perth Zoo, frozen in a domestic freezer, then thawed and air dried to minimise the risk of disease transmission. Water quality was tested weekly using a Fresh and Saltwater Professional Liquid Test Kit (Aquarium Pharmaceuticals Inc., USA) and water changes were done at least once a week; one third of the water was drained off and replaced with aerated tap water into the front portion of the filter. Pieces of polystyrene were placed on the surface of the water for the metamorphs to climb on to and prevent drowning.

2.4 Diet From hatching to the end of the study at 13 weeks, tadpoles were kept in the presence of detritus (eucalyptus leaf litter) and fed daily with boiled/frozen lettuce (Tyler, 1999). In addition, tadpoles were fed on a supplementary diet of (a) nil which acted as a control, (b) Wardley® Premium Spirulina Discs, (c) Sera™ GVG-mix tropical fish food, or (d) a combination of Wardley® Premium Spirulina discs and Sera GVG-mix fish food. The Wardley® Premium Spirulina discs (The Hartz Mountain Corporation, New Jersey, USA) contained a minimum of 32% crude protein, and the Sera GVG-mix (Sera, Heinsberg, Germany) contained a minimum of 46.4% crude protein. The quantity of food given was determined by the staff so that it was eaten in a reasonable time with no remainder to foul the water; typically the quantity of lettuce given was eaten overnight whilst the Wardley® Premium Spirulina discs and Sera GVG-mix fish food were eaten in approximately 10 minutes.

2.5 Measurements Daily minimum and maximum temperatures of each tadpole aquarium were kept, and the average of these two values used as a factor in the modelling. All tadpoles were caught 3 weeks after the eggs were laid and weighed individually using an analytical balance (Ohaus, Australia). From week 9, development was monitored daily by looking for the presence or absence of hind and fore limbs. Hind limbs were judged as present when the digits were clearly visible by the naked eye from the bud (approximately Gosner stage 38), but only erupted fore-limbs were counted (Banks and Leyden, 1990). Metamorphs were removed and weighed on the analytical balance. The date was recorded for any tadpole found dead or euthanized. The study was terminated 13 weeks after egg release.

2.6 Statistical analyses Descriptive statistics are presented as median and 1st and 3rd quartiles for weight at 3 weeks and metamorphosis and time to metamorphosis, and as counts and percentages for categorical variables.

A linear mixed effects model (Pinheiro and Bates, 2000; Pinheiro *et al*., 2009) was used to evaluate tadpole weight at three weeks and at metamorphosis, against the available covariates. Separate intercepts for each breeding pair were fitted in order to reflect the different weight results within each pair. The anticipated correlation among the weights of tadpoles from the same breeding pair was adjusted for by including a compound symmetry correlation structure within the model, which allows for uniform correlation between the tadpoles from the same pair. Explanatory variables included in the model were age at metamorphosis, diet, aquarium location and temperature (average of minimum and maximum daily temperatures). Weight was log-transformed to normalise the error distribution of the linear mixed model; consequently, differences in the weight response were interpreted in multiplicative terms, as a median fold increase. The same linear mixed effects approach (without transformation of the response) was used to evaluate the differences in mean time to metamorphosis under the different diets.

Death rates in three weeks were compared across diets by constructing a permutation distribution against which to compare the test statistic from a standard χ^2 test of the counts in each diet group. As observations were not independent, this bootstrap method made it possible to avoid any bias introduced by the correlations within the breeding pairs of tadpoles. The distribution of the test statistic was constructed from 10000 random permutations of the data.

Death and metamorphosis rates by the end of the study were assessed via logistic regression modelling. A Generalised Linear Mixed Effects Model (Bates and Maechler, 2009) was used to generate odds ratios of each outcome under the different diets. This model performs logistic regression for a binary response in the usual manner, but additionally allows the fitting of separate intercepts and slopes for explanatory variables that require this, and allows for the incorporation of a correlation structure for non-independent observations, as described in the previous paragraph. Odds ratios were adjusted for diet, breeding pair and aquarium location.

