

EXPERIMENTAL RESPONSE TO DENSITY LEVELS IN THE DEVELOPMENT OF TADPOLES AND FROGLETS OF *Boana pulchella* (ANURA: HYLIDAE)

Raúl Maneyro ^{1*} & Gabriel Francescoli²

¹ Laboratorio de Sistemática e Historia Natural de Vertebrados, Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay. E-mail: rmaneyro@fcien.edu.uy.

² Sección Etología, Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay. E-mail: gabo@fcien.edu.uy.

* Corresponding author: rmaneyro@fcien.edu.uy

ABSTRACT.

Density has been proposed as a factor that could determinate larval growth in anurans. We studied such effects on *Boana pulchella*. After hatching, we reared the tadpoles during 90 days and 93 tadpoles with similar size and developmental stages were chosen. We placed them in 800 cm³ cans with dechlorified water at five densities (1, 2, 4, 8 and 16 individuals/can) with three replicates for each treatment. In all the cans the feeding was *ad libitum*, and they were maintained until the end of the Gosner's developmental stage 42. Each time that a tadpole was extracted, a proportional water volume was too, in order to maintain constant densities. We found significant differences among treatments for froglets' length, mandibular width and mass, while these differences were not significant for metamorphic time. Since the tadpoles were feeding *ad libitum* an explanation by competitive trophic interactions for that differences could be discarded. These differences could be evidence that density has significant effects.

KEYWORDS: Density, metamorphosis, tadpoles, *Boana pulchella*.

RESUMEN.

Respuesta experimental a niveles de densidad en el desarrollo de larvas y metaformos de *Boana pulchella* (Anura: Hylidae).

La densidad ha sido propuesta como un factor que puede determinar el crecimiento larvario en los anuros. Se estudió estos efectos en *Boana pulchella*. Luego de la eclosión, se criaron a las larvas durante 90 días y se seleccionaron 93 renacuajos con tamaños y estados de desarrollo similares. Luego se colocaron en recipientes de 800 cm³ con agua dechlorada en cinco densidades (1, 2, 4, 8 y 16 individuos/ recipiente) con tres réplicas por cada tratamiento. En todos los recipientes la alimentación fue *ad libitum*, y las larvas fueron mantenidas hasta el final del estado de desarrollo 42 de Gosner. Cada vez que una larva era extraída, un volumen proporcional de agua era extraído también, para mantener constante las densidades. Se encontraron diferencias significativas entre tratamientos para las variables: tamaño de los metaformos, ancho mandibular y masa, al tiempo que las diferencias en el

tiempo de metamorfosis no fueron significativas. Debido a que las larvas fueron alimentadas *ad libitum* se descarta que las diferencias se relacionen con interacciones tróficas competitivas. Estas diferencias podrían evidenciar que la densidad tiene efectos significativos.

PALABRAS CLAVE: Densidad, metamorfosis, renacuajos, *Boana pulchella*.

INTRODUCTION

Phenotypic plasticity could be defined as the variation in one or more characters, in response to variations in environmental conditions (Via & Lande, 1985; Nespolo, 2000; Whitman & Agrawal, 2009). This phenomenon has been studied in many organisms, because it is a practical tool to elucidate potential ecological interactions, like inter or intraspecific competition and predation. Tadpoles (anuran larvae), are one of the most widespread model organisms for the study of this ecological interactions (Brockelman, 1969; Alford, 1999; Relyea 2001).

Tadpoles respond to variation in resources, and many factors have been reported as determinant of amphibian's development (Leips & Travis, 1994; Newman, 1994; Touchon *et al.*, 2013; Fan *et al.*, 2014). Among the biotic ones, the relationship between predator presence and performance of tadpoles and froglets has been well studied (Werner & McPeck, 1994; Relyea, 2001). Additionally, changes in food quantity (Wilbur, 1977a; Steinwascher, 1979) and presence of competitors (Griffiths *et al.*, 1993) have been signaled as other relevant causes for changes in tadpoles performances. Finally, density levels are another known biotic factor influencing amphibian survivorship and development (i.e.: Brockelman, 1969; Denver *et al.*, 1998; Laufer & Maneyro, 2008). In fact, intraspecific competition caused by density in exotrophic anuran larvae negatively affects size and performance of tadpoles (DeBenedictis, 1974; Dash & Hota, 1980; Semlitsch & Caldwell, 1982). However, density levels had also been signed as a factor that could positively influence the development of anuran larvae too, mainly through facilitated access to food (Eterovick, 2000).

