

# **Chemical communication in the reproductive behaviour of Neotropical poison frogs (Dendrobatidae)**

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I dedicate my thesis to my grandparents, Maria and Josef Schulte, Agnes and Hubert Koch, as well as my grandaunt, Martha Hörster. They had a profound impact on my interest in nature, travelling, and studying, and supported me with their love and pride.



# Table of contents

Acknowledgements .....	1
Preliminary remarks .....	3
Introduction .....	5
Chapter I     A danger foreseen is a danger avoided: how chemical cues of different tadpoles influence parental decisions of a Neotropical poison frog .....	34
Chapter II     The chemistry of parental care: the characterization of chemical communication between poison frogs and tadpoles .....	54
Chapter III    Avoiding predation: the importance of chemical and visual cues in poison frog reproductive behaviour .....	80
Chapter IV    Do poison frogs recognize chemical cues of the other sex or do they react to cues of stressed conspecifics? .....	98
Chapter V     The power of the seasons: rainfall triggers parental care in poison frogs .....	110
Chapter VI    Feeding or avoiding? Facultative egg-feeding in a Peruvian poison frog ( <i>Ranitomeya variabilis</i> ) .....	131
Chapter VII   The response of a Neotropical poison frog ( <i>Ranitomeya variabilis</i> ) to larval chemical cues is not influenced by kinship .....	147
Conclusions .....	157
Summary/Zusammenfassung .....	161
Curriculum Vitae .....	167
Erklärung .....	169

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## Preliminary remarks

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The thesis consists of a general introduction including a summarized overview about the scientific background and the aims of the thesis, a general conclusion as well as seven chapters written as scientific papers. Six of the latter are listed below as published or accepted for publication in peer-reviewed scientific journals or represented as unpublished manuscripts, one is published as a chapter of a peer-reviewed book. The authors and their specific contributions to the collection and analysis of data, along with the preparation of the manuscripts are represented in the following list, as well.

**Schulte, L.M.** & Lötters, S. A danger foreseen is a danger avoided: how chemical cues of different tadpoles influence parental decisions of a Neotropical poison frog. *Animal Cognition*, doi 10.1007/s10071-013-0659-2 (online first) – published

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*Performance of the experiments: LMS*

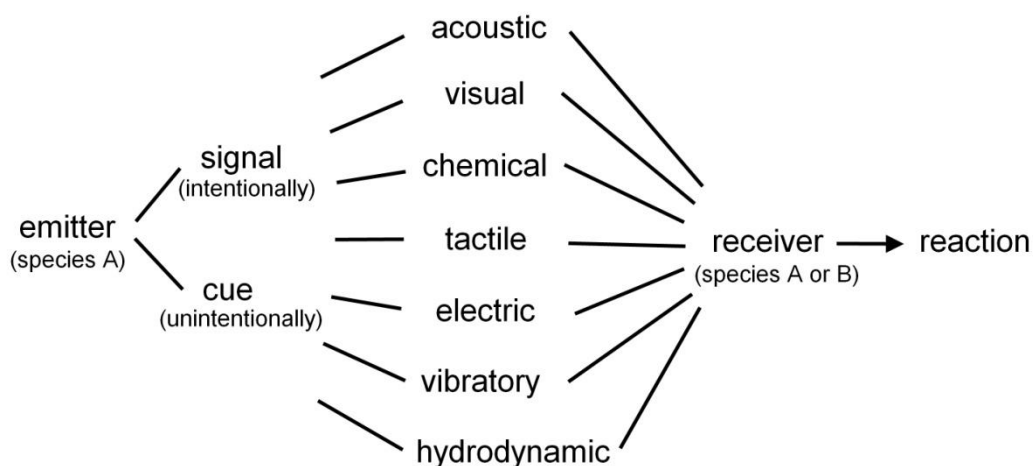
*Analysis of the data: LMS*

*Writing of the paper: LMS and MV*

# Introduction

A crucial aspect of animal behaviour is the interaction between animals on the intra- and interspecific level. Such interactions are triggered through communication, defined by Wilson (1970) as action on the part of one organism that alters the probability pattern of behaviour in another one. The evolution of communication systems allows individuals to make decisions that are based on the behaviour, morphology or physiology of other organisms (Endler, 1993). Those communication systems have evolved between animals belonging to the same as well as to different species (Bradbury and Vehrencamp, 2011; Peake, 2005) and the interests of the communicating individuals are not necessarily in accordance with each other, i.e. communication can be honest or dishonest (Dawkins and Krebs, 1978; Searcy and Nowicki, 2005).