The times to metamorphosis and death were analysed simultaneously with non-parametric competing risks survival analysis methods(http://cran.r-project.org/web/packages/survival/index.html), allowing for right-censored data. While the event of interest is metamorphosis, there are more deaths than successful metamorphoses, which may skew the final assessment of metamorphosis incidence if the former are treated as censored observations. A competing risks model balances these two outcomes.

A 5% significance level was used in all analyses, which were performed in the R statistical computing environment, version 2.9.1 (http://www.r-project.org/).

3. Results

The development and survival of tadpoles throughout the course of the study up until the close at weeks 13 is shown in Table 1. The proportions of tadpoles that did not survive differed across the four dietary groups. The rate of loss for the control group was lowest at 60% compared with the highest rate of 78% for the spirulina plus fish food diet. The results of logistic regression analysis in Table 2 indicate that after adjusting for breeding pair, aquarium location and mean temperature, the odds ratio of death were significantly lower in the control group compared with the groups supplemented with spirulina, either alone or in combination with fish food (both $p<0.001$), but not significantly different from the group supplemented with fish food only ($p=0.163$). The odds ratio of death was approximately 3 times greater under the spirulina-supplemented diets compared to the control group (ORs 2.9, 95% CI 1.8–4.9 and 3.0, 95% CI 1.8–4.9). The odds ratio of death in the two spirulina-supplemented groups was also significantly (approximately two-fold) higher than in the Sera™ GVG-mix fish food only group ($p=0.004$ and 0.006).

Some loss of tadpoles had occurred even at 3 weeks, and there were significant differences between the groups ($p<0.001$). The death rate was higher than the lettuce only control group (2.1%) for all groups receiving dietary supplements (all $p<0.005$), namely, Wardley® Premium Spirulina discs alone (18.0%) or with Sera™ GVG-mix fish food (17.2%), or with Sera™ GVG-mix fish food alone (7.7%). The loss seen with the Wardley® Premium Spirulina discs, either alone or with the Sera™ GVG-mix fish food, was also higher than for the Sera™ GVG-mix fish food (both $p<0.005$).

Among the tadpoles surviving to 3 weeks, there were significant differences in weight between the groups (Tables 3 and 4) ($p<0.001$). From a linear mixed effects analysis adjusting for aquarium location, mean temperature and differences between breeding pairs, it was found that compared to the tadpoles on no diet supplement, median tadpole weight at 3 weeks was 3.4 (95% CI 3.1–3.7) times greater on the fish food diet, 6.1 (95% CI 5.5–6.8) times greater on the spirulina diet and 4.8 (95% CI 4.4-5.3) times greater on the mixed diet. There were also significant differences between median weights of the tadpoles in the two groups fed with the Wardley® Premium Spirulina discs, alone or with Sera™ GVG-mix fish food, and both the groups were significantly heavier than those fed with Sera™ GVG-mix fish food alone (all $p<0.001$).

Of the 30% of tadpoles that survived the full study period, the rate of metamorphosis was 90%. After adjusting for breeding pair, aquarium location and mean temperature, the odds ratio of metamorphosis within the full study group was 45%–49% lower in the groups supplemented with spirulina compared with the control group ($p=0.008$ and 0.012), and also 42% lower for the spirulina only group when compared with the fish food only group ($p=0.028$). The odds ratio of metamorphosis was not significantly different between the control and fish food fed tadpoles ($p=0.620$), nor between the two spirulina supplemented diets ($p=0.844$).

Figure 1 illustrates the *cumulative* incidence of both death and metamorphosis events throughout the study, and clearly shows that the times to each of these events are accelerated under the supplemented diets. The cumulative incidence of death outcomes outweighs metamorphosis for all diet groups; however, the spirulina and

Table 1 The development and survival of *L. moorei* tadpoles fed on leaf litter and lettuce supplemented with either nil (control), Wardley Premium Spirulina discs, Sera GVG-mix or Wardley Premium Sprirulina discs plus Sera GVG-mix.