The duration of the metamorphic period affects froglets' size (Wilbur & Collins, 1973; Werner, 1986), and consequently their post-metamorphic performance (Clarke, 1974; Pough & Kamel, 1984; Werner, 1986; Smith, 1987). Indeed, many trade-offs could be identified, mainly between remaining in the aquatic habitat and size at metamorphosis. This fact could be obvious for amphibians inhabiting variable environments, where the duration of an ephemeral pond is the main determinant of metamorphic timing (Denver, 1997; Denver *et al.*, 1998) and some species accelerate metamorphosis as a response to habitat desiccation (Newman, 1988; 1989). However, in species that live in permanent ponds those trade-offs are less evident.

The hylid frog, *Boana pulchella* (Duméril & Bibron, 1841) is one of the most common species among Uruguayan anurans (Maneyro & Carreira, 2012). This species has reproductive activity along the whole year (Langone, 1994) but some populations use to show high reproductive activity during cold seasons (Ximenez & Tozetti, 2015). Males call from sites with dense vegetation (Santos *et al.*, 2016) and the eggs are laid in a gelatinous mass, attached to aquatic vegetation, mainly in lentic water bodies (Maneyro & Carreira, 2012). The tadpoles are exotrophic thus having a non-specialized behavior that can be described as a Type I reproductive mode (*sensu* Duellman & Trueb, 1994). Previous experimental works on this species have demonstrated that density and food levels affect tadpoles' growth (i.e. Kehr, 1989).

The aim of this work is to assess the variation in size (tadpoles and froglets) and duration of metamorphosis of this frog as a response to different density levels.

METHODS

Experimental design

A clutch of *Boana pulchella* was collected (27 April 2004) in Coronilla (Rocha, Uruguay). After hatching, the larvae were maintained in an aquarium for three months (to avoid mortality during the experiment), and they fed on a mix of boiled *Lactuca* sp. and Beta-fish commercial food. After this period, 93 similar-size tadpoles at the 26th developmental stage (Gosner, 1960) were randomly selected and the experiment began on July 25th, 2004.

The tadpoles were reared in the laboratory in one-liter containers with 800 cc of dechlorinated water each one. The experimental design consisted in five density levels (1, 2, 4, 8 and 16 tadpoles by each 800 cc container) with three replicates each. The containers were randomly arranged to avoid microenvironmental variation to affect treatments. Food consisted in a mix of boiled *Lactuca* sp., boiled spinach and Beta-fish commercial food and was

provided *ad libitum*. The water was changed three to four times a week.

When a tadpole reached the 42nd developmental stage (Gosner, 1960), it was extracted from the container and a proportional water volume was extracted too in order to maintain a constant density in each container.

Data acquisition

Each tadpole was measured three times during the experiment, with a digital caliper (accuracy 0,05 mm). The measurements taken were (following McDiarmid & Altig, 1999): body length (BL), tail length (TAL), maximum width (MW) and maximum height (MTH). First measures were taken thirty days after the beginning of the experiment (August 25th, 2004). The last two were on September 2nd, 2004 and September 18th, 2004.

At metamorphic climax (stage 42nd), the froglets were sacrificed and weighed (M) with a Pesola dynamometer (accurate 0,1 g). Two measures were taken with a digital caliper (accuracy 0,05 mm): Snout-Vent length (SVL) and Mandibular width (MdW). Additionally, the number of days from the beginning of the experiment to metamorphic climax (T) was recorded.

Statistical Analysis

An index (IT) was developed to analyze the performance of tadpoles related to time. Its expression is: $IT = [(BL * MW * MTH)^{1/3} + (TAL * MTH)^{1/2}] / DY$, where BL, MW, MTH and TAL corresponds to the morphometric variables explained above, while DY is the number of days from hatching (so its results could be expressed as mm/day). After verification of normality and homoscedasticity in IT, differences among density treatments were tested using ANOVA.

The effects of density on tadpoles' growth along time were tested using MANOVA. In this case the independent variables were density (D) and date (DT), while the dependent variables were: BL, TAL, MW and MTH. A \log_{10} -transformation was used when a variable had not homogeneous variance (heteroscedasticity). Differences in tadpole size among density treatments were tested using ANOVA for each date.

A second index was developed for the froglets (IF). Its expression is $IF = M/T$, where M is the mass (grams) at the end of the experiment, and T is the number of days from hatching to end of the experiment (then its results could be expressed as gr/day).

