Rhinella icterica and *Rhinella ornata* (Anura: Bufonidae) tadpoles do not recognise siblings

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Benefits conferred to animals living in groups may be greater if groups are formed by relatives rather than non-relatives, because cooperating with relatives increases the probability of their own genes being passed on to group offspring (inclusive fitness). Non-social aggregations are formed in response to environmental characteristics, while social aggregations are formed from the attraction among individuals. The attraction or repulsion between individuals is mediated by recognition mechanisms, which mediate important ecological processes and behaviours. Here, we conducted laboratory experiments to test if tadpoles of two sympatric bufonids, *Rhinella icterica* and *R. ornata*, are able to recognise siblings. We collected eggs of the two species in the field and raised them in laboratory settings, according to three different methods: siblings and non-siblings reared in separated containers; siblings and non-siblings reared in the same container separated by a plastic net; and eggs from the same spawn reared separately, each one in an individual container. Later, we tested if tadpoles could choose between groups of siblings and non-siblings. The results indicate that tadpoles of neither species were able to discriminate between siblings and non-siblings, regardless of the rearing methods. Therefore, kinship is less important than environmental factors in tadpole aggregation behaviour of these species, and it may be dependent on the balance between costs and benefits. Our results can be used as a start point to better understand tadpole aggregation behaviour and recognition mechanisms in these species.

Keywords: kin recognition, aggregation behaviour, chemical communication, Atlantic Forest

INTRODUCTION

Tadpoles of many anuran species live in groups, which increases individual survival by decreasing predation rate, and increasing foraging and thermoregulation efficiency (Watt et al., 1997; Hoff et al., 1999; Eterovick, 2000; Hero et al., 2001). However, when resources are limited, there are some costs of group formation, as increasing competition, cannibalism, predation, disease susceptibility, and inbreeding (Hamilton & May, 1977; Shykoff & Schmid-Hempel, 1991; Pfennig et al., 1993; Goater et al., 1994).

Non-social groupings are formed in response to environmental characteristics (e.g., feeding microhabitats and temperature gradients), while social groups are formed from attraction between individuals (Wassersug, 1973; Hoff et al., 1999). An aggregation can be formed by genetically related or unrelated individuals (Waldman, 1982; Glos et al., 2007), but benefits conferred to animals living in groups may be greater if groups are formed by relatives than non-relatives, because cooperating with relatives increases the probability of their own genes being passed on to group offspring (inclusive fitness; Hamilton, 1964).

In this context, species that live in groups of related individuals tend to show adaptations that allow kin recognition (Blaustein & O'Hara, 1983; Waldman, 1988). Thus, association between siblings may act in aggregation maintenance through sharing spatial and temporal distribution (indirect recognition), through phenotypic matching (direct recognition), or both (Blaustein & O'Hara, 1983; Waldman, 1988).

Tadpoles of some anuran species discriminate between siblings and non-siblings (reviewed in Blaustein & Waldman, 1992). This discrimination consists of behaviour differences toward relatives of different kinship levels and non-relatives (Waldman, 1988). The adaptive values of this behaviour may be related to increasing and developing the tadpoles' coexistence in related groups (Waldman, 1988; Blaustein & Waldman, 1992). Mechanisms that allow siblings recognition in tadpoles can give them adaptive advantages, as in tadpoles of some species that have more rapid development when living among relatives (Jasienski, 1988; Twomey et al., 2008), and as some cannibalistic tadpoles that prevent predation of relatives (Pfennig et al., 1993).

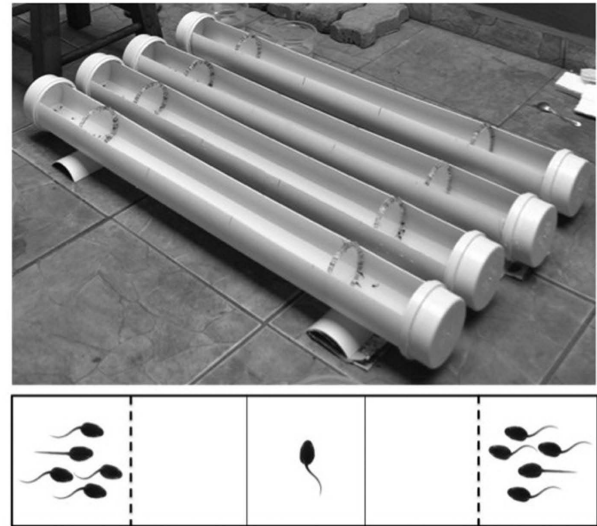Recognition mechanisms in tadpoles are developed

