

# The effect of food availability on phenotypic plasticity and phenotypic integration in the hyloid frog *Hypsiboas pulchellus*

Magdalena Carabio, Gabriel Perazza, Fernando Larrañaga  
and Daniel E. Naya

*Departamento de Ecología y Evolución, Facultad de Ciencias, Universidad de la República,  
Montevideo, Uruguay*

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## ABSTRACT

**Background:** Changes in food availability usually have an effect on the fitness-related traits of anuran larvae, such as body shape, developmental rate, and body size.

**Organism:** *Hypsiboas pulchellus*, the South American common tree frog.

**Aim:** Analyse the effect of food availability on larval survival, developmental rate (Gosner stage at the end of the experiment), body size (body mass and total length), body shape (the ratio between body length and total length), and intestine size (gut dry mass and gut length).

**Methods:** We exposed tadpoles of *H. pulchellus*, all from a single clutch, to different levels of food availability. We set up eight food availability groups of five individuals each. After 39 days, we measured all phenotypic variables studied. We used linear and logistic regressions to analyse the data. We also used a structural equation modelling approach to evaluate the likelihood of ten causal models of covariation among the phenotypic traits (derived from four major biological hypotheses).

**Results:** Three traits were observed to vary positively with food availability: developmental stage, body size, and gut size. Tadpole survival did not change with food availability. The results of structural equation modelling indicate that: (1) food availability acts directly on body size and gut size, (2) body size directly affects developmental rate, and (3) developmental rate acts directly on body shape.

**Conclusions:** The present study supports the idea that anuran larvae are strongly affected by food availability. It also illustrates how structural equation modelling can increase our understanding of phenotypic integration among several traits that respond to changes in food availability.

**Keywords:** competition, food availability, larvae, path analysis, phenotypic integration, phenotypic plasticity, tadpoles.

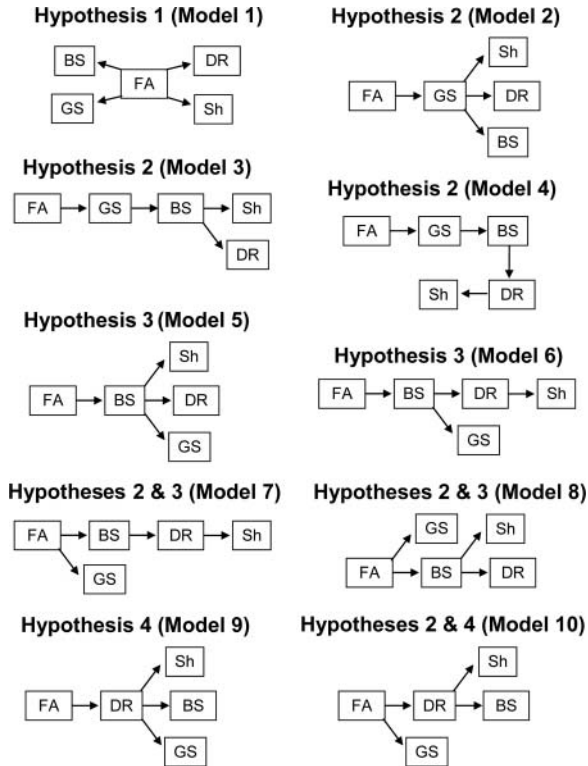
## INTRODUCTION

Phenotypic plasticity is defined as the ability of a genotype to produce different phenotypes in response to changes in internal or external environmental conditions (Pigliucci, 2001; Piersma and van Gils, 2010). Phenotypic plasticity is a widespread phenomenon in nature, and several lines of evidence suggest that it usually has an adaptive value (Naya *et al.*, 2009). Many studies of phenotypic plasticity have been conducted in aquatic animals, especially ones with complex life cycles (Withman and Ananthakrishnan, 2009). These studies have shown that plasticity at the larval stage is important because it not only affects individuals' performance in the aquatic environment (Laforsch and Tollrian, 2004; Benard, 2006), but also performance during the later terrestrial phase of the life cycle (Gomez-Mestre *et al.*, 2010; Crespi and Warne, 2013).