Communication between two (or more) individuals of the same or different species starts with the emitter producing either a signal or a cue (Fig. 1). Signals are intentionally emitted in order to be recognized by another animal and are advantageous to the emitting individual (Otte, 1974; Steiger et al., 2011). Cues on the other hand are unintentionally released to the environment and the recognition by another animal might have a neutral or damaging effect to the emitter (Steiger et al., 2011).



**Fig. 1** Fundamental steps of communication. The emitting individual generates a signal or a cue that triggers a reaction of the receiving animal. The stimulation of the receiver can occur in one of the seven ways shown in the illustration. Emitter and receiver may belong to the same or to different species. Own scheme.

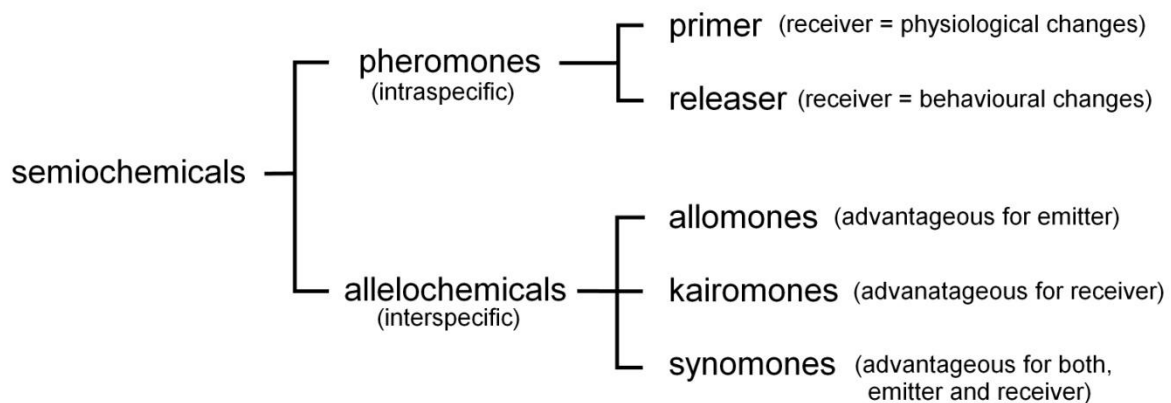


However, if the reaction of the receiving animal is of advantage to the emitter, the evolution of a cue towards signalling is expected (Wyatt, 2010). The other important aspect for the functioning of communication is the ability of the receiving organism to be able to recognize the emitted signal or cue. Recognition ranges from the identification of prey and predators to the identification of conspecifics, gender, group members, kin or offspring and it implies that the receiver has an innate or learned memory for the relevant message that triggers a correspondent response (Bradbury and Vehrencamp, 2011; Guilford and Dawkins, 1991).

The nature of the message, i.e. the signal or cue emitted or received can be acoustic (e.g. avian songs or anuran calls; Narins et al., 2006; Todt and Naguib, 2000), visual (e.g. colour changes in chameleons or bioluminescence in anglerfishes; Stuart-Fox and Moussalli, 2008; Wilson, 1937), tactile (e.g. allogrooming in primates or antennating in ants; Mailleux et al., 2011; Weber, 1973), electric (e.g. prey detection in platypus or conspecific recognition in knifefishes; Aguilera et al., 2001; Proske et al., 1998), vibrational (e.g. courtship behaviour in wolf spiders or fiddler crabs; Aicher and Tautz, 1990; Kotiaho et al., 1996), hydrodynamic (e.g. prey chasing in catfish or seals; Dehnhardt et al., 2001; Pohlmann et al., 2001) or chemical (e.g. offspring recognition in sheep or scent marking in bumble bees; Burger et al., 2011; Saleh et al., 2007), as well as a combination of several of these components (Johnstone, 1996; Narins et al., 2003; Uetz and Roberts, 2002).

## Chemical communication

Of all communication systems listed above, chemical communication is regarded as the oldest and the most widespread form, even present in unicellular organisms (Agosta, 1992; Wyatt, 2003). Chemical compounds involved in chemical communication are summarized as semiochemicals and can be divided into pheromones, active between individuals of the same species (changing the physiology or the behaviour of the receiver), and allelochemicals, active between individuals of different species. The latter can be divided into allomones, kairomones and synomones, depending on who benefits: the emitter, the receiver or both emitter and receiver (Fig. 2).



**Fig. 2** Classification of semiochemicals. Modified after Nordlund et al. (1981). Examples for primer pheromones can be shown in caste systems of social insects (e.g. pheromonal repression of ovary development in bees; Wossler and Crewe, 1999) and releaser pheromones are for instance aggregation pheromones that trigger the clustering behaviour of marine animals (e.g. in barnacles; Clare and Matsumura, 2000). Allomones can be imitated pheromones produced by predators to attract prey (e.g. in spiders; Haynes et al., 2002), while kairomones are usually not produced on purpose by the emitter, since they might be used for example by prey to detect predators (e.g. in fish; Chivers and Smith, 1993). An example for synomones is the attraction of symbionts in mutualistic systems (e.g. attraction of anemone fish by sea anemones; Murata et al., 1986).