during the embryonic phase or shortly after hatching (Waldman, 1981, 1882; Blaustein & O'Hara, 1982) and it may persist following metamorphosis (Blaustein et al., 1984; Waldman, 1989; Graves et al., 1993). There are three basic types of kin recognition mechanisms (Blaustein & O'Hara, 1983). First, recognition may originate from social or familiar learning mechanisms, a process by which individuals from some familiar groups learn to recognise others from early development stages, even if they have not developed a mechanism to identify their siblings (Waldman, 1982). Second is phenotypic matching, which occurs when an individual learns and remembers a specific characteristic of their own or their relatives (e.g., odour, colour, or particular mark), which may be a similar feature or a noticeable difference. Phenotypic matching is fundamentally different from familiar recognition because they provide recognition of unfamiliar individuals (Blaustein & O'Hara, 1983). The third one relies on specific genes recognition, also provides kin and non-kin recognition. However, this mechanism is innate and is expressed by a phenotypic characteristic (e.g., odour) and different mechanisms can operate isolated or simultaneously (Blaustein & O'Hara, 1983).

Here, we conducted laboratory experiments to test if tadpoles of two toad species, *Rhinella icterica* and *R. ornata* can recognise siblings. *Rhinella icterica* belongs to the *R. marina* group (Maciel et al., 2010), while *R. ornata* is a member of the *R. crucifer* group (Baldissera Jr. et al., 2004). These species have schooling behaviour (Eterovick, 2000; Simon, 2010; pers. obs.), likely living in groups of siblings, because spawn consists of thousands of eggs. They often co-occur in sites within the Atlantic Forest of south-eastern Brazil, where they have a well-known reproductive season, laying eggs in shallow waters (Bertoluci, 1992, 1998; Bertoluci & Rodrigues, 2002; Narvaes et al., 2009). We addressed the following questions: (1) do tadpoles prefer to associate with siblings than non-siblings (kin recognition)?; and (2) does familiarity (prior social contact with non-siblings tadpoles) influence recognition mechanisms?

METHODS

We collected eggs of both species between July and August 2017 at the Boracéia Biological Station (23°38' S, 45°52' W), an Atlantic Forest reserve, São Paulo, south-eastern Brazil. We collected two spawns each of both *Rhinella icterica* and *R. ornata* (ca. 600 eggs of each spawn). Spawn could be easily assigned to species in the field because *R. ornata* has smaller eggs arranged in a single string, while *R. icterica* deposits larger eggs arranged in a double string (Simon, 2010; pers. obs.). We transported eggs to the Laboratório de Zoologia de Vertebrados, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, in plastic pots containing water from ponds where spawns were collected.

Spawn were raised in the laboratory at room temperature, with a natural photoperiod, and with aeration by aquarium pumps. Embryos were between

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

stages 16 and 18 (Gosner, 1960) when they were separated from the rest of the spawn. We used three different rearing methods: (1) tadpoles without prior contact with non-siblings: 300 eggs reared with siblings only, from the same spawn allocated in two 50 L opaque container (one container for each spawn) (2) tadpoles of two different spawns reared in the same container, enabling contact with chemical and visual cues of non-siblings: 150 eggs from each spawn in an opaque 50 L container and each group of tadpoles separated by a plastic net (0.5 mm mesh); and (3) eggs from the same spawn reared separately (n=120), each in a 0.5 L opaque container. Tadpoles were fed once daily with ornamental fish food. Water in each container was changed twice a week to keep the water clean. After metamorphosis, we kept the juveniles in a terrarium, and prior to release at the locations where the eggs were collected.

Experimental trials were conducted between August and September 2017 between 0800 and 1800, using tadpoles between stages 25 and 38. Trials were carried out in four plastic containers (100 × 15 × 10 cm) filled with spring water (pH = 6.3; Fig. 1). At each end of a container a 0.5 mm mesh plastic net was placed, 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 areas.

