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Is the response of a Neotropical poison frog (*Ranitomeya variabilis*) to larval chemical cues influenced by relatedness?

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Offspring recognition via chemical cues is widely known among vertebrates. In order to test this capacity in the poison frog *Ranitomeya variabilis*, we analysed whether parental frogs deposit their tadpoles with closely related rather than unrelated tadpoles. We conducted pool-choice experiments with one pool presenting chemical cues of a tadpole previously found at the same location. Tadpoles were most frequently deposited in pools without tadpole cues and results of genetic analyses did not lead to the conclusion that frogs consider relatedness when choosing where to deposit tadpoles. We suppose that frogs discriminate possible tadpole deposition sites by location rather than by offspring recognition.

Key words: chemical recognition, Dendrobatidae, offspring identification, parental care, phytotelmata

In species that show parental care, the discrimination between offspring and unrelated young is of high importance for an individual's fitness (Beecher, 1990; Holmes, 1990; Stynoski, 2009). Neotropical poison frogs (Aromobatidae, Dendrobatidae) demonstrate highly evolved forms of parental care (e.g., Summers, 1990; Brust, 1993; Summers & McKeon, 2004, 2006; Brown et al., 2008), often including the transport of single tadpoles from egg deposition sites to phytotelmata (small water bodies in plants such as leaf axils of bromeliads; Varga, 1928). In several species, tadpoles show cannibalistic behaviour towards smaller conspecifics, which benefits the tadpole both by eliminating competitors as well as by compensating for limited food resources (Caldwell & de Araújo, 1998; Summers, 1999). Parental frogs of the species *Ranitomeya variabilis* are able to recognise and avoid phytotelmata already occupied by cannibalistic larvae for tadpole deposition through chemical cues (Schulte et al., 2011). However, while chemical cues of cannibalistic tadpoles led to avoidance of phytotelmata in the rainy season, they were preferred for tadpole depositions during the dry season (Schulte & Lötters, 2014). This could be interpreted as an attempt to feed older tadpoles by sacrificing younger conspecific larvae to them.

In the present study, we tested whether parents preferentially deposit younger tadpoles with older tadpoles when chemical cues of their own offspring are present. Kin recognition mediated by chemical cues is widely known among vertebrates (e.g., Porter & Moore, 1981; Gustin & McCracken, 1987; Levy et al., 2004; Burger et al., 2011; Krause et al., 2012). In some anuran species, larvae prefer grouping with siblings over non-siblings (Blaustein & O'Hara, 1982; Waldman, 1985; Eluvathingal et al., 2009). We hypothesise that adult *R. variabilis* discriminate their own tadpoles via chemical cues, and thus deposit younger tadpoles with related older tadpoles rather than with non-related tadpoles.

Between 21 June and 23 August 2010 and 25 April and 7 June 2011 (i.e. in the dry seasons) we placed 54 artificial phytotelmata in a premontane late-stage secondary forest at km 32 on the Tarapoto-Yurimaguas road, close to the upper Cainarachi River, Región San Martín, Peru. We used opaque polypropylene plastic cups (200 ml volume, 10 cm height, 7 cm in diameter), fixed pairwise to trees at 0.5–1.5 m above the ground. They were filled with 50 ml water each and one cup per pair received a tadpole hidden either in an empty tea bag (Cilia® paper filter bags, article number 1576645002) or underneath a second opaque plastic floor perforated with tiny holes (two methods were used in case one method failed). This way the chemical cues of the tadpole could reach into the water, but the tadpole could not be seen or otherwise sensed by the frogs. The other cup was equipped with a tea bag or a second floor, but no tadpole. The tadpoles in the experiment were previously deposited by a male frog in a single artificial phytotelm hanging at the spot that was chosen for a pair of cups. They were left in the cup during the whole experiment (hereafter referred to as "stationary tadpoles"). Stationary tadpoles were fed once a week ad libitum with flaked fish food (Tetramin Tetra®, article number 329155). The food was placed within the areas where the tadpoles were hidden (i.e. in the tea bags or under the second floors). Some fish food was also placed in the cups not containing tadpoles.