The effects of density on size of froglets, IF and metamorphic timing (number of days until metamorphic climax) were analyzed using ANOVA. For the variable "number of days" (T) a \log_{10} -transformation of T was necessary to obtain homogeneity of variances. Additionally, a MANCOVA was performed in order to include possible effects of metamorphic timing (covariance) in the variation of froglets size among treatments (density).

RESULTS

Effects of density and time on tadpoles size

An ANOVA performed on IT showed significant differences ($F = 13.33$, $gl = 4$, $p < 0.001$). The size of *Boana pulchella* tadpoles analyzed by MANOVA and measured as body length, tail length, maximum width, and maximum height varied among treatments (density) and among dates (Table 1). There was a decrease for each of the four morphometric variables as a response to an increase in density, and an increase among the three dates when the tadpoles were measured (Figure 1). Those differences in morphometric variables, analyzed by ANOVA, were significant among treatments too (Table 2).

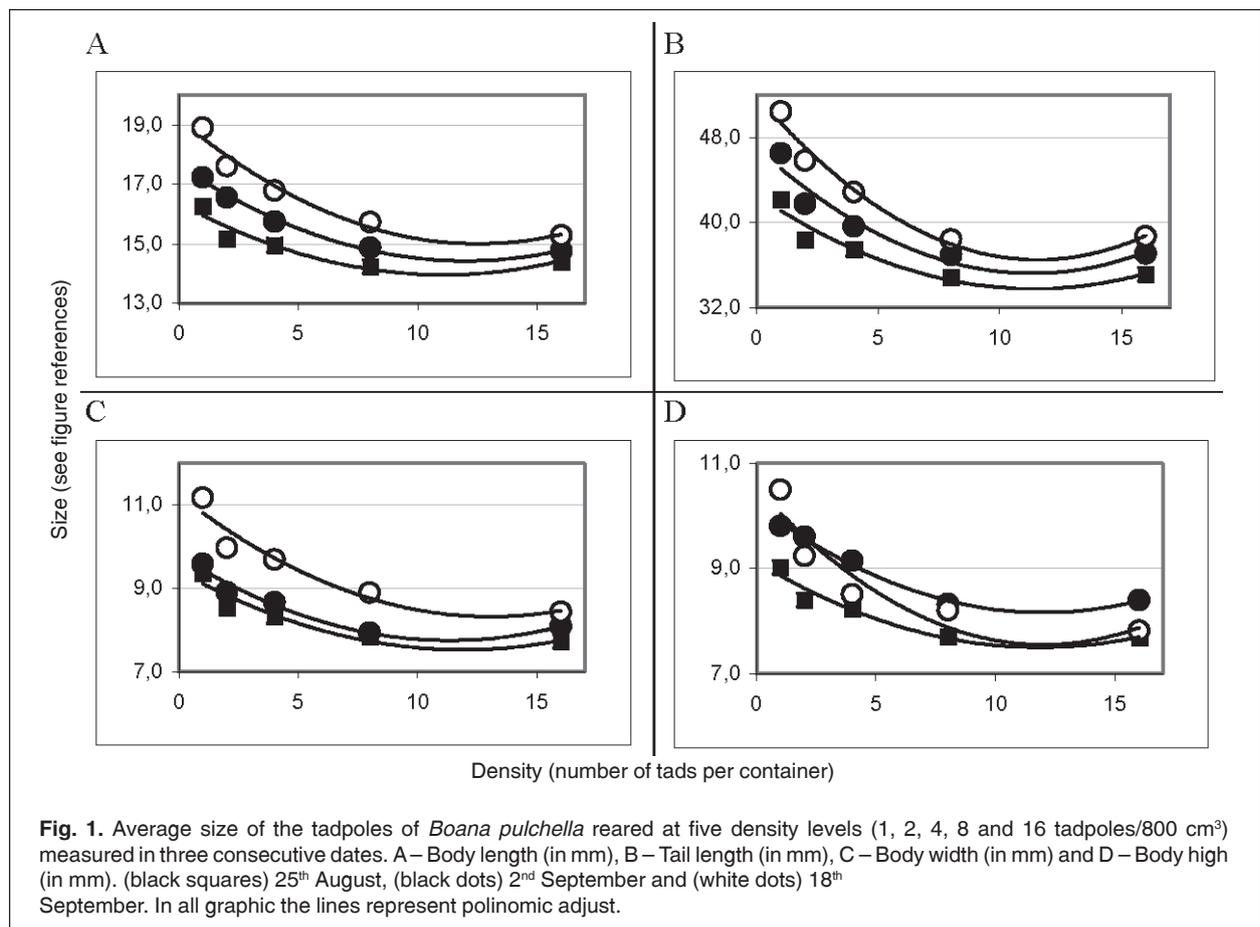
Effects of density and timing on froglets size