At the beginning of each trial, one tadpole was placed at the centre of each of the four containers (see similar designs in O'Hara & Blaustein, 1981, 1982; Blaustein & O'Hara, 1982, 1986; Cornell et al., 1989; Saidapur & Girish, 2000; Leu et al., 2013; Rajput et al., 2014; and Pizzato et al., 2016). After 10 minutes of acclimation, we observed tadpole behaviour using a video recording

Table 1. Synthesis of association and recognition tests. Familiar = tadpoles reared in contact with tested tadpole; non-familiar = tadpoles reared without contact with tested tadpole

Experiment	Stimulus group 1	Tested tadpole	Stimulus group 2
Control	familiar siblings	1st rearing method	familiar siblings
1	familiar siblings	1st rearing method	non-familiar non-siblings
2	familiar siblings	2nd rearing method	familiar non-siblings
3	non-familiar siblings	3rd rearing method	non-familiar non-siblings
4	Familiar siblings	1st rearing method	non-familiar siblings

camera (Kodak z990) for 29 minutes, and then measured the amount of time each tadpole remained in the region next to each stimulus group. Each tadpole was tested only once and after each test containers were cleaned and water changed. At each test we turned containers at 90° and inverted the side of each stimulus group, in order to avoid possible environmental influences. Each trial was replicated 32 times during the daytime period on successive days. Four replicates were filmed at a time. The same procedures were repeated for both species.

For each trial, both stimulus groups were chosen considering kinship and familiarity (prior contact) with test-tadpole (Table 1; familiar = reared in contact with test-tadpole; unfamiliar = reared without contact with test-tadpole):

Control: siblings with prior contact vs. siblings with prior contact. All tadpoles from the same spawn and reared together in one container. We expect no difference in tadpole preference to aggregate with either group.

Experiment 1: siblings with prior contact vs. non-siblings without prior contact. Test tadpoles reared without prior contact with non-siblings. One stimulus group formed by tadpoles from the same spawn reared together with test-tadpoles. The other stimulus group is formed by non-siblings of the test tadpole. Through this experiment we tested if the tadpoles of these species prefer to associate with siblings than non-siblings.

Experiment 2: siblings with prior contact vs. non-siblings with prior contact. Siblings and non-siblings reared in the same container, separated by a plastic net. One stimulus group formed by siblings reared together with test-tadpoles. The other stimulus group formed by non-siblings reared with chemical and visual contact of test tadpole. Through this experiment we tested if the contact between siblings and non-siblings during development influences association choice to one of the groups by test tadpoles.

Experiment 3: siblings without prior contact vs. non-siblings without prior contact. Test tadpoles from the same spawn reared separately (isolated). One stimulus group formed by tadpoles from the same spawn as test-tadpoles. The other stimulus group formed by tadpoles from a different spawn of test-tadpole. Through this experiment we tested if the lack of prior contact with other tadpoles influences in test-tadpole choice.

Experiment 4: siblings with prior contact vs. siblings without prior contact. Test tadpoles reared without prior contact with non-siblings. One stimulus group formed by tadpoles reared together with test-tadpoles. The other

stimulus group formed by siblings of test tadpole reared in another container. Through this experiment we tested if familiarity is required to sibling association.

Data consisted of differences between the time spent by the test-tadpole in the compartments located near stimulus groups 1 and 2. The differences between time spent by test-tadpoles near each stimulus group, as well as the mean of differences and the pseudo median of differences, when negative, indicate a longer time spent by tadpoles near stimulus group 2, whereas, when positive, they indicate a longer time spent by tadpoles near stimulus group 1. We verified if data of each experiment corresponded to normal distribution by Shapiro-Wilk test. We used a paired t-test to analyse data of Control, and experiments 1 and 4 with *R. icterica* tadpoles and in Control, experiments 2, 3 and 4 with *R. ornata*; and Wilcoxon signed-rank test to analyse data of experiments 2 and 3 with *R. icterica* and experiment 1 with *R. ornata*. Tests were two-tailed. Analyses were performed in R platform (R Core Team, 2017).

RESULTS

Data varied more for *R. ornata* than *R. icterica*, but all experiments for both species exhibited random pattern or non-significant differences between the time spent by tadpoles close to siblings or non-siblings (Figs. 2 and 3). In Experiment 1 with *R. ornata*, tadpoles remained considerably longer near non-siblings, but the difference was not significant. Results of experiments 2 and 3 further confirm this pattern.

In the Control, experiments 1 and 4 with *R. icterica* and in the Control, experiments 2, 3 and 4, with *R. ornata*, the mean of differences did not differ (Tables 2 and 3). Similarly, in experiments 2 and 3 with *R. icterica* and in experiment 1 with *R. ornata*, the pseudomedian of differences did not differ (Tables 2 and 3). These results indicate that regardless of previous contact with siblings the tadpoles of *Rhinella icterica* and *Rhinella ornata* do not exhibit spatial attraction to siblings. This suggests that kinship in these tadpoles is not relevant for aggregation behaviour.
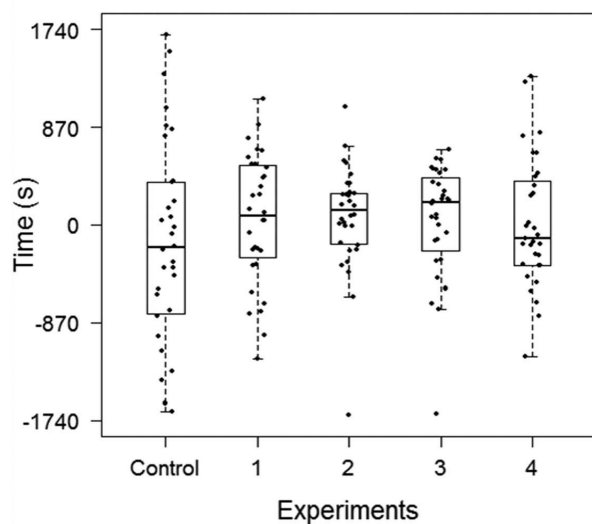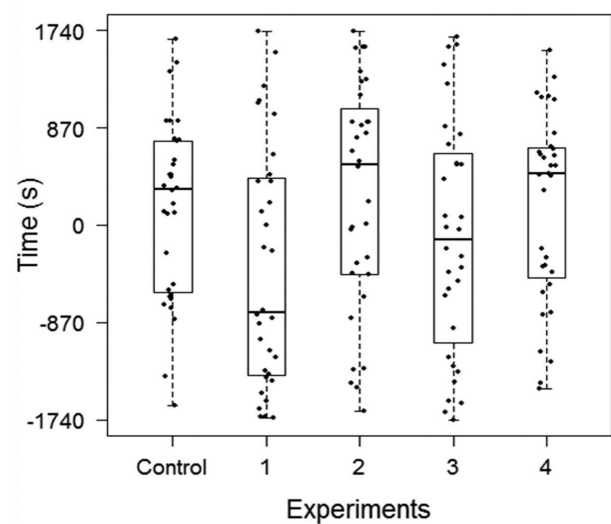
DISCUSSION

Tadpoles of *Rhinella icterica* and *R. ornata* may aggregate in response to factors other than sibling association. Other stimuli to aggregate can be related with reduction of

Table 2. Statitstic tests results for each experiment with *Rhinella icterica* tadpoles. df = degrees of freedom; CI = confidence interval

Experiment	Shapiro-Wilk test	Paired-t test (t) or Wilcoxon (V)	Mean of differences	Pseudomedian of differences	CI (95 %) Inf. Lim	Sup. Lim.
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 = 0.72	t = 0.44 ; df = 31; p= 0.66	44,8	-	-162.26	252.01
2	W = 0.89 p = 0.003	t = 348 ; df = 31; p= 0.12	-	111	-36	235
3	W = 0.88 p = 0.002	t = 295 ; df = 30; p= 0.36	-	87.63	-110	273
4	W = 0.96 p = 0.37	t = 0.11 ; df = 31; p= 0.91	11.31	-	-195.11	217.73

Table 3. Statitstic tests results for each experiment with *Rhinella ornata* tadpoles. df = degrees of freedom; CI = confidance interval

Experiment	Shapiro-Wilk test	Paired-t test (t) or Wilcoxon (V)	Mean of differences	Pseudomedian of differences	CI (95 %) Inf. Lim	Sup. Lim.
Control	W = 0.96 p = 0.47	t = 1.08; df = 31; p = 0.28	154.37	-	-135.44	444.19
1	W = 0.91 p = 0.01	V = 160; df = 31; p = 0.052	-	-408.5	-853	1
2	W = 0.93 p = 0.059	t = 1.49; df = 31; p = 0.14	270.68	-	-97.81	639.19
3	W = 0.95 p = 0.18	t = -0.60; df = 31; p = 0.55	-111.31	-	-488.86	266.24
4	W = 0.94 p = 0.07	t = 1.09; df = 31; p = 0.28	164.68	-	-141.92	471.30

Figure 2. Box plot with dots, representing experiments 1, 2, 3, 4 and control executed with *Rhinella icterica* tadpoles. In each plot, points correspond to the difference between time spent by tested tadpole in each trial close to stimulus group 1 and 2. Positive values correspond to a longer time spent by test-tadpole close to stimulus group 1, while negative values correspond to a longer time spent by the test-tadpole close to stimulus group 2.

Figure 3. Box plot with dots, representing experiments 1, 2, 3, 4 and control executed with *Rhinella ornata* tapoles. In each plot, points correspond to the difference between time spent by tested tadpole in each trial close to stimulus group 1 and 2. Positive values correspond to a longer time spent by the test-tadpole close to stimulus group 1, while negative values correspond to a longer time spent by the test-tadpole close to stimulus group 2.

predation risk and response to predator cues (Watt et al., 1997), thermotaxy (Wassersug, 1973), facilitating access to food particles (as in tadpoles of *Rhinella pombali*; Eterovick, 2000), and reinforcement of aposematism (Wassersug, 1981).

Because there is very little information about larval ecology and schooling of these species, information from genetically similar species may help explain the absence of sibling attraction in these species. In *Rhinella marina*, which belongs to the same group as *R. icterica* (Maciel et al., 2010), there was a weak tendency of association with siblings (Raven et al., 2017). In tests of choice between a siblings group and an empty compartment, tested tadpoles spent significantly more time near sibling group, whereas when submitted to choice between non-siblings and an empty compartment, tested tadpoles exhibited a random distribution. However, in a third test tadpoles failed to discriminate between siblings and non-siblings. In combination with the results of other experiments, they conclude that tadpoles of *R. marina* aggregate in response to abiotic factors such as light levels, temperature and structural complexity.

Although kin recognition among tadpoles occurs in several bufonids (e.g., Waldman, 1981, 1982; O'Hara & Blaustein, 1982; Saidapur & Girish, 2000; Gramapurohit et al., 2006; Eluvathingal et al., 2009), species of *Rhinella* do not discriminate kin (Raven et al., 2017; present study). In tadpoles of other anuran families, presence of this behaviour is also variable even within the same genus, such as *Lithobates* (Ranidae; Waldman, 1984; Fishwild et al., 1990) and *Spea* (Scaphiopodidae; Pfennig, 1990; Hall et al., 1995).

Tadpoles of two bufonid species, (*Anaxyrus americanus* and *A. boreas*) recognise siblings when it was reared only with siblings, but not when it was reared with siblings and non-siblings together (Waldman, 1981; O'Hara & Blaustein, 1982). In the present study the results were similar for both *R. icterica* and *R. ornata* even with different rearing methods, indicating that previous contact does not influence the choice of aggregation with more or less related tadpoles. In Experiment 4, results were also similar for both species: tadpoles were randomly distributed, indicating that prior contact is not an important factor to sibling association in tadpoles of these species.

The absence of kin recognition in tadpoles of *R. icterica* and *R. ornata* suggests that kinship is less important than environmental factors in the aggregation behaviour. However, even with the presence of recognition, the decision of which action to take is often context-dependent, in other words, it is expected that an action (attraction or repulsion) will only occur whether its cost does not exceed the benefits (Waldman, 1987, 1988; Reeve, 1989).

For some authors the absence of sibling discrimination among tadpoles in laboratory tests is due to absence of stimuli to aggregation behaviour (Blaustein et al., 1993). When there are few selective pressures that lead to aggregation, sibling association tend to be weak, because tadpoles get few benefits from this behaviour (Blaustein and O'Hara, 1986).

Both recognition processes and schooling may vary within the same species depending on some factors, such as presence and density of predators (Wrona, 1991; Fitzgerald, 1992; Watt et al., 1997), diets (Gamboa et al., 1990; Pfennig, 1990), development stage (Blaustein & O'Hara, 1986; Rautio et al., 1991; Blaustein et al., 1993; Nicieza et al., 1999), resource distribution, and temperature variation (Hokit & Blaustein, 1997). For example, *Lithobates sylvaticus* tadpoles recognised and were attracted to relatives in laboratory experiments, but in natural environments they demonstrated both attraction and repulsion to relatives in different ponds (Waldman, 1984; Halverson et al., 2006).

Our experiments controlled most environmental variables that could influence spatial preference by tadpoles, thus focusing only on presence or absence of kin recognition traits. Therefore, the lack of attraction to siblings by these tadpoles could be due to a lack of stimulus and selective pressures for schooling behaviour. Another explanation could be that the recognition mechanisms in these species act in high levels, as conspecifics groups. Polettini Neto & Bertoluci (2021) found that tadpoles of *Rhinella icterica* have preference to associate with conspecifics, while tadpoles of *R. ornata* do not show any discrimination between conspecifics and heterospecifics. Our results can be used as a start point to better understand tadpole aggregation behaviour and recognition mechanisms in these species, and more information on larval ecology of these species will contribute for more accurate interpretations of these behaviours.

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