Fate	Lettuce/leaf litter with				Total
	Control	Wardley Premium Spirulina discs	Sera GVG-mix	Wardley Premium Spirulina discs + Sera GVG-mix	
Found dead in tank	4 (2%)	41 (21%)	3 (2%)	54 (27%)	102 (13%)
Euthanized	52 (27%)	77 (40%)	108 (55%)	79 (40%)	316 (40%)
Not found	61 (31%)	34 (17%)	17 (9%)	22 (11%)	134 (17%)
Remaining tadpole	15 (8%)	0 (0%)	5 (2%)	0 (0%)	20 (3%)
Metamorphosis	63 (32%)	43 (22%)	62 (32%)	43 (22%)	211 (27%)
Total	195	195	195	198	783

Table 2 The odds ratios of metamorphosis and death under each diet, for the supplemented diet groups compared to the control group.

	Metamorphosis		Death	
Diet	Odds ratio (95%CI)	*p*-value	Odds ratio (95%CI)	*p*-value
Spirulina	0.51 (0.31, 0.84)	0.008	2.95 (1.78, 4.87)	<0.001
Fish food	0.88 (0.54, 1.44)	0.620	1.41 (0.86, 2.30)	0.163
Mixed	0.54 (0.33, 0.87)	0.012	2.97 (1.81, 4.86)	<0.001

Table 3 Time to metamorphosis, tadpole weight at 3 weeks and metamorph weight for the different diets. Results are presented as median (1st and 3rd quartiles).

Parameter	Lettuce/leaf litter supplemented with			
	Control	Spirulina	Fish food	Mixed
Weight at 3 weeks (g)	0.08 (0.06, 0.10)	0.34 (0.22, 0.61)	0.27 (0.13, 0.53)	0.33 (0.24, 0.50)
Weight at metamorphosis (g)	3.6 (3.0, 4.8)	7.1 (5.3, 8.7)	4.0 (3.5, 4.7)	6.8 (5.2, 7.8)
Time to metamorphosis (days)	80 (75, 87)	58.0 (53, 64)	64 (58, 70)	56 (51, 59)

Table 4 Regression analyses of tadpole weight at 3 weeks, and metamorph weight and time to metamorphosis: Fold increase for weight corresponds to the regression coefficient of the explanatory variable when weight is converted back from the log-transform to its original scale (e^{β}).

	Weight at 3 weeks		Weight at metamorphosis		Time to metamorphosis	
Explanatory variable	Fold increase (95%CI)	*p*-value	Fold increase (95% CI)	*p*-value	β (95% CI)	*p*-value
Spirulina	6.14 (5.54, 6.81)	<0.001	2.19 (1.86, 2.57)	<0.001	−21.9 (−25.1, −18.7)	<0.001
Fish food	3.37 (3.08, 3.68)	<0.001	1.23 (1.07, 1.40)	00.004	−16.5 (−19.3, −13.6)	<0.001
Mixed	4.82 (4.40, 5.29)	<0.001	2.11 (1.77, 2.52)	<0.001	−26.2 (−29.4, −23.0)	<0.001
Age	–	–	1.01 (1.00, 1.01)	<0.001	–	–
Location	1.06 (0.99, 1.14)	00.091	0.99 (0.90, 1.10)	00.910	−1.39 (−4.02, 1.23)	00.298

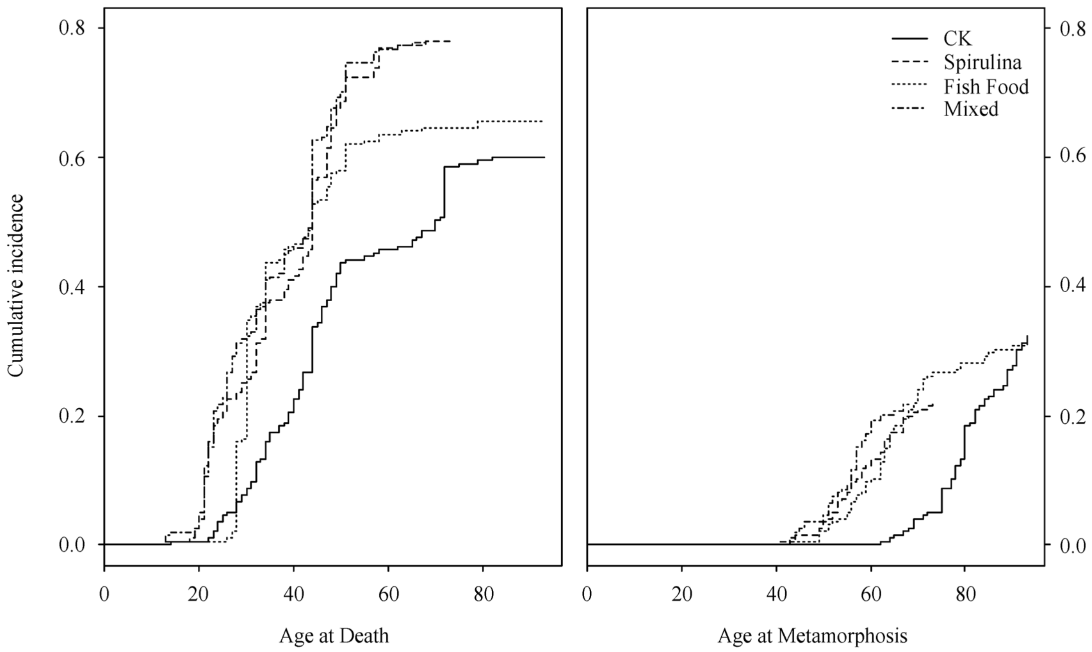

Figure 1 Cumulative incidence of death (left) and metamorphosis (right) under the 4 diets. The legend applies to both graphs.

mixed diets have a greater incidence of death (78%) than the nil and fish food diets (60% and 66%). In contrast, the cumulative incidence of tadpole metamorphosis is a little higher on the control and fish food diets (32%) than on the spirulina and mixed diets (22%).

Notwithstanding the reduced chances of reaching metamorphosis, time to and weight at metamorphosis were significantly different under diet supplementation compared to the control group (Tables 3 and 4). The overall median time taken to reach metamorphosis was 66 days, and the median weight of tadpoles at that point was 5.1 g. Within diet groups, the median time to metamorphosis ranged from 56 days for the tadpoles on the mixed diet to 80 days for the tadpoles on the lettuce only diet. After adjustment for breeding pairs, aquarium location and temperature within a linear mixed effects model (Table 4), this mean time was found to be significantly lower under all supplemented diets when compared with the lettuce only diet (all $p<0.001$), with mean decreases (95% CI) of 16.5 (13.6, 19.3) days for the fish food diet, 21.9 (18.7, 25.1) days for the spirulina diet and 26.2 (23.0, 29.4) days for the mixed diet. Aquarium location was not found to be a significant predictor of time to metamorphosis ($p=0.298$).

Significant differences in weight at metamorphosis were found between the supplemented and lettuce only diet groups (all $p<0.005$, see Table 4), after accounting for age at metamorphosis, breeding pair, aquarium location and temperature. The group fed on Wardley® Premium Spirulina discs alone was associated with a two-fold median increase in weight compared with controls (median fold change 2.2, 95% CI: 1.9–2.6), as was the mixed diet group (median fold change 2.1, 95% CI: 1.8–2.5). Median weight for both groups was also significantly higher than the fish food group, with fold increases of 1.8 (95% CI: 1.6–2.0) and 1.7 (95% CI: 1.5–2.0). Median tadpole weight on the fish food diet was associated with a very modest fold increase of 1.2 compared with the lettuce only diet (95% CI 1.1–1.4). Age was a significant predictor of weight at metamorphosis ($p<0.001$), while aquarium location was not ($p=0.910$).

4. Discussion

Tadpoles are known for their plasticity of development which allows them to cope with a changing habitat and thereby maximise survival (Denver *et al*., 1998; Doughty and Roberts, 2003). When presented with competition and drying ponds, some species choose to eat foods that favour growth (Richter-Boix *et al*., 2007) including carnivory and cannibalism (Tejedo, 1991). Factors such as diet and temperature are often linked in affecting phenotypic plasticity, for example, by temperature influencing intestinal morphology and physiology (Castaneda *et al*., 2006). Genetic divergence between separated populations can also affect the growth rate of tadpoles in response to environmental factors (Uller *et al*., 2002). The present study was therefore conducted in a controlled environment using animals from one location in breeding tanks and aquaria designed for systematic study (Gaikhorst *et al*., 2008) in an attempt to restrict the effects seen to the diet, with variables controlled including temperature, population density and staple diet.