Especially in invertebrates chemical communication is widespread and extensively studied both on land (e.g. in moth: Butenandt and Hecker, 1961; snails: Croll, 1983; ants: Hölldobler et al., 2001; earthworms: Jiang et al., 1989; ticks: Louly et al., 2008; bees: Nunes et al., 2012; beetles: Raffa, 2001) and in water (e.g. in sanddollars: Burke, 1984; sea cucumbers: Hamel and Mercier, 1996; polychaetes: Hardege et al., 2004; sea hares: Painter et al., 2004; sponges and corals: Porter and Targett, 1988; damselfly larvae: Wisenden et al., 1997; shrimp: Zhang et al., 2012). But there are also innumerable examples for chemical communication in vertebrates. In terrestrial vertebrates, mammals in particular are known for the utilisation of chemical signals, for example for mate recognition (e.g. Talley et al., 2001; Wedekind and Furi, 1997), territorial scent marking (e.g. Gosling and Roberts, 2001; Lledo-Ferrer et al., 2011) and offspring or group recognition (e.g. Brennan and Kendrick, 2006; Hurst et al., 2001; Porter et al., 1991). But there is also evidence that chemical communication is of importance for example during mate choice in reptiles (e.g. Martín and López, 2013; Mason et al., 1989) as well as during sex and breeding behaviour in birds (e.g. Balthazart and Taziaux, 2009; Bonadonna and Sanz-Aguilar, 2012; Caspers and Krause, 2010). In fishes, the

only purely aquatic vertebrate group, chemical communication is particularly widespread. This might be a consequence of the fact that chemicals are ideally dissolved and dispersed in aquatic environments and that their use is especially advantageous in turbid water and highly structured habitats or for species with poor developed visual senses (Wisenden, 2000). Fishes are able to locate potential mates (e.g. Fisher and Rosenthal, 2006) and avoid injured conspecifics (e.g. Mirza and Chivers, 2003) or predators (e.g. Mathis et al., 1993) by means of chemical signals and cues.

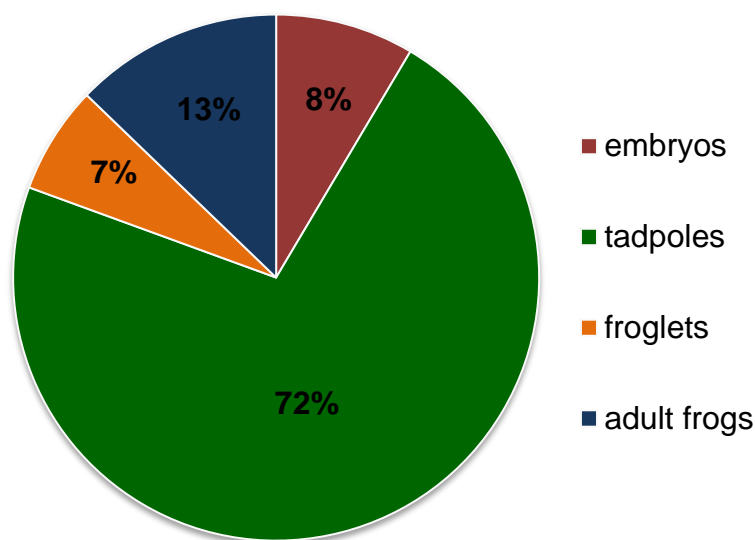
In amphibians, the transition group between terrestrial and aquatic vertebrates, research is highly focused on the order Urodela, i.e. newts and salamanders. Their usage of courtship pheromones is widely known and well-studied (e.g. Caspers and Steinfartz, 2011; Feldhoff et al., 1999; Houck, 1986; Rollmann et al., 2000). But also conspecific recognition (e.g. Secondi et al., 2005), territorial marking (e.g. Gautier and Miaud, 2003) and predator recognition (e.g. Orizaola and Brana, 2003) based on chemical compounds is documented for this group. For the order Gymnophiona (caecilians) there is only one species known to use chemicals signals (Warbeck, 2002; Warbeck et al., 1996; Warbeck and Parzefall, 2001), but since the animals belonging to this order are mostly blind and probably deaf, they almost certainly depend on chemical (and tactile) communication (Wells, 2007).

The anurans, i.e. frogs and toads, forming the third amphibian order, are particularly known for acoustic communication (e.g. Gerhardt and Huber, 2002; Narins et al., 2006). But both, larval and adult anurans are also shown to use and produce a wide range of chemical cues and signals.