The artificial phytotelmata were checked every 3–8 days for newly deposited tadpoles (hereafter referred to as "secondary tadpoles"). Secondary tadpoles were removed from cup pairs and a tissue sample (i.e. a tail

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clip) was collected from each tadpole. Tissue samples of stationary tadpoles were taken at the end of the experiments. To be able to define the proportion of genetic relatedness, embryos were randomly collected from egg clutches assumed to be full siblings (*n*=7 clutches, 19 tadpoles). Furthermore, toe clips were taken from presumably unrelated adult frogs (five individuals each from two forest sites separated by a coffee plantation).

Tissue samples were stored in 99% ethanol at room temperature. DNA was extracted with the Qiagen DNEasy blood and tissue kit. All specimens were genotyped using seven polymorphic microsatellite markers developed for *R. variabilis* (RvarA09, RvarB01, RvarD01+, RvarE04, RvarF01, RvarF08, RvarG12; Brown et al., 2009a). The number of loci corresponds to the average of six to ten loci used in studies related to kinship, parentage and behaviour (Rieseberg et al., 2012; e.g. compare Ringler et al., 2012). For PCR reactions, DNA was diluted 1:10 with ultrapure water and amplifications were performed in a Multigene Gradient Thermal Cycler (Labnet) using the Qiagen Multiplex Mastermix. Multiplex PCRs were run with combinations of two loci with similar annealing temperatures (see Brown et al., 2009a), using 10 µl reaction mixtures containing 1.4 µl diluted DNA, 5.0 µl MultiplexMasterMix, 2.6 µl water and 0.5 µl of each primer. PCR conditions were those recommended by the manufacturer. PCR products were genotyped on a MegaBACE 1000 automated sequencer (GE Healthcare) and alleles were sized using FragmentProfiler 1.2 (Amersham Biosciences) with ET 550-R as the size standard. The quality of the genotypic dataset (i.e. occurrence of null alleles, allelic dropout and stuttering) was investigated using the program MICRO-CHECKER (v.2.2.3; van Oosterhout et al., 2004).

Pairwise relatedness between grouped individuals was analysed with the KINSHIP relatedness estimator (Queller & Goodnight, 1989; Goodnight & Queller, 1999) implemented in the program KINGROUP (v.2; Konovalov et al., 2004). Values of calculated relatedness (r_{QG}) range from -1 to +1, with positive/negative values indicating that the two individuals under consideration have a higher/lower probability of kinship than random pairs of the dataset. Pairwise relatedness should be on average r_{QG}=0.5 for full siblings, r_{QG}=0.25 for half siblings, and r_{QG}=0.0 for unrelated individuals. The significance level for each comparison was calculated with a simulation routine (simulating 17,000 pairs) integrated into the program. For further clarity the average relatedness among the putatively unrelated individuals as well as among the embryos from the same clutches was specified. To determine if secondary tadpoles were deposited differently based on relatedness to the stationary tadpole (i.e. alone vs. with the stationary tadpole) we calculated a contingency table using the Fisher's exact test (Fisher, 1922).

Table 1. Deposition sites of secondary tadpoles (alone in empty cups or in cups with stationary tadpoles) and relatedness (r_{QG}) between stationary and secondary tadpoles. For relatedness between secondary tadpoles, r_{QG} is only shown for significantly related individuals. *p<0.05, **p<0.01, ***p<0.001. Stationary tadpoles 1–4 were kept underneath a second floor, stationary tadpoles 5–9 within a tea bag.