Anuran larvae, in particular, are widely used as a biological model for the study of phenotypic plasticity (Miner *et al.*, 2005; Ledon-Rettig and Pfennig, 2011). Current evidence indicates that anuran tadpoles exhibit considerable plasticity in diverse traits (e.g. gene expression profile, morphology, physiology, behaviour, life history) in response to changes in different abiotic and biotic environmental factors, such as ambient temperature (e.g. Walsh *et al.*, 2008; O'Regan *et al.*, 2014), hydroperiod (e.g. Lind and Johansson, 2007; Gomez-Mestre *et al.*, 2013), food quality (e.g. Liess *et al.*, 2013; Stoler and Relyea, 2013), water quality (e.g. Barth and Wilson, 2010; Katzenberger *et al.*, 2014), intensity of competition (e.g. Relyea and Hoverman, 2003; Relyea and Auld, 2004), predation pressure (e.g. Relyea and Hoverman, 2003; Orizaola *et al.*, 2012), and parasitism (e.g. Marino *et al.*, 2014; Preston *et al.*, 2014). In the case of competition, it is known that an increase in tadpole density and/or a decrease in the *per capita* amount of food will usually result in changes in body shape, developmental rate, and body size at metamorphosis (e.g. Crump, 1981; Kehr, 1989; Relyea and Hoverman, 2003; Morey and Reznick, 2004; Enriquez-Urzelai *et al.*, 2013; Searcy *et al.*, 2015). However, information on the effect of changes in competition intensity on other phenotypic traits that can also affect tadpoles' performance is limited. For instance, to our knowledge, only one study has analysed the effect of competition on the characteristics of the digestive system (Relyea and Auld, 2004), a model system for the study of phenotypic flexibility in vertebrates (Secor and Diamond, 1998; Naya *et al.*, 2007; Karasov *et al.*, 2011). In addition, no study has explored the causal relationships between traits that respond plastically to changes in tadpole density and/or food availability.

Within this general context, the aim of the present study was to assess the effect of food availability on the survival, developmental rate, body size, body shape (defined as the ratio between body length and overall length), and intestine size of tadpoles of the South American common tree frog (*Hypsiboas pulchellus*). In addition, using a structural equation modelling approach we analysed the causal relationship between food availability and the above phenotypic variables. Specifically, we wished to evaluate different causal models derived from four major biological hypotheses.

The first, the null hypothesis, states that food availability acts independently on developmental rate, body size, body shape, and intestinal size (Model 1 in Fig. 1). The second hypothesis is based on the fact that the digestive system usually represents the functional link between food intake and metabolizable energy (Secor, 2001). Thus, it states that an increase in food availability directly affects gut size, which, in turn, affects developmental rate, body size, and body shape (Models 2–4 in Fig. 1). The third hypothesis is derived from the mass threshold hypothesis (Wilbur and Collins, 1973), and posits that changes in body mass 'drive' changes in all the other variables. That is, food availability has a direct effect on body mass, which, in turn, affects gut size, developmental rate, and (then) body shape (Models 5 and 6 in Fig. 1). Note that a combination of the second and third hypotheses is also possible,



**Fig. 1.** Path diagrams showing the ten causal models tested in the present study. FA = food availability, GS = gut size, BS = body size, DR = developmental rate, Sh = body shape.

whereby food availability acts directly on gut size and body size, while body size acts directly on developmental rate and (then) body shape (Models 7 and 8 in Fig. 1). The fourth hypothesis states that developmental processes lead to changes in body size, body shape, and intestine size – that is, food availability directly affects developmental rate, which, in turn, affects gut size, body size, and body shape (Model 9 in Fig. 1). Finally, a combination of the second and fourth hypotheses is also possible, whereby food availability directly acts on gut size and developmental rate, while developmental rate acts directly on body size and body shape (Model 10 in Fig. 1).