Boiled lettuce has been found to support the growth and development of tadpoles from a number of species (Tyler, 1999). However, preliminary work with our rearing system using lettuce alone noted approximately 30% *L. moorei* tadpoles dying of natural causes or were euthenised when they developed scoliosis (Gaikhorst *et al*., 2008). We therefore investigated the relative merits of dietary supplementation on the growth, development and survival of *L. moorei* tadpoles. The need for such supplementation was suggested by a number of previous reports in a range of species, showing (i) a leafy diet can cause slow tadpole growth, delayed metamorphosis and body deformities (Mohanty and Dash, 1986); (ii) lettuce gives the poorest growth compared to other diets (Hirschfeld *et al*., 1970; Martinez *et al*., 1994); (iii) dietary deficiencies have been implicated in causing skeletal malformations (Martinez *et al*., 1992); and (iv) different species have varying protein requirements (Carmona-Osalde *et al*., 1996; Kupferberg, 1997). The two foods chosen (Wardley® Premium Spirulina Discs and Sera™ GVG-mix fish food) were selected for their ease of supply, their high crude protein content (both >32%), and a range of other ingredients thought to possibly be beneficial (e. g., vitamins). The tadpoles fed with the other diets compared to those on lettuce/leaf litter alone had a shorter metamorphosis time, a greater metamorph weight, and greater mortality. The high mortality appeared associated with spirulina in the diet. However, it did come as a great surprise that there was an increase in mortality with any of the dietary supplements, but the cause is unclear. A dietary deficiency seems unlikely given the complexity of the supplementary foods compared to lettuce/ leaf litter alone, and yet regular water changes ensured that waste products such as nitrates and nitrites were kept at low levels as confirmed by the testing of water quality.

Rana catesbeiana tadpoles have been shown to easily digest ingredients of plant origin whereas blood meal and rice bran were not so digestible (Secco *et al*., 2005), and *Rana perezi* Seoane larvae show that fresh diets are digested most readily although tadpole growth is slowest (Martinez *et al*., 1993). However, the tadpoles fed on compound or compound/fresh mixed diets rich in protein, fats and carbohydrates gave the shortest metamorphosis period (Martinez *et al*., 1994; Martinez *et al*., 1993). They also had the highest rate of skeletal abnormalities when fed on the compounded diets at 22–25°C (Martinez *et al*., 1993). In addition, different but related species may respond differently to enriched food as shown by Buchholz and Hayes (2000) who found that the spadefoot toad *Scaphiopus couchi* did very poorly on high protein diet (fish food) but their congener, *Scaphiopus multiplicate*, performed best on this diet. The increased mortality in the present study with diets other than the lettuce alone shows that complex interactions can occur among habitats, diets and tadpole survival. Whilst compound diets may be generally regarded as beneficial (Martinez *et al*., 1992), the varied habitats and dietary needs of tadpoles may require specific dietary compositions and improve carriers to prevent leaching. Good water quality is essential when amphibians are raised (Odum and Zippel, 2008), and the possibility of increasing nitrogenous waste as a consequence of the increased protein content of the dietary supplements is considered. Whilst the testing of the water reveales low levels of ammonia, nitrites and nitrates, other toxic compounds such as various metals (Andren *et al*., 1988; Wall, 1999) not tested for cannot be excluded. We did not test the tadpoles or water for pathogens and similarly their effect on tadpole survival cannot be discounted.

In conclusion, any benefit of the food supplements in terms of increasing the rates of growth and development of the tadpoles is outweighed by greater mortality. There is now a need for the efficient rearing of tadpoles, many from novel species that need complex habitats, and further studies on diet are required due to the current conservation crisis.

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