Stationary tadpole	Secondary tadpole	Deposition of secondary tadpole	r_{QG} stationary and secondary tadpoles	Related secondary tadpoles	r_{QG} related secondary tadpoles
1	1	alone	0.182		
	2	alone	0.110		
	3	with stationary	0.198		
2	1	alone	-0.133		
	2	alone	-0.034	1, 2	0.285 *
3	1	alone	-0.223		
	2	alone	-0.106		
	3	alone	-0.100	2, 3	0.465 ***
4	1	alone	-0.004		
	2	alone	-0.005		
	3	alone	0.495 ***		
	4	alone	0.341 *	3, 4	0.487 ***
5	1	alone	0.410 **		
	2	alone	-0.206		
6	1	alone	-0.121		
7	1	alone	0.048		
8	1	alone	0.037		
9	1	alone	-0.204		

Relatedness was estimated as r_{QG}=-0.025 (±0.120 SD) among putatively unrelated individuals and r_{QG}=0.391 (±0.173 SD) among embryos (i.e. full siblings). Tadpoles from the same pair of cups that were estimated as significantly related had a range of r_{QG}=0.285–0.495, and those that were estimated as unrelated had a range of r_{QG}=-0.223–0.198. Among the pairs of cups installed in 2010 and 2011, only nine received secondary tadpole depositions, but some were used more than once (after previously deposited tadpoles were removed and the water was changed). In total, 18 secondary tadpoles were deposited, of which only one was deposited in the cup containing a stationary tadpole. This secondary tadpole was not significantly related with the stationary tadpole (r_{QG}=0.198). Of those secondary tadpoles that were placed alone, 14 were unrelated and three were related to the stationary tadpole (Table 1). Three of the four pairs of the secondary tadpoles placed consecutively in the same cup pairs were related to each other (Table 1).

Because nearly all of the secondary tadpoles were deposited in the empty cups and only three of them were related to the stationary tadpole, we did not find a significant difference of deposition patterns among related and non-related tadpoles (Fisher's exact test: *p*=0.222).

Although our study was conducted during the dry season, tadpoles were generally deposited alone rather than in the cups containing a hidden tadpole. This might be due to pseudo-replication, since the cups were used more than once. While the only secondary tadpole deposited together with a stationary tadpole was not a sibling, three secondary tadpoles that were deposited in the empty cup were siblings of the associated stationary tadpole. Therefore we cannot draw conclusions about whether parental *R. variabilis* are able to distinguish between their own offspring and unrelated tadpoles by means of chemical cues. However, some of the secondary tadpoles placed consecutively in the same cup were related to each other, which might indicate that frogs use location rather than chemical cues for discrimination. The use of location rather than chemical cues was however improbable for the secondary tadpoles that were deposited first, because the arrangement of the artificial phytotelmata was changed by replacing the originally chosen cup with a pair of new cups.

Stynoski (2009) and Poelman & Dicke (2007) showed for other poison frog species (*Oophaga pumilio* and *Ranitomeya amazonica*) that parents were unable to discriminate between kin and non-kin, instead using indirect recognition via location. Because the breeding resources used by poison frogs are spatially separated and therefore preclude the possibility that the offspring change sites, direct recognition of offspring may be less important than, for example, in free-ranging species living in big flocks or colonies (Gustin & McCracken, 1987; Burger et al., 2011). Nevertheless, *R. variabilis* repeatedly deposit clutches in the same phytotelm that already contains a tadpole (Schulte, 2014). When the tadpole in the phytotelm regularly used for clutch deposition dies and is replaced by a new, non-familiar tadpole, offspring recognition would be advantageous to prevent parents from depositing eggs that are likely eaten by the new tadpole (see Brown et al., 2009b).

Although we did not find evidence that *R. variabilis* can discriminate between offspring, our study does not exclude that it is possible. Further clarification could come from an ex-situ choice test with chemical cues of related offspring versus chemical cues of an unrelated tadpole. Given that many species are able to discriminate their own offspring by means of chemical cues (e.g. McKaye & Barlow, 1976; Kaitz et al., 1987; Loughry & McCracken, 1991; Lévy et al., 1996; Neff & Sherman, 2003), the possibility that anurans also possess this ability persists, even if so far kin-discrimination has only been shown between siblings (i.e. tadpoles; Blaustein & O'Hara, 1982; Waldman, 1982, 1985; Cornell et al., 1989; Pfennig & Frankino, 1997; Pfennig, 1999; Eluvathingal et al., 2009).

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