## MATERIALS AND METHODS

### Experimental design

Widely distributed in South America, the hylid frog *Hypsiboas pulchellus* inhabits temporary and permanent water bodies, reproducing all year around (Canavero *et al.*, 2008). In late July 2009, an egg mass of this species was collected from Barra Grande, Rocha, Uruguay (34°28'S, 54°19'W) and brought to the aquarium of the Faculty of Sciences (Montevideo, Uruguay), where the eggs were reared until the experiment began. Given that tadpoles'

plastic responses may be affected by clutch identity due to genetic or maternal effects (Gibbons and George, 2013; Bennett and Murray, 2014), we used individuals from a single clutch in order to reduce potential sources of variation among experimental units. Seventy-two hours after hatching (on 13 August 2009), 40 individual larvae were randomly assigned to one of the eight experimental treatments (i.e. five individuals in each treatment). These experimental groups were established by manipulating food availability: 3.0, 5.0, 8.0, 10.0, 15.0, 20.0, 25.0, and 30.0 mg of standard fish food (Hikari®) supplied every 3 days. Then, larvae were individually reared in two-litre pools, at a temperature of 20°C and photoperiod of 12 hours light/12 hours dark. To reduce pool pollution, 25% of the water was changed every 3 days. The experiment ended after 39 days (20 September 2009), the average time taken by *H. pulchellus* to metamorphose in spring (Kehr and Basso, 1992).

At the end of the experiment, each tadpole was sacrificed with an overdose of benzocaine and two photographs (side and dorsal views) were taken with a digital camera (Nikon Digital Sight DS-5MC) to determine body length and total length of each individual. Body shape was calculated as the ratio between body length and total length. Then, the body mass of tadpoles was measured using an analytical balance ( $\pm 0.0001$  g; AND HR-200), and their developmental stage was determined according to Gosner (1960). We dissected each tadpole and measured the length of its intestine using a plastic ruler ( $\pm 0.1$  cm). The contents were then removed and the intestine was washed and rinsed using a 0.9% NaCl solution. Finally, the intestine and carcass were individually weighed ( $\pm 0.0001$  g), dried in an oven until of a constant weight (4 days at 60°C), and then re-weighed ( $\pm 0.0001$  g).

Collection of specimens was conducted in a public space, outside of protected areas, and involved a non-endangered species. División Fauna, MGAP, Uruguay (Permit #18/09) approved animal collection, while the procedures of the study conformed to national and institutional guidelines for research on live animals. The experimental protocol was approved by Comisión de Ética de Facultad de Ciencias, Udelar, Uruguay (Permit #05-02-08). All data used in the analyses are available online (Table S1, [evolutionary-ecology.com/data/3053Appendix.xlsx](http://evolutionary-ecology.com/data/3053Appendix.xlsx)).

### Data analysis

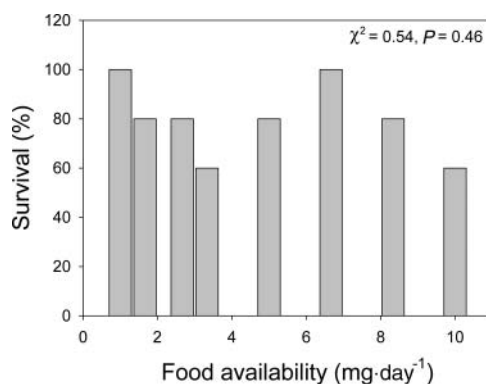
The relationship between food availability and larval survival was examined using a logistic regression. Relationships between food availability and all the other phenotypic variables were evaluated separately by means of the Pearson product-moment correlation coefficient. To make comparisons among the ten statistical models described above (Fig. 1), we used a structural equation modelling approach (Shipley, 2000). In these analyses, carcass dry mass was included as a proxy of body size in order to avoid a part-to-whole statistical problem between body mass and intestine mass (note that the correlation between body mass and carcass dry mass was very high:  $r = 0.99$ ,  $P < 0.0001$ ). We used a maximum likelihood method to estimate the general fit of each model as well as model parameters. The significance of each model was assessed using the  $\chi^2$  statistic, which compares the fit between the observed and predicted elements of the covariance matrix (Shipley, 2000). A significant  $\chi^2$  value means that the predicted model is not supported by the data. In the case that more than one model could not be discarded using this criterion (i.e. its  $\chi^2$  value), we used the root mean square error of approximation (RMSEA) index for model selection. RMSEA values below 0.06 are considered to indicate a good fit of the model to the data, while values above 0.1 indicate a poor fit of the model to the data (Hu and Bentler, 1999). Statistical significance was

set at 0.05 and all analyses were performed using the General Linear Models and Structural Equation Modelling modules of the statistical package Statistica v.7.0.

## RESULTS

Thirty-two of the 40 experimental animals survived the experiment, with mortality being evenly distributed among the treatments (Fig. 2). Developmental stage, body mass, total length, body length, gut dry mass, and gut length were all positively correlated with food availability (Fig. 3). In contrast, the ratio between body length and total length (i.e. body shape) was negatively correlated with food availability ( $r = -0.71$ ,  $P < 0.00001$ ).

The results of the structural equation modelling indicate that, based on  $\chi^2$  values, one model derived from our third hypothesis (Model 6) and two models derived from the combination of our second and third hypotheses (Models 7 and 8) were supported by the data (Table 1). In addition, the RMSEA values indicate that Model 7 was better

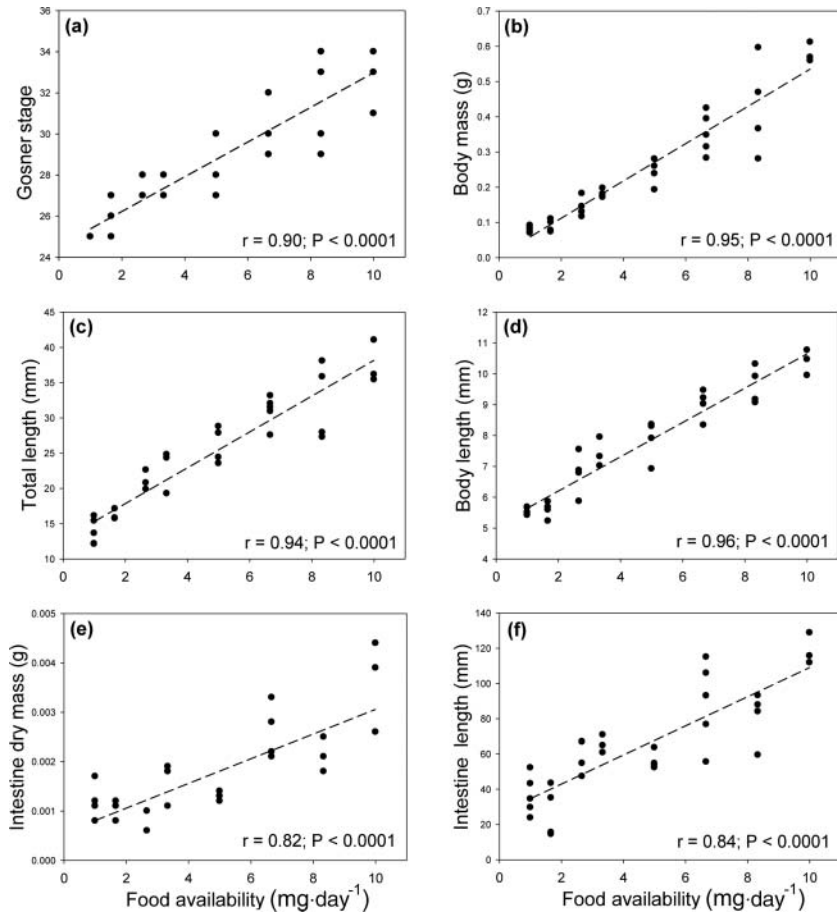


**Fig. 2.** Percentage of individuals that survived the experiment by treatment group.

**Table 1.** Chi-square goodness of fit ( $\chi^2$ ), degrees of freedom (d.f.), and associated probability ( $P$ -value), together with the root mean square error of approximation (RMSEA) index, for each causal model evaluated

Model	Paths	$\chi^2$	d.f.	$P$ -value	RMSEA
1	FA → DR; FA → BS; FA → DR; FA → Sh	35.6	6	<0.00001	0.304
2	FA → GS; GS → BS; GS → DR; GS → Sh	112.0	6	<0.00001	0.704
3	FA → GS; GS → BS; BS → DR; BS → Sh	47.7	6	<0.00001	0.372
4	FA → GS → BS → DR → Sh	42.9	6	<0.00001	0.332
5	FA → BS → DR; BS → Sh; BS → GS	13.2	6	0.04	0.180
6	FA → BS → DR → Sh; BS → GS	8.4	6	0.21	0.103
7	FA → BS → DR → Sh; FA → GS	6.2	6	0.40	0.018
8	FA → BS → DR; FA → GS; BS → Sh	11.0	6	0.09	0.156
9	FA → DR → BS; DR → GS; DR → Sh	33.5	6	<0.00001	0.379
10	FA → DR → BS; FA → GS; DR → Sh	22.9	6	0.001	0.241

*Note:* FA = food availability, GS = gut size (gut dry mass), BS = body size (carcass dry mass), DR = developmental rate (Gosner stage), Sh = body shape (ratio between body length and total length).

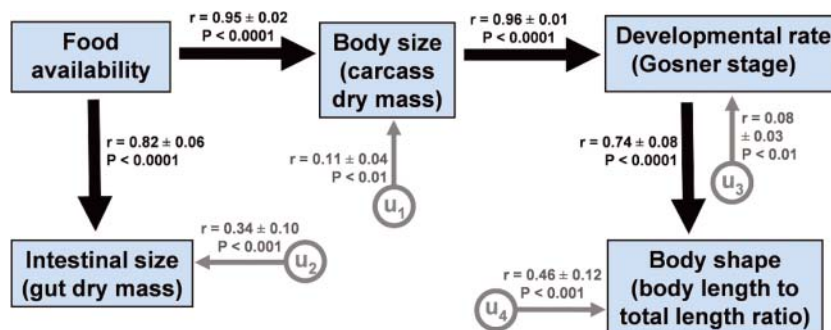


**Fig. 3.** Relationship between food availability and (a) developmental stage, (b) body mass, (c) total length, (d) body length, (e) gut dry mass, and (f) gut length.

supported by the data than were Models 6 and 8 (Table 1). Thus, the causal relationships among food availability and phenotypic variables may be stated as follows: (1) food availability directly affects body size (carcass dry mass) and gut size (intestine dry mass), (2) body size (carcass dry mass) directly affects developmental rate (Gosner stage), and (3) developmental rate (Gosner stage) directly affects body shape (the ratio between body length and total length) (Fig. 4).

## DISCUSSION

Phenotypic plasticity is high on current research agendas in ecology and evolution. First, plastic responses usually represent adaptations to cope with environmental variability, and hence they are central to understanding current phenotypic evolution and also to predict future evolutionary trajectories (Pigliucci, 2001; Handelsman *et al.*, 2014). Second, phenotypic plasticity could represent a major source of phenotypic novelty, leading to the colonization



**Fig. 4.** Path diagram for the best causal model (Model 7) based on  $\chi^2$  and RMSEA values. Partial correlation coefficients ( $\pm$ SE) are provided for each path;  $u_x$  represents residual (unknown) effects not ascribed to endogenous variables.

of new environments (West-Eberhard, 2003; Pigliucci and Muller, 2010). This is important not only in understanding the origin of phenotypic variation, but also in the contemporary context of biological invasions (Pujol-Buxo *et al.*, 2013; Nunes *et al.*, 2014). Third, several recent studies have suggested that the ability of different species to deal with accelerated changing conditions will be closely related to the current amount of plasticity for fitness-related traits (Charmentier *et al.*, 2008; Valladares *et al.*, 2014). Fourth, theoretical analyses at the food web level indicate that phenotypic plasticity may represent a major source of community stability, and hence of ecosystem functioning (Kovach-Orr and Fussmann, 2013). Thus, studies aimed at determining the levels and limits of plastic responses in natural populations, as well as the integration processes among plastic traits, are timely.

In the present study, we observed that changes in food availability resulted in plastic responses in several traits at different organizational levels, resulting in a high rate of survival in all the experimental groups. Specifically, our results suggest that food availability directly affects body mass and gut mass, that body size directly affects developmental rate, and finally, that developmental rate determines body shape. All these paths have, to some extent, received empirical support. For instance, the fact that food availability is positively related to body size at metamorphosis has been reported for several species of anuran (Morey and Reznick, 2004), including hyloid frogs (Crump, 1981; Beck, 1997; Browne *et al.*, 2003). Similarly, a common observation among ectothermic animals is that food availability is positively related to the size of the gastrointestinal tract (Naya *et al.*, 2008; Zaldua and Naya, 2014), including in post-metamorphic anurans (Secor, 2001; Cramp and Franklin, 2005; Naya *et al.*, 2009). As for the relationship between body mass and developmental rate, our results support the existence of mass developmental thresholds (Wilbur and Collins, 1973; Day and Rowe, 2002). According to this idea, each species has a minimum body size that must be reached, and a maximum body size that should not be exceeded, to move from one developmental stage to the next. When resources are abundant, most individuals reach the maximum threshold size before moving on to the next developmental stage (although this can happen at different times because of, for example, individual variation in digestive and energy conversion efficiencies). In contrast, when resources are scarce, the average size for a given developmental stage and also the range of variation around this variable decreases, since all larvae reach each developmental stage at or near the minimum threshold size. In line with this, we not only observed a positive correlation between body size and developmental rate, but also that the variability

around the average developmental stage tends to increase with food availability (Fig. 3a). However, note that our experimental design does not allow a formal test of the different developmental models that have been proposed for anuran tadpoles (see Beck, 1997). Finally, that developmental rate can affect body shape is in line with previous studies showing that tadpoles experiencing low food availability or high levels of competition usually exhibit relatively short tails and long bodies (Relyea and Hoverman, 2003; Bennett *et al.*, 2013). As mentioned above, all these plastic adjustments resulted in a high survival rate in all the experimental groups. This is important because, even though artificial diets are usually of a better quality than natural diets, our experimental design involved a ten-fold variation in food availability, and we did not observe any sign of saturation in the plastic response for any phenotypic trait (Fig. 3). In any case, the fact that the survival rate did not differ among groups does not mean that individual future performance (and maybe the future survival rate itself) will be the same for individuals in each of the experimental groups. For instance, some studies indicate that early metamorphosing individuals usually show deficiencies in their immune response (Gervasi and Fofopoulos, 2008) and predator avoidance (Ficetola and De Bernardi, 2006) after metamorphosis.

In summary, phenotypic plasticity and phenotypic integration are concepts central to contemporary ecological and evolutionary theory, as well as being fundamental to predict the responses of animals to global environmental change. Although some researchers have investigated the causal relationships among traits that respond plastically to change in predation pressure (Johnson *et al.*, 2008; Calsbeek and Kuchta, 2011) and water quality (Barth and Wilson, 2010), we know of no previous work that studied the integration of plastic responses to changes in food availability. From this perspective, the causal model proposed in the present study may represent a novel and relevant contribution to our understanding of phenotypic integration processes under variable environmental conditions.

#### ACKNOWLEDGEMENTS

Thanks to Claudio Borteiro for help with animal collection. The study was funded by Comision Sectorial de Investigación Científica (PAIE 2008), Universidad de la Republica, Uruguay.

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