## Chemical communication in anurans

That anurans have a sense of smell and are able to detect chemical compounds was already proved in 1914 by Jonathan Risser. He showed that adult *Anaxyrus americanus* (Bufonidae) were able to distinguish between different aromatic oils and that this ability disappeared when he cut the toads' olfactory tracts. He further demonstrated that tadpoles of the same species were only able to find hidden food when their nares were not plugged with vaseline. Other studies about the general sense of smell in anurans were conducted for *Xenopus laevis* (Pipidae) by Altner (1962) and for *Incilius valliceps* (Bufonidae) by Grupp (1976). The latter

furthermore showed that *I. valliceps* as well as other species are able to find their breeding pools by the sense of smell (Grubb, 1970, 1973a,b). Similar findings for olfactory location of breeding pools or the own home range could be shown for other anurans, as well (e.g. Dole, 1968; Forester and Wisnieski, 1991; Ishii et al., 1995; Oldham, 1967).



**Fig. 3** Percentage of studies about the usage of chemical cues or signals in different anuran stages. Until September of 2013 more than 200 studies about behavioural or morphological responses of anurans towards con- or heterospecific chemical cues or signals were published (including the already published studies of this thesis). Studies where the influence of non-chemical cues could not be excluded (e.g. visual cues from caged sender animals) were not included here. Own scheme.

Regarding only semiochemicals (i.e. pheromones and allelochemicals) that trigger physiological or behavioural reactions in anurans, there are more than 200 studies to date. The majority of these studies refer to the influence of chemical cues or signals on tadpoles, and only few publications are available for embryos, adults or juvenile frogs (own unpublished data; Fig. 3). The first study treating anuran semiochemicals was published in the late 1940s. Eibl-Eibesfeldt (1949) showed that *Bufo bufo* tadpoles reacted with avoidance behaviour towards crushed conspecifics (or conspecific “skin juice”), but not towards crushed heterospecifics. Hrbáček independently observed the same behaviour and successfully extracted the chemical compounds responsible for this reaction with ether (Hrbáček, 1950). Since that time a great deal of studies focused on alarm cues. Several authors confirmed the

effect of alarm substances on *B. bufo* tadpoles (e.g. Kulzer, 1954; Manteifel, 2001; Pfeiffer, 1966) as well as on other bufonid tadpoles (e.g. Hews and Blaustein, 1985; Mirza et al., 2006). Some authors further confirmed that bufonid tadpoles only avoided crushed conspecifics or congeners (Petranka, 1989; Pfeiffer, 1966; Summery and Mathis, 1998). Similar experiments with *Rana* tadpoles did not result in the same flight or avoidance behaviour as shown for bufonids, but in decreased activity and earlier metamorphosis (Ferrari et al., 2007; Kiesecker et al., 2002; Kiesecker et al., 1999). Only few other than bufonid or ranid tadpoles were also tested for alarm responses towards hurt conspecifics (e.g. *Discoglossus*: Eibl-Eibesfeldt, 1962; *Hyla*: Schoepfner and Relyea, 2005; *Smilisca*: Summery and Mathis, 1998).

Use-oriented research is conducted with alarm substances produced by *Rhinella marina* (formerly *Bufo marinus*). In Australia (where *R. marina* is invasive), tadpoles of this species are negatively affected by chemicals of crushed conspecifics (Hagman et al., 2009; Hagman and Shine, 2009b), while native Australian species (e.g. *Limnodynastes peronii*, *Litoria dentata*, *Pseudophryne coriacea*) do not show a negative response to this substance (Hagman and Shine, 2008). For this reason it is discussed to use the alarm cues of *R. marina* as a possible pest control (Crossland and Shine, 2012; Hagman and Shine, 2009a). But not only tadpoles show reactions towards alarm substances. Touchon et al. (2006) showed that embryonic *A. americanus* accelerated hatching when confronted with crushed conspecific eggs as well as with crushed con- or heterospecific tadpoles. Mandrillon and Saglio (2007; 2009) on the other hand showed for *Rana temporaria* (Ranidae) that embryos delayed hatching when exposed to crushed conspecific tadpoles. The avoidance of injured conspecifics in just metamorphosed froglets as well as in adult frogs could be shown for *Pseudacris regilla* (Chivers et al., 2001; Wirsing et al., 2005).

While in most studies the chemical alarm substances are described as unintentionally released alarm cues, Fraker et al. (2009) showed that the release of these chemicals is actually an active secretory process during which two different peptides are combined with each other. When already dead or euthanized tadpoles were crushed, no behavioural effect could be elicited in conspecific individuals, i.e. the alarm substance was not released. This means the right denomination would be alarm signals rather than alarm cues. Besides the study of Fraker et al. (2009) and a recent study of Mirza et al. (2013), to the best of my knowledge there have

been no further attempts to decode the alarm pheromones since the first attempts of Hrbáček (1950).