The effects of density were significant for froglets size (measured as mass, snout-vent length and mandibular width) and not significant for the duration of the metamorphosis (Table 3). When the effect of density on froglets size was analyzed using the duration of the metamorphosis as covariable, significant effects were detected in each morphometric variable (Table

Table 1. Summary of MANOVA at five levels of density (D) and three dates (DT) on tadpoles size of *Boana pulchella* (dependent variables: body length, tail length, maximum width, maximum height). Significant effects are marked with *

	Wilks' Lambda	Rao's R	df D	dfDT	p
D *	.684436	6.59050	16	798	.000000
DT *	.682681	13.72170	8	522	.000000
D + DT	.885780	1.00736	32	964	.456907

4). Additionally, the slope in the scatter plots of size (mass, snout-vent length and mandibular width) vs. duration of metamorphosis (\log_{10} transformed), decreased as the density increased (Figure 2). Additionally, an ANOVA performed on the IT showed significant differences too ($F = 11.38$, $gl = 4$, $p < 0.001$).



DISCUSSION

Effects of density and time on tadpoles size

Several works have highlighted that first developmental stages are critical to determinate the fitness of individuals during metamorphosis and also at its end (Leips & Travis, 1994; Newman, 1994). In this experiment, there was a response to differential density levels although tadpoles were maintained in the same conditions during three months (until the 26th developmental stage) and after that similar size individuals were randomly selected. As would be expected, during the experiment the tadpole sizes increased along time. Size (measured as body length, maximum width, maximum height and tail length) differed significantly among dates and treatments (Table 1), as did growth rate (based on IT). Additionally, in Figure 1 those differences in the morphometric variables between treatments can be observed. Tadpoles reared at low densities were bigger than those reared at higher densities for all morphometric variables. When these variables were analyzed independently, they showed significant differences among treatments too (Table 2).

In previous work, differences in growth rate in tadpoles were interpreted as a response to competition for resources (Brockelman, 1969; Wilbur, 1977b). In laboratory conditions, at low food levels, body size increased with food supply, while as the food supply increases on critical levels, growth could decrease. This was interpreted as a consequence of water pollution, because this phenomenon was not observed

in nature (Wilbur, 1977b). Additionally, at low food levels, exploitative mechanisms are less important, and interference (chemical, mechanical) seems to have the main effect on feeding ability in tadpoles reared under experimental conditions (Steinwascher, 1978). Density is one of the determinants of tadpoles' development, at least in non-aggregative species; since it was demonstrate that food level is more important than density for larval development in *Leptodactylus latrans* (Laufer & Maneyro, 2008).

Since in the present work tadpoles were fed *ad libitum* and the water was frequently changed, direct competition for resources or due to water pollution not occurred. So, differences in size among density treatments could be attributable to competition by interference (probably mechanical), as was proposed in previous works (Kher, 1989), however differences in oxygen level among treatments can not be discarded.

Effects of density and timing on froglets size

After the metamorphic climax, the effects of density were relevant too. Effects in froglets sizes as a response to density levels were significant, but the duration of the metamorphosis was not (Table 3). However, significant differences in size were found among treatments when duration of the metamorphosis was used as a covariable (Table 4). This observation can be complemented by differences among slopes of each treatment for the growth rate (morphometric variables vs. duration of metamorphosis), as can be observed in Figure 2. Moreover, the growth rate for

Table 2. Effects of five density levels on size of tadpoles of *Boana pulchella* at three dates (ANOVA results). BL – Body length, TL – Tail length, MW – Maximum width, MH – Maximum height. Number of days since the beginning of the experiment in brackets. Significant effects are marked with *.

DATE	Variable	SS	df	MS	F	P
25 august (30)	BL *	0.164609	4	0.041152	3.133762	0.018503
	TL *	2.413034	4	0.603259	6.307502	0.000165
	MW *	0.124361	4	0.031090	4.784805	0.001538
	MH *	0.095402	4	0.023851	2.692078	0.036081
02 setpember (38)	BL *	0.380604	4	0.095151	6.713191	0.000092
	TL *	4.021413	4	1.005353	8.329538	0.000010
	MW *	0.136793	4	0.034198	5.746085	0.000372
	MH *	0.179468	4	0.044867	4.031951	0.004756
18 setpember (54) _§	BL *	0.442474	3	0.147491	5.992997	0.000930
	TL *	4.374006	3	1.458002	8.671316	0.000043
	MW *	0.242352	3	0.080784	8.847148	0.000036
	MH *	0.138158	3	0.046053	6.233944	0.000701

_§ The lowest density treatment was not considered for this date.

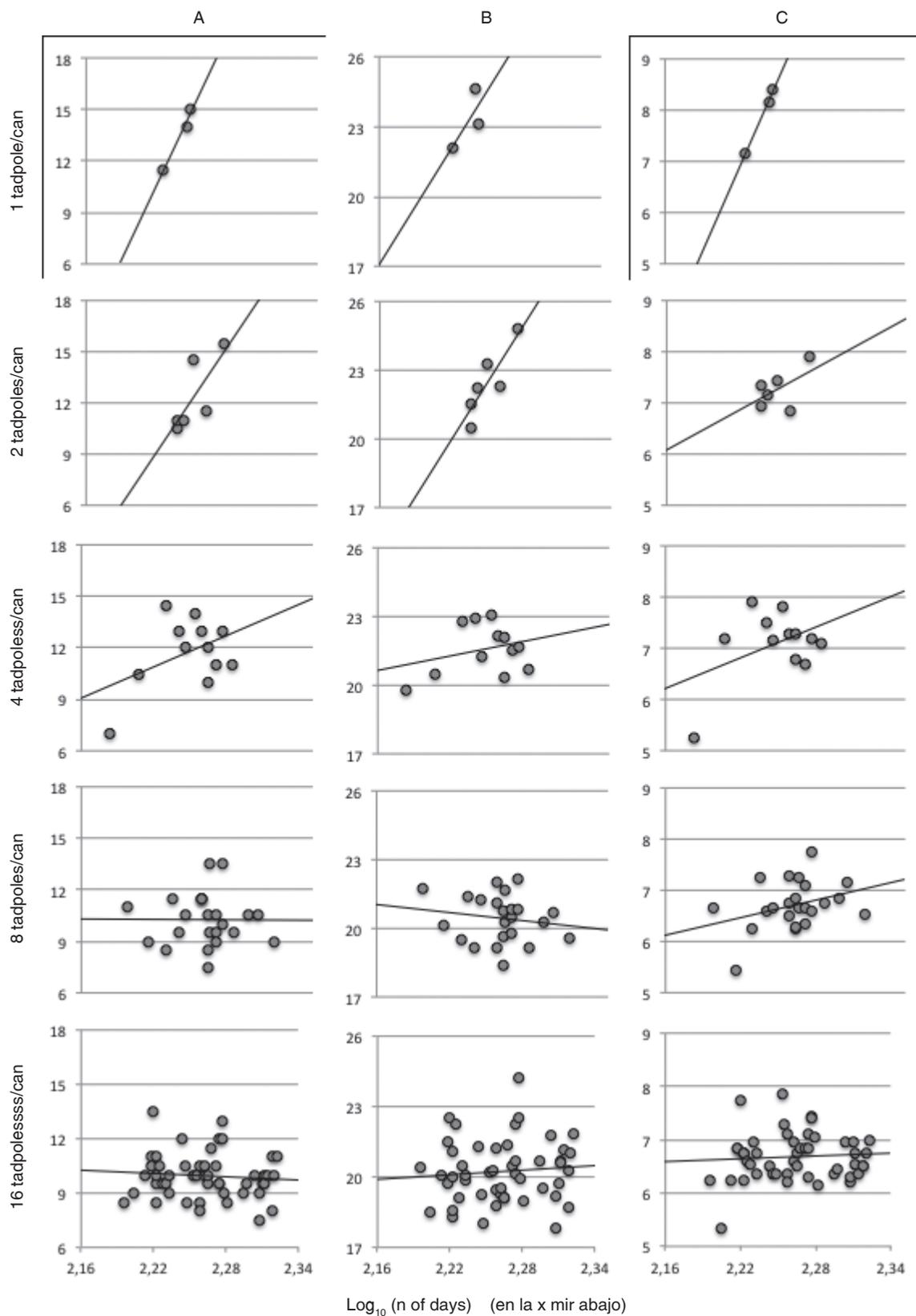


Fig. 2. Lineal regressions between metamorphic duration (\log_{10} transformed) and mass (in grams) (A), snout-vent length (as SVL, in mm) (B) and mandibular width (as MW, in mm) (C) for *Boana pulchella* froglets reared at five density levels (1, 2, 4, 8, and 16 tadpoles per can).

Table 3. Summary of the ANOVA at five levels of density on *Boana pulchella* froglets size and timing. M – mass, SVL – Snout-vent length, MdW – Mandibular width, log T – logarithmic transformation of time of metamorphosis. Significant effects are marked with *.

Variable	SS	df	MS	F	p
M *	81.97967	4	20.49492	9.145660	.000003
SVL *	59.69226	4	14.92306	9.844669	.000001
MdW *	6.87056	4	1.71764	7.253699	.000043
logT	.00655	4	0.00164	1.674122	.163134

froglets (measured as IF) exhibited significant differences among density treatments.

Metamorphosis has been signaled as influenced by a trade off between time and size (Duellman & Trueb, 1994; Stebbins & Cohen, 1997). Positive effects of intraspecific interactions were reported in laboratory studies. Among other consequences, intraspecific interactions may increase metamorphic duration (Breden & Kelly, 1982) and may produce bigger froglets (Wilbur, 1977a). Additionally, these interactions may decrease metamorphic synchrony (Breden & Kelly, 1982). The latter is relevant for tadpoles with aggregative behavior, since synchronic metamorphosis has been suggested as an antipredatory mechanism (d'Heursel & Haddad, 2002; Mares, 1972). In the present work, the size of the froglets seemed to be determined by the duration of metamorphosis at lower densities, but for density itself at higher ones (Figure 2). *Boana pulchella* has not aggregative behavior, so differences in the slopes in Figure 2, could be interpreted as a response to the above-mentioned trade-off. This means that in optimal conditions (low density and high food levels) metamorphic time is expected to be greater and, consequently, froglets would be bigger. Conversely, at high densities the possible facilitation of access to food (positive effect) could not compensate the negative effects of interference, and at similar metamorphic time, the froglets are smaller.

Even if tadpoles were provided with food *ad libitum* it is possible to think about some other advantageous reasons of increasing in size. One of those advantages is that bigger (healthier, stronger) tadpoles and froglets could avoid predation more easily than others (Crump, 1984). Additionally, bigger froglets can be less susceptible to pathogens (like *Batrachochytrium dendrobatidis*) since they maintain homeostasis easier than smaller individuals (Wu *et al.*, 2018). Other advantage of the increased size is the possibility of maintaining a higher body mass in

Table 4. Summary of MANCOVA at five levels of density (treatments) on *Boana pulchella* froglets size. Co-variable = Metamorphosis time (log transformed). M – mass, SVL – Snout-vent length, MdW – Mandibular width. Wilks' Lambda = .635296, Rao's R = 3.509943, df = 12, 225, p = .000089. Significant effects are marked with *.

Var.	MS	F(df = 4,87)	p
M *	19.96276	8.832767	.000005
SVL *	14.96437	9.841393	.000001
MdW *	1.90705	8.295430	.000010

the next stage of development, thus enhancing the competing abilities of bigger individuals, as was demonstrated for *Lithobates sphenoccephalus* (Steinwascher, 1978). Another advantage could be, in the same vein, that being bigger allows fully developed animals to capture and consume bigger prey, a proved fact in *Boana pulchella* (Maneyro & da Rosa, 2004).

The effect of predation could be considered as one of the main possibilities influencing the results here obtained on an evolutionary scale. However, that influence should be tested in order to assess its importance. If predation pressure is not found to be the main reason of the increase in size when other conditions are the same, we could be facing a scenario showing some kind of "anticipative" behavior by the tadpoles. This behavior would have an advantage only after metamorphosis and when the animals change their living environment and way of life.

Finally, the date when the froglets finished their metamorphosis in the experiment (November) is coincident with that observed in nature. These observations were made in two wild Uruguayan populations: one in the South (Balneario Solís, departamento de Maldonado) where froglets arrived to stage 42nd in December, and the other in the North (Parque Gran Bretaña, departamento de Rivera), where they did it in November (Maneyro *Obs. Pers.*). However, during these field observations the froglets seemed to be bigger than the biggest tadpole in the experiments here described. This fact suggests that in spite of some factors (like food availability) were compensated during the experiment, other potential factors could be responsible for these differences (size in nature bigger than size in low density experiments). So, it could be interesting to search which are these effects and how they act on individuals in wild populations inhabiting natural environments.

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