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Article

Attraction to conspecifcs in *Rhinella icterica* and *R. ornata* tadpoles (Anura: Bufonidae)

Alexandre Polettini Neto*[1] *& Jaime Bertoluci*[1]

[1]*Universidade de São Paulo, Escola Superior de Agricultura Luiz de Queiroz, Departamento de Ciências Biológicas, Av. Pádua Dias, 11, 13418-900, Piracicaba, SP, Brasil*

**Corresponding author: Jaime Bertoluci, e-mail: jaime.bertoluci@usp.br*

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Abstract: Tadpoles are able to perceive and discriminate signals from environment and they may use this ability in behaviors and ecological processes. Recognition mechanisms may be involved in schooling by means of attraction among individuals, characterizing a social process. By means of laboratory experiments the present study investigated the presence or absence of attraction to conspecifics in tadpoles of *Rhinella icterica* and *R. ornata*, two sympatric species from the Atlantic Forest of southeastern Brazil. We collected eggs of these two species in the field and reared them in laboratory according to two different methods (isolated or in groups of siblings). Tadpoles were submitted to experiments of choice between conspecifics, heterospecifics, and an empty compartment. *Rhinella icterica* tadpoles preferred to associate with conspecifics rather than *R. ornata* tadpoles, and we verified this is an innate behavior. *Rhinella ornata* tadpoles failed to discriminate between conspecifics and *R. icterica* tadpoles. When submitted to choice between a group of tadpoles of the other species and an empty compartment, *R. icterica* tadpoles presented random distribution, while *R. ornata* tadpoles preferred to associate with heterospecifics. Our results indicate *R. icterica* tadpoles have preference to associate with conspecifics, while *R. ornata* tadpoles may school indiscriminately. This study contributes for a better understanding of larval anuran social behavior.

Keywords: *anuran larvae; behavior; communication; sympatric species; Atlantic Forest; schooling; southeastern Brazil.*

Atração por coespecíficos em girinos de *Rhinella icterica e R. ornata* (Anura: Bufonidae)

Resumo: Os girinos são capazes de perceber e discriminar sinais do ambiente e podem usar essa capacidade em comportamentos e processos ecológicos. Mecanismos de reconhecimento podem estar envolvidos na agregação por meio da atração entre indivíduos, caracterizando um processo social. O presente estudo investigou, por meio de experimentos de laboratório, a presença ou ausência de atração por coespecíficos em girinos de *Rhinella icterica* e *R. ornata*, duas espécies simpátricas, da Mata Atlântica do sudeste do Brasil. Coletamos ovos dessas duas espécies no campo e os cultivamos em laboratório de acordo com dois métodos diferentes (isolados ou em grupo de irmãos). Os girinos foram submetidos a experimentos de escolha entre coespecíficos, heteroespecíficos e compartimento vazio. Girinos de *Rhinella icterica* preferiram associar-se a coespecíficos do que com girinos de *R. ornata*, e verificamos que este é um comportamento inato. Os girinos de *Rhinella ornata* não conseguiram discriminar entre coespecíficos e girinos de *R. icterica.* Quando submetidos à escolha entre um grupo de girinos da outra espécie e um compartimento vazio, os girinos de *R. icterica* apresentaram distribuição aleatória, enquanto os girinos de *R. ornata* preferiram associar-se a girinos heteroespecíficos. Nossos resultados indicam que girinos de *R. icterica* preferem associar-se a coespecíficos, enquanto girinos de *R. ornata* podem agregar-se indiscriminadamente. Este estudo contribui para uma melhor compreensão do comportamento social de larvas de anuros.

Palavras-chave: *larvas de anuros; comportamento; comunicação, espécies simpátricas; Mata Atlântica; agregação, Sudeste do Brasil.*

Introduction

Recognition mechanisms (of relatives, conspecifics, mates, neighbours, rivals, prey or predators) are essential for survival, reproduction and social interactions between organisms (Sherman et al. 1997). Among anuran larvae, chemical communication plays a key role in several behaviors, such as communication between a female and its offspring (Kam & Yang 2002; Stynoski & Noble 2012), detection of alarm cues in prey-predator contexts (Hews 1988; Hokit & Blaustein 1995; Summey & Mathis 1998; Kiesecker et al. 1996, 1999), intraspecific competition (Glennemeier & Denver 2002), microhabitat and food discrimination (Pfening 1990; Gamboa et al. 1991; Hall et al.1995; Sontag et al. 2006), and recognition of predators (Petranka et al. 1987; Lawler 1989; Kiesecker et al. 1996), conspecifics (Leu et al. 2013; Chapman et al. 2015; Raven et al. 2017), and kin (Blaustein & O'Hara, 1982; Waldman 1986; Rajput et al. 2014; Pizzatto et al. 2016; Raven et al. 2017).

In tadpoles, recognition abilities based on chemical signals are developed during the embryonic phase or shortly after hatching (Waldman 1981, 1882; Blaustein & O'Hara 1982; Hepper & Waldman 1992) and may persist after metamorphosis (Blaustein et al.1984; Waldman 1989; Graves et al. 1993; Flowers and Graves 1997). Due to vulnerability to predators at this stage, some species have developed strategies such as metamorphosis synchronization and aggregation (Pulliam & Caraco 1984; Hews, 1988). In this case, spatial aggregation may be based primarily on conspecific attraction rather than mediated by environmental factors (Graves et al. 1993). Non-social groups are formed in response to attractive environmental characteristics (e.g., feeding microhabitats and temperature gradients), while social groups are formed from attraction between individuals (Wassersug 1973; Hoff et al.1999). Thus, communication characterizes social behavior (Townsend et al. 2003).

In this context, the present study sought to analyse, through laboratory experiments, the presence or absence of attraction to conspecifics in tadpoles of two congeneric species (*Rhinella icterica* and *R. ornata*) that occur in sympatry and show a wide distribution in the Atlantic Forest of southeastern Brazil. These species are able to school during larval phase (Eterovick 2000; Simon 2010).

These species belong to the family Bufonidae, which is composed by 53 genera and 629 species (Frost 2020). In the genus *Rhinella* Fitzinger, 1826, there are 92 species distributed from southern United States to southern South America (Frost 2019). *Rhinella icterica* is included in *Rhinella marina* Group (Maciel et al. 2010), while *R. ornata* belongs to the *Rhinella crucifer* Group (Baldissera Jr. et al. 2004).

Reproductive isolation between *R. icterica* and *R. ornata* may not be effective, due to sympatric occurrence, overlapping of reproductive sites and seasons of reproduction and close relationship between them (Bertoluci 1998; Bertoluci & Rodrigues 2002; Abrunhosa et al. 2006), enabling interspecific amplexes and the occurrence of non-viable hybrids (Haddad & Cardoso 1990). The same habitat is shared by these two tadpole species of different kinship degrees and development stages, thus, an intra and interspecific communication system would has an important adaptive value for them.

Until now, only one study has sought to analyze attraction to conspecifcs in Brazilian tadpoles (*Rhinella marina*; Raven et al. 2017), but this was done with invasive populations in the Australian territory. Thus, the present study contributes to an important knowledge gap of Brazilian tadpoles' behavior.

Therefore, we aimed to answer, for each species, the following questions: (1) do tadpoles prefer to associate with conspecifics rather than heterospecifics? (2) Does prior social experience (isolation or group rearing) influence recognition mechanism?

Material and Methods

1. *Egg collection*

We collected eggs from two spawns of *R. icterica* and two spawns of *R. ornata*, between 24 July and 14 August 2017, encompassing the reproductive season of the target species (Bertoluci & Rodrigues 2002). We identified spawns based on size and arrangement of eggs within the gelatinous strings: smaller eggs arranged in a single string in *Rhinella ornata*, larger eggs arranged in a double string in *R. icterica* (Simon 2010). We transported eggs to laboratory in plastic pots containing local water.

We collected eggs in the Estação Biológica de Boracéia (EBB), a well-preserved Atlantic Forest reserve of Serra do Mar in São Paulo state (23°38' S, 45°52' W). EBB has an area of 16,450 ha and is located at altitudes around 900 m a.s.l. The area is covered by Dense Ombrophylous Forest, where the presence of palm trees, ferns and giant bamboos is common (Travassos & Camargo 1958; Heyer et al.1990; Bertoluci & Rodrigues 2002). Rainy season runs from September to March, and temperature can reach 24°C in the hottest months (September to March) and 5°C in the colder months (April to August) (Bertoluci & Rodrigues 2002).

2. *Rearing methods*

We conditioned eggs of two spawns (spawn A and spawn B) of each species, in laboratory, according to two different methods. They were separated from the rest of the embryos in the spawn between stages 16 and 18 (Gosner 1960).

At the 1st rearing method (group of siblings), we placed 300 eggs of each spawn of *R. icterica* in two different 50L opaque containers and we did the same with eggs of *R. ornata*. At the 2nd method (isolation rearing), we placed 50 eggs from spawn A, of each species, each egg in a 0.5L opaque container, in 50 different containers.

We maintained eggs and tadpoles at room temperature and natural photoperiod. Tadpoles were fed once daily with ornamental fish food in large quantities so that food was always available. Aeration pumps for aquariums provided oxygenation. We changed the water in each container twice a week to keep the environment clean. After the metamorphosis, we released juveniles at the egg collection sites.

3. *Experiments*

Tests were carried out in four plastic containers (100 × 15 × 10 cm), filled with spring water (Fig. 1). At each end of a container we adapted a plastic net with a mesh of 0.5 mm, delimiting the stimulus groups areas (20 tadpoles in each group). The central part of each container was marked with a permanent pen, dividing it into three equal-sized compartments.

We performed the tests between 25 August and 27 September 2017, during daytime, between 08:00 a.m. and 6:00 p.m. Tested tadpoles were between stages 25 and 38 (Gosner 1960).

We placed test tadpoles in the center of each container and submitted to choice tests between two groups (Fig. 1) (as in Blaustein & O'Hara 1982; Cornell et al. 1989; Leu et al. 2013; Rajput et al. 2014; Pizzatto et

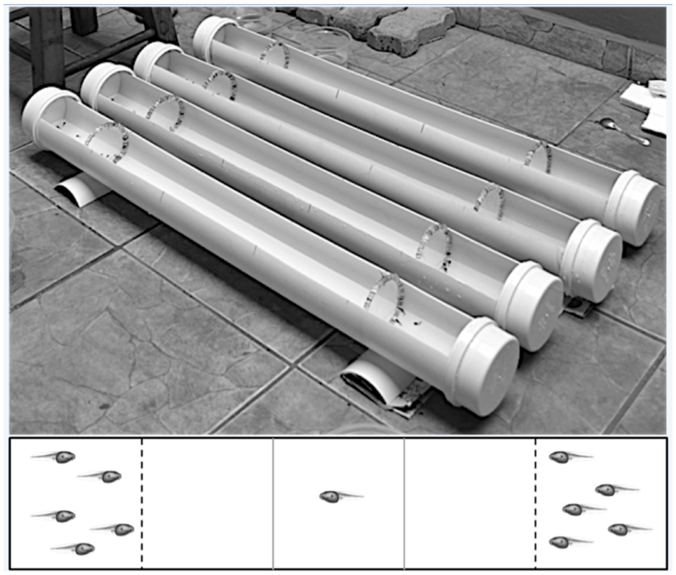

Figure 1. Test arena. Stimulus groups (20 tadpoles in each group) are represented in the right and left extremities, and test tadpole is represented in central area. Dashed line represents a net, which delimits stimulus groups areas, but allows chemical and visual stimuli flow to central region. Vertical gray lines represent demarcation of areas close to each stimulus group. Each division is 20 cm long, totalling 100 cm of arena total length.

al. 2016). We filmed the experiments (Kodak z990 camera), so researcher presence did not influence tadpole behavior. After 10 minutes of test tadpole acclimation, we filmed each experiment for 29 minutes (1740 s), and then we measured the time each tadpole remained in the compartment next to each stimulus group. Each tadpole was tested only once and, after each test, containers were cleaned and the water changed. At each test we turned the containers to 90° and inverted the side of each stimulus group. Each experiment was replicated 32 times. Four replicates were filmed at a time. All procedures were repeated for both species.

We organized the experiments as follows:

Control: all tadpoles (test tadpoles and stimulus groups) from the same spawn and reared together in one container. With this experiment we were able to analyse if there were errors in experimental design. The expected was that test tadpoles had no preference for association with one of the two groups.

Experiment 1: test tadpole from spawn B reared according to 1st rearing method. Stimulus group 1 formed by tadpoles of spawn A (conspecifics tadpoles, but not siblings of test tadpole). Stimulus group 2 formed by tadpoles of the other studied species (heterospecifics). With this, we sought to analyse if there was preference of association with conspecifics by the species tested.

Experiment 2: test tadpoles from spawn A reared according to the 2nd rearing method (in isolation). Stimulus group 1 formed by tadpoles of spawn B, reared according to 1st rearing method. Stimulus group 2 formed by heterospecific tadpoles. With this, we sought to analyse if the rearing method in total isolation would influence association choice by tested tadpoles with one of the stimulus groups.

Experiment 3: test tadpole from spawn A, reared according to the 1st rearing method. Stimulus group 1 formed by heterospecific tadpoles. Stimulus group 2 empty (tadpoles absence). With this experiment we sought to analyse sociability of tested tadpoles (preference for grouping or isolation), and attraction or repulsion by tested tadpoles to heterospecific tadpoles.

4. *Data analyses*

We performed statistical analyses with R platform (R Core Team 2017). We consider level of significance $\alpha = 0.05$. Tests were two-tailed. Confidence level used was 95%. For each experiment, data considered in the statistical tests were the differences between the time spent by the test tadpole in the compartments located near stimulus groups 1 and 2 (T1– T2). We verified if data of each experiment corresponded to normal distribution by Shapiro-Wilk test (H_o = data correspond to normal distribution). To data that reached the normal distribution we applied paired t-test, due to dependence of samples, since two measurements were made for the same individual in each experiment (paired data). Non-parametric alternative to t-paired test is Wilcoxon signed-rank test, which was applied to data that did not reach normal distribution. With the tests, we were able to analyse whether the difference between time spent by test tadpoles near each of two stimulus groups was statistically significant. The null hypothesis was that mean (or median) of the differences is null (H_o: $\Delta = 0$), while the alternative hypothesis was that mean (or median) of the differences is not null (H1: $\Delta \neq 0$). When the null hypothesis was rejected, we used binomial test to verify whether the number of tadpoles that spent the most time near a stimulus group was statistically significant in relation to total number of replicates.

Data obtained in experiments Control, 1 and 3 with *Rhinella icterica* tadpoles and in experiments Control, 1 and 3 with *R. ornata* tadpoles corresponded to normal distribution (Shapiro-Wilk test; Table 2 and 3); in these cases, we used paired t-test to detect differences between time spent by test tadpoles next to each stimulus group. However, in experiments 2 with *R. icterica* data did not correspond to normal distribution (Shapiro-Wilk test; Tables 1 and 2), so we used Wilcoxon test.

Results

The differences between time spent by test tadpoles close to each stimulus group, as well as the mean of the differences (value used in the Paired t-test) and pseudomedian differences (value used in the Wilcoxon test), when negative, indicate a longer permanence time of test tadpoles close to stimulus group 2, whereas, when positive, they indicate a longer permanence time of the tadpoles test near to stimulus group 1 (Tables 2 and 3, Figs. 2 and 3). In general, data obtained with experiments varied more for *R. ornata* than for *R. icterica* (Figs. 2 and 3).

In experiments Control and 3 with *R. icterica* and in experiments Control and 1 with *R. ornata*, the mean differences did not differ statistically from hypothetical mean obtained by paired t-test (Tables 2 and 3). Therefore, for these experiments, we did not reject the null hypothesis and concluded that there was no statistically significant difference between time spent by tadpoles next to each stimulus group (H_o: $\Delta = 0$).

In experiment 1 with *R. icterica* and in experiment 3 with *R. ornata*, the mean differences differed statistically from hypothetical mean obtained by paired t-test (Tables 2 and 3). Similarly, in Experiment 2 with *R. icterica*, the pseudomedian differences differed statistically from the hypothetical median by Wilcoxon test (Tables 2 and 3). Therefore, for these experiments, we rejected the null hypothesis and concluded that there was a statistically significant difference between time spent by tadpoles next to each stimulus group (H1: $\Delta \neq 0$). In these experiments, we confirmed, with the binomial test, that the number of tadpoles that showed preference for group 1 was significant in relation to the total number of replicates (Tables 2 and 3).

Table 1. Synthesis of experiments and control. In the first rearing method, tadpoles were reared with its siblings, and in the second method each tadpole was reared in isolation. The test tadpoles were submitted to choice between two stimulus groups of conspecifcs, heterospecifics or empty compartment.

Experiment	Test tadpole	Stimulus Group 1	Stimulus Group 2
Control	1st rearing method	Conspecifcs	Conspecifics
1	1st rearing method	Conspecifcs	Heterospecifics
2	2nd rearing method	Conspecifcs	Heterospecifics
3	1st rearing method	Heterospecifics	Empty

Table 2. Statistical tests results for each experiment with *Rhinella icterica* tadpoles. When P < 0,05, there is statistically significant difference between time spent by tadpoles next to each stimulus group. df = degrees of freedom; CI = confidence interval.

Exper.	Shapiro-Wilk test	Paired-t test (t) or Wilcoxon (V)	Mean of differences	Pseudomedian of differences	CI (95%)		Binomial test
					Inf. Limit	Sup. Limit	
Control	W = 0.97 *P* = 0.58	T = -0.98; df = 31; *P* = 0.33	-159.68	-	-490.68	171.3	
1	W = 0.97 *P* = 0.53	T = 4,89; df = 31; *P* < 0.001	430.93	-	251.49	610.38	N = 24 *P* < 0.001
2	W = 0.92 *P* = 0.02	V = 384; df = 31; *P* = 0.02	-	176	31.99	325.99	N = 22 *P* < 0.001
3	W = 0.98 *P* = 0.91	T = 0.57; df = 31; *P* = 0.57	55.68	-	-142.55	253.93	

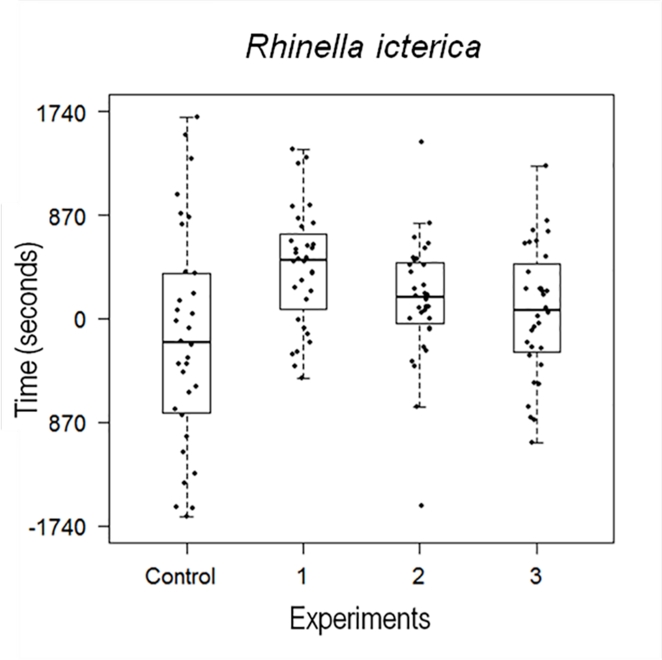

Figure 2. Boxplot of data resulting from the experiments with *Rhinella icterica* tadpoles. Each point represents the difference between time spent by tested tadpole, in each trial, close to stimulus groups 1 and 2.

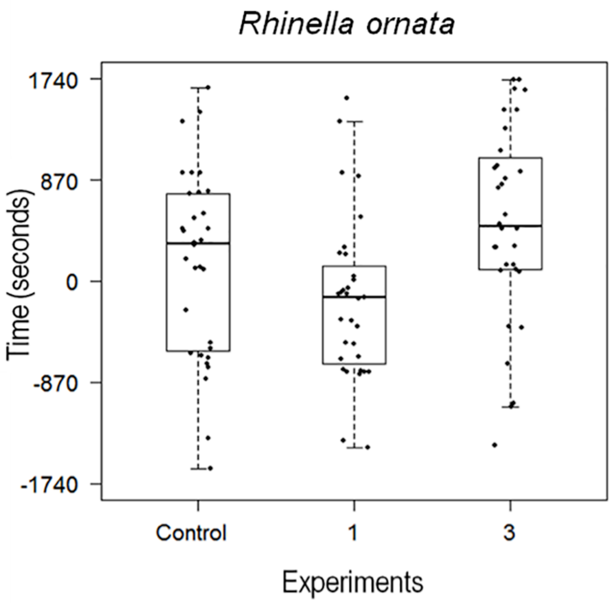

Figure 3. Boxplot of data resulting from the experiments with *Rhinella ornata* tadpoles. Each point represents the difference between time spent by tested tadpole, in each trial, close to stimulus groups 1 and 2.

Unfortunately, we made a mistake when choosing test tadpole in Experiment 2 for *Rhinella ornata*, and we realized in time to not use these data, but was not possible to repeat the experiment. Therefore, we were unable to determine whether isolation rearing would influence attraction to conspecifcs in tadpoles of *R. ornata.*

Discussion

Rhinella icterica tadpoles demonstrated a strong attraction to conspecifics. This result suggests that these tadpoles possess the ability to discriminate between conspecifics and heterospecifics. We could conclude

Table 3. Statistical tests results for each experiment with *Rhinella ornata* tadpoles. When P < 0,05 there is statistically significant difference between time spent by tadpoles next to each stimulus group. df = degrees of freedom; CI = confidence interval.

Exper.	**Shapiro-Wilk test**	**Paired-t test (t)**	**Mean of differences**	**CI (95%)**		**Binomial test**
				Inferior Limit	**Superior Limit**	
Control	W = 0.96 *P* = 0.47	T = 1.08; df = 31; *P* = 0.28	154.37	-135.44	444.19	-
1	W = 0.94 *P* = 0.10	T = -1.41, df = 31 *P*= 0.16	-174.12	-425.26	77.01	-
3	W = 0.95 *P* = 0.20	T = 3.32; df = 31; *P* = 0.02	496.5	192.14	800.85	N= 25 *P*< 0.001

that this attraction behavior is innate, since tadpoles reared in isolation, from the embryonic stage, also demonstrated preference to associate with conspecific tadpoles. The preference to conspecifics is a result of attraction to conspecifics rather than repulsion to heterospecifics, because tadpoles of *R. icterica* have been randomly allocated when submitted to a choice between heterospecifics and an empty compartment.

Rhinella marina tadpoles, that belong to the same phylogenetic group as *R. icterica* (*Rhinella marina* Group; Maciel et al. 2010), when submitted to choice tests between a non-siblings group and an empty compartment, showed no attraction for conspecific when these were non-siblings (Raven et al. 2017). However, *R. marina* tadpoles diminish activity and avoid chemical cues of injured conspecifics, characterizing a recognition and discrimination process, but this does not lead to aggregation, which is formed in response to environmental factors such as light, temperature and habitat structural complexity (Hagman & Shine 2008; Raven et al. 2017). There are different reactions of another bufonid tadpoles to injured conspecifics cues: *Anaxyrus boreas* also exhibit repulsion to these signals, but with increased activity (Hews & Blaustein 1985), while *Sclerophrys danielae* answer to these cues aggregating (Spieler & Linsenmair 1997).

Sontag et al. (2006) demonstrated that tadpoles of *Anaxyrus americanus* recognize conspecifics signals to find food sources and even to discriminate food quality. Similarly, tadpoles of *R. marina* have a stronger attraction to conspecific cues feeding than to cues directly from food (Raven et al. 2017).

Conspecific recognition mechanisms can be used in cannibalism context. Crossland & Shine (2011) found that *R. marina* tadpoles are attracted by chemical cues from conspecific eggs and they eat them. This behavior is not a result of indiscriminate foraging, as these tadpoles rarely eat eggs of other species present in the same habitat (Crossland & Shine 2010). This type of cannibalism can provide an important nutrition source for tadpoles and reduce future intraspecific competition (Crossland et al. 2011). In addition, by finding conspecific eggs, *R. marina* tadpoles can release chemicals that suppress the development of embryos (Crossland & Shine 2012, Clarke et al. 2015).

Crossland et al. (2011) have demonstrated that the substances involved in attraction process of *R. marina* tadpoles by conspecific eggs are toxins used in defence against predators, known as bufadienolids. Thus, it is possible that these substances are involved in attraction to conspecifics in tadpoles of *R. icterica* and other bufonids. These examples of behaviors based on attraction to conspecific cues in species genetically related to *R. icterica* suggest that attraction to conspecifics in this species may be related to similar behaviors. However, further studies are needed to verify if this attraction to conspecific is related with some recognition mechanism.

We found a notable difference between *R. icterica* and *R. ornata* tadpoles in relation to attraction to conspecifics. *Rhinella ornata* tadpoles did not prefer any of the groups, formed by either conspecifics or heterospecifics, indicating that attraction to conspecifics in this species may not occur. *Rhinella ornata* tadpoles spent more time near heterospecifics than the empty compartment, which suggests that *R. ornata* tadpoles prefer to associate with tadpoles of another species than to be isolated.

Some species of tadpoles exhibit schooling behavior (e.g., Beiswenger 1975, 1977; Wassersug et al. 1981; Eterovick 2000; Heursel & Haddad 2002). Functions and benefits attributed to this behavior include predation rate decrease—due to predator confusion and aposematism enhancement—, foraging rate increase, and greater efficiency in thermoregulation (Watt et al.1997; Hoff et al. 1999; Eterovick 2000; Hero et al. 2001). However, when resources are limited, some costs of group formation may be evident, such as increasing competition, cannibalism and predation, as well as increasing disease and inbreeding susceptibility (Hamilton and May 1977; Bateson 1983; Shykoff & Schmid-Hempel 1991; Pfening et al.1993; Goater 1994). Spatial attraction of tadpoles of *R. ornata* by tadpoles groups of *R. icterica*, as well as the non-repulsion of tadpoles of *R. icterica* to tadpoles groups of *R. ornata* tadpole, suggests that spatial interaction between these tadpoles in the natural environment does not involve large competition costs.

Some authors have attempted to explain the absence of discrimination among siblings by tadpoles under laboratory conditions as a consequence of lack of stimuli to reproduce the aggregation behavior (Blaustein & Waldman 1992). The aggregation formation, with relatives or not, may be dependent on the balance between the costs and benefits of this behavior (Hamilton 1964). Both, recognition processes and aggregation can vary within the same species depending on some factors, such as presence and density of predators (Wrona & Dixon 1991; Fitzgeral 1992; Watt et al. 1997) differences in tadpole diet (Gamboa et al. 1991; Pfenning 1990), development stage (Rautio et al. 1991; Blaustein & Waldman 1992; Nicieza 1999), and resource distribution and temperature variation (Hokit & Blaustein 1997). *Lithobates sylvaticus* tadpoles, for example, demonstrated kin recognition and attraction in laboratory experiments, but in natural environments they demonstrated both attraction and repulsion to kin in different ponds (Waldman 1984; Halverson et al. 2006). We were not able to consider the variable stage of development in our study, because of the rapid development and the great variation of development of tadpoles from the same spawn. This research opens a pathway for further studies analyse each variable that may be related to attraction to conspecifics in tadpoles of these two bufonids.

In this study, when opting for laboratory tests, we sought to avoid much of these variables, which could influence the results. Thus, our results suggest that attraction to conspecific may be a important factor for aggregation or association with other tadpoles in *R. icterica*, while tadpoles of *R. ornata* may aggregate indiscriminately or due to other variables than attraction to conspecifics. The behavior differences between two ecologically and genetically closely related species indicate that there may be no generalizations in anuran larvae behavior.

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Author Contributions

Alexandre Polettini Neto. Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intelectual content.

Jaime Bertoluci. Substantial contribution in the concept and design of the study; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intelectual content.

Conflicts of interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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