To examine the effects of chemical alarm substances on anurans in a more concrete ecological context, research was further focused on a combination of alarm signals and predator cues. While various studies have shown that kairomones of potential predators alone trigger avoidance (e.g. Petranka et al., 1987; Swart and Taylor, 2004), decreased activity (e.g. Burgett et al., 2007; Gallie et al., 2001; Sharma et al., 2008) or morphological changes (e.g. Barry, 2011; Gómez and Kehr, 2012), others found that predators were only recognized after they fed on con- (or hetero-) specifics, i.e. after inducing the release of alarm substances. Examples for the latter are *Rana* spp. tadpoles reacting with activity reduction towards chemicals released by predatory damselfly larvae when fed with tadpoles (Fraker, 2008a, b; Peacor, 2006; Takahara et al., 2008; Winkler and van Buskirk, 2012) but not (or much less) when fed with invertebrates (Chivers and Mirzal, 2001; Ferland-Raymond and Murray, 2008). These findings were confirmed by Wilson (1993) using newts and Marquis et al. (2004) using crayfish as predators. Eklöv (2000) further showed that the avoidance behaviour was stronger the more conspecifics were consumed. A similar finding was documented for *Agalychnis* tadpoles (Hylidae) that showed stronger phenotypic reactions the more conspecific biomass was consumed by its larval dragonfly predators (McCoy et al., 2012). A reaction to predators that fed on heterospecific tadpoles could be confirmed for several species, too (Ferland-Raymond and Murray, 2008; Laurila et al., 1998), but sometimes not to the same extent as to conspecific tadpoles (Fraker, 2009).

Gomez-Mestre and Díaz-Paniagua (2011) as well as Nunes (2013) restricted the requirement of tadpole digestion for predator cue detection in different anuran species to exotic predators, while native predators were recognized also when not fed beforehand. The finding that only native but not exotic predators could be recognized by frog larvae was confirmed by several studies using predator kairomones only (e.g. Pearl et al., 2003; Polo-Cavia et al., 2010; Smith et al., 2008). However, tadpoles have the possibility to learn the recognition of kairomones from formerly unknown predators via classical conditioning with alarm signals as unconditioned stimulus (e.g. Ferrari et al., 2012; Ferrari et al., 2009; Gonzalo et al., 2009, 2010; Mandrillon and Saglio, 2005). Also embryonic anurans are already able to learn the recognition of predators with the help of tadpole alarm signals and accordingly react to the

kairomones after hatching (Ferrari and Chivers, 2009, 2010; Mathis et al., 2008). The recognition of chemical cues and signals across developmental stages, as shown between embryos and tadpoles, could not be confirmed for recently metamorphosed anurans. While froglets were able to avoid chemical cues of snakes that fed on conspecifics of the same stage (Chivers et al., 1999), snakes feeding on tadpoles did not evoke the same reaction (Belden et al., 2000).

Despite the numerous studies on the topic of chemicals released by predators fed on conspecifics, the approach to analyse the responsible compounds has just started in the last years. Ferland-Raymond et al. (2010) fractionated the water containing a dragonfly predator fed with tadpoles of either *Litobates septentrionalis* or *Lithobates catesbeianus* (Ranidae). This way the cue could be restricted to a single ion that triggered the response in the test animals. It could only be found when the predators digested one of the two tadpoles and was not produced by an unfed predator. This means that the cue is probably released by the consumed tadpole and defecated as a by-product. Takahara et al. (2013) further showed that tadpoles of different species reacted to different chemical components when confronted with cues of predators fed with conspecifics. While *Glandirana rugosa* (Ranidae) detected chemical cues with hydrophobic properties, *Hyla japonica* (Hylidae) responded only to hydrophilic chemicals.

Even if generally fewer studies are available, the decoding of sex pheromones in anurans is already more advanced. The earliest evidence of chemical sexual communication in frogs was provided by Rabb and Rabb (1963) showing that *P. pipa* males became agitated when confronted with water previously housing a mating pair. Choice tests revealed that males of different species were attracted to chemical signals of conspecific females (*Ascaphus truei*: Asey et al., 2005; *Pseudophryne bibronii*: Byrne and Keogh, 2007; *A. americanus*: Forester and Thompson, 1998), while *Hymenochirus* sp. (Pipidae) females were attracted to chemical signals of conspecific males (Burns and Thomas, 1997; Chan et al., 1999). Pearl et al. (2000) investigated this behaviour of female *Hymenochirus* sp. more detailed and found that the attracting pheromones originated from male breeding glands and were restricted to trigger reactions in conspecific females. Wabnitz et al. (1999, 2000) were the first to decode an anuran sex pheromone. Splendipherin was isolated from male *Litoria splendida* (Hylidae)

skin secretions and triggers attraction behaviour in conspecific females. The peptide is produced especially during the breeding season.

Another sex-specific pheromone was described by King et al. (2005). They isolated a peptide (LASP) from skin secretions of male *Leptodactylus fallax* (Leptodactylidae) that did not attract females but did attract males and stimulated aggressive behaviour in the latter. More recently Poth et al. (2012) characterized the third anuran pheromone. It was found in the femoral glands of male *Mantidactylus betsileanus* (Mantellidae) and while its effect on conspecific females was not tested, the attraction of conspecific males could be confirmed. The recognition and attraction of adult or juvenile frogs to conspecifics based on chemical substances was also investigated for various other species, but rather in the sense of aggregation than with a sexual or aggressive purpose (e.g. Delaney and Bishop, 2007; Gonzalo et al., 2006; Graves et al., 1993; Hamer et al., 2011; Waldman and Bishop, 2004). A similar aggregation behaviour was also shown for tadpoles being attracted to chemical signals of conspecifics (e.g. Buttermore et al., 2011; Eluvathingal et al., 2009; Kiseleva, 1995, 1996) or siblings (e.g. Blaustein and O'Hara, 1982a; Blaustein and O'hara, 1982b; Waldman, 1985).

Although anuran amphibians exhibit a greater diversity of reproductive modes than any other tetrapod vertebrate group (Haddad and Prado, 2005), in this context only a handful of studies have investigated the influence of chemical communication. Spieler and Linsenmayer (1997) as well as Takahashi (2007) showed for anurans that deposit their clutches in lentic water bodies (*Hoplobatrachus occipitalis*, *Hyla versicolor*) that breeding sites were examined for predator kairomones before egg deposition. For *Kurixalus eiffingeri* (Rhacophoridae), a species that breeds in phytotelmata (small water bodies in plants such as leaf axils or bamboo stumps; Varga, 1928) and exhibits parental care in form of uniparental egg-feeding, Kam and Yang (2002) showed interspecific communication between female frogs and their tadpoles. Tadpoles recognized females by chemical (not visual) signals and thereupon started begging for nutritive eggs. A similar begging behaviour was shown for *Oophaga pumilio* (Dendrobatidae, poison frogs) tadpoles, but it was not triggered only by chemical but by a combination of chemical, visual and tactile cues (Stynoski and Noble, 2012). Another species in the same family that was shown to use both inter- and intraspecific chemical cues during parental care behaviour is the Amazonian *Ranitomeya variabilis*. As other dendrobatids, both



*O. pumilio* and *R. variabilis* show complex sociobiological and reproductive behaviours including parental care.

## Parental care in poison frogs

Poison frogs belong to the superfamily Dendrobatoidea that comprises the families Dendrobatidae and Aromobatidae (Grant et al., 2006) with currently next to 300 described species (Frost, 2013). These mainly diurnal frogs occur in the Neotropics and many of them are brightly coloured and possess skin alkaloids (e.g. Lötters et al., 2007; Saporito et al., 2012). Besides their colouration, poison frogs are especially known for their remarkably diverse parental care behaviours (e.g. Summers and McKeon, 2004; Summers and McKeon, 2006; Weygoldt, 1987). Except of very few species with nidicolous endotrophic tadpoles (see below), all poison frogs have one strategy in common: that is the transport of their tadpoles on their backs from the eggs to aquatic habitats. However, there are many differences, starting with the egg deposition sites, the amount of tadpoles transported at once, the sex of the parent that conducts the transportation, the sites where tadpoles are deposited, if tadpoles are provisioned with nutritive eggs or not and if such feeding behaviour is conducted by male and female or by females only:

(1) Eggs are deposited terrestrially and nidicolous endotrophic tadpoles develop within the eggs. — The only poison frogs that do not transport their tadpoles from the terrestrial clutch (deposited in the leaf litter) to a water body can be found in the family Aromobatidae (e.g. *Allobates nidicola*: Caldwell and Lima, 2003; *Anomaloglossus stepheni*: Juncá et al., 1994). However, since the males regularly attend their eggs until hatching, it can still be spoken of parental care in these species.

(2) Eggs are deposited terrestrially and males transport tadpoles collectively to streams; no egg provisioning. — The deposition of clutches on the ground (e.g. in the leaf litter) is the most common egg deposition strategy conducted by poison frogs. Eggs are usually attended regularly by the male, in order to moisten them (e.g. Wells, 2007). In the case of for example *Hyloxalus nexipus* (Dendrobatidae; Frost, 1986) or *Epipedobates anthonyi* (Dendrobatidae; Lötters et al., 2007) all tadpoles of the clutch are transported together and deposited in little streams. After deposition in the water, tadpoles are left on their own.

(3) Eggs are deposited terrestrially and females transport tadpoles collectively to streams; no egg provisioning. — Even if in most cases males are responsible for the transportation of tadpoles, there are few species in the genus *Colostethus* sensu stricto (Dendrobatidae) where the females transport all tadpoles at once to a stream (e.g. *C. inguinalis*; Wells, 1980). Whether females also care for the eggs beforehand is unknown.

(4) Eggs are deposited terrestrially and males transport tadpoles collectively to large ground phytotelmata or puddles; no egg provisioning. — Large ground phytotelmata are for example water-filled palm bracts. Due to their hard structure they are durable and usually house a number of insect larvae (Greeney, 2001; Patrick et al., 2002). Numerous poison frog species use those water bodies for the deposition of their tadpoles, though many of them are also found to use puddles in the same way (e.g. *Hyloxalus azureiventris* (Dendrobatidae): Lötters et al., 2000; *Ameerega trivittata* (Dendrobatidae): Roithmair, 1994).

(5) Eggs are deposited terrestrially and both sexes (i.e. male or female) transport tadpoles collectively to large ground phytotelmata or puddles; no egg provisioning. — While in some stream-breeding species, females alone are responsible for the transportation of their offspring (see 3), in those species using big ground phytotelmata or puddles, only some cases are known, where females conduct this behaviour on an irregular basis. For example in the Aromobatidae *Allobates brunneus* or *A. femoralis* females could be observed to take over their males' job and transport their tadpoles to such pools (Caldwell and de Araújo, 2005; Weygoldt, 1987).

(6) Eggs are deposited terrestrially and males transport tadpoles singly to small phytotelmata; no egg provisioning. — Many poison frog species use small phytotelmata (e.g. water filled leaf axils) for the deposition of their offspring. This has the advantage that the risk of predation is minimized, but due to their small size and accordingly scarce food resources, (often compensated by larval cannibalism), phytotelmata commonly cannot sustain more than one tadpole (e.g. Aspbury and Juliano, 1998). Thus, males of the dendrobatid species *Ranitomeya benedicta* (Brown et al., 2008c) or *Dendrobates auratus* (Summers, 1990) for example transport their tadpoles one by one, each to a different phytotelm.

(7) Eggs are deposited vertically above phytotelmata and males transport tadpoles singly to small phytotelmata; biparental egg provisioning. — Species that use very small phytotelmata like *Ranitomeya imitator* (Brown et al., 2008a) or *Ranitomeya vanzolinii* (Caldwell and de Oliveira, 1999) do not only need to separate their tadpoles (if they have more than one), they also have to provision them with food to avoid starvation. In the mentioned species males first deposit their tadpoles in a very small water body and then call their females in regular intervals to provision the tadpoles with eggs. Those nutritive eggs are not fertilized by the

males. Fertilized eggs are not deposited into the water but rather 10-20 cm above the phytotelm. For *R. imitator* it is furthermore known that pairs are monogamous, so females only feed their own offspring (Brown et al., 2010).

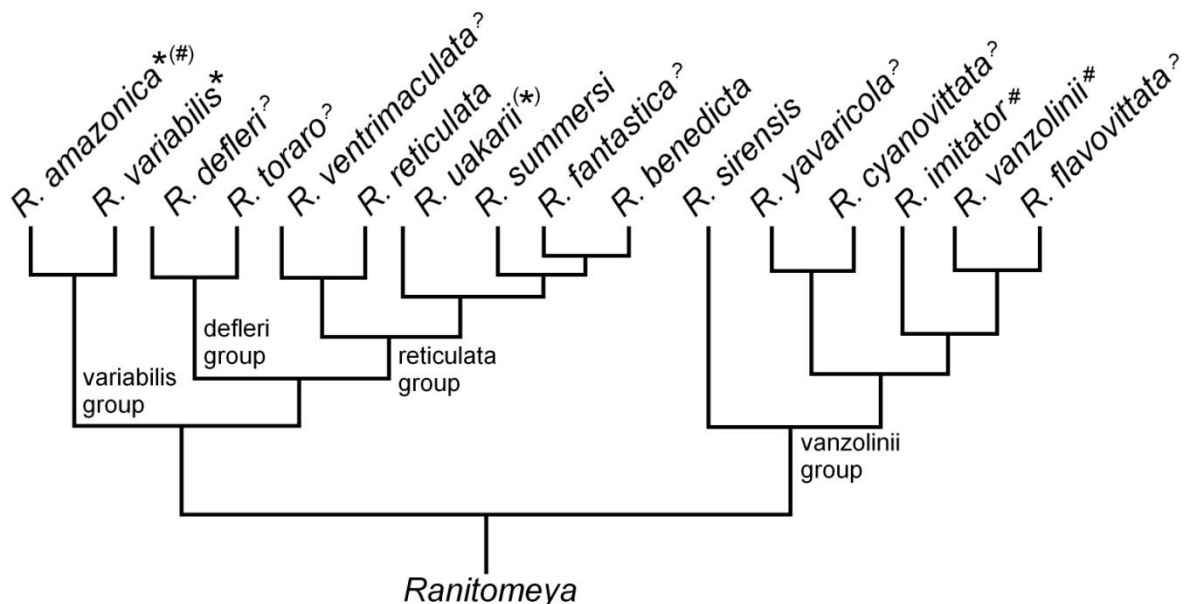
(8) Eggs are deposited terrestrially and females transport tadpoles singly to small phytotelmata; uniparental egg provisioning. — The only species known where females transport their tadpoles individually from terrestrial clutches into phytotelmata and thereafter provision them independently with nutritive eggs, belong to the genus *Oophaga* (Dendrobatidae). Examples are *O. pumilio* (Brust, 1993) or *O. granulifera* (van Wijngaarden and Bolaños, 1992).

(9) Eggs are deposited in phytotelmata at the water surface and males transport tadpoles singly to medium/small phytotelmata; no or sporadic egg provisioning. — Nearly all poison frogs deposit their eggs terrestrially and only few species of the genus *Ranitomeya* (Dendrobatidae) are known to deposit their eggs on the water surface within the phytotelmata instead. While *R. uakarii* conducts this behaviour irregularly (alternating with egg deposition in the leaf litter; Brown et al., 2006), *R. variabilis* (Brown et al., 2008b) and *R. amazonica* (Poelman and Dicke, 2007) exclusively use phytotelmata for egg deposition. Even if the latter is known to deposit fertilized eggs with tadpoles during the dry season (in order to feed them), usually both species avoid phytotelmata already occupied. For *R. variabilis* the recognition of conspecific cannibalistic tadpoles occupying a phytotelm was shown to be due to chemical cues (Schulte et al., 2011; see following section).

### ***Ranitomeya variabilis* – focal species to study chemical communication during parental care**

*Ranitomeya variabilis* is one of 16 *Ranitomeya* species which are divided into four species groups (Fig. 4; Brown et al., 2011). It occurs in the rainforests east of the Andes in Peru, Ecuador and Colombia and uses *Heliconia*, *Dieffenbachia* and bromeliad (*Aechmea* and *Guzmania* spp.) leaf axils as phytotelmata for both clutch and tadpole depositions (Brown et al., 2008b). Eggs are attached to the inner wall of a phytotelm, just at the water surface (Brown et al., 2008a). After developing, males retrieve tadpoles by either removing them from the eggs or out of the phytotelm they hatched into. Tadpoles are usually transported individually to a new phytotelm, but sometimes males transport several tadpoles at once, deposit them first communally, then return and retrieve each tadpole individually to a separate

pool (Brown et al., 2008b). Because tadpoles of this species are highly cannibalistic and feed on eggs as well as on conspecific tadpoles when deposited together (Summers, 1999, but see Brown et al., 2009), frogs do not only deposit their own tadpoles separated from each other, they particularly avoid depositions with tadpoles of conspecific males. Schulte et al. (2011) investigated how adults were able to recognize a tadpole occupying a phytotelm, since they are often hidden on the ground, i.e. not visible to the frogs. It turned out that *R. variabilis* only requires chemical cues of conspecific tadpoles to locate and avoid them for both tadpole and clutch deposition. Offering them chemical cues of non-predatory bufonid tadpoles only elicited avoidance behaviour for egg, but not tadpole depositions.



**Fig. 4** *Ranitomeya* species tree modified after Brown et al. (2011). Legend: \*clutch deposition at the water surface in phytotelmata, # parental care includes egg-feeding, ( ) behaviour is only sporadically conducted, ? behaviour is not or only partially known.

## Objectives and structure of this thesis

The central goal of this thesis was to gain a deeper insight into intra- and interspecific chemical communication conducted by anurans. More specifically, chemical communication in the context of parental care, as well as between adult frogs, was studied in our model species *Ranitomeya variabilis* and the results were used for the identification and categorical classification of the responsible chemical cues and signals. The findings were further used to expand the knowledge about parental care in the focal species.

In **Chapter I** the ability of *R. variabilis* to identify and distinguish the chemicals released by tadpoles of different species was analyzed. The aim of this study was to discover which pattern these frogs use when they screen phytotelmata for the possible presence of other species. In-situ pool-choice experiments were conducted, presenting chemical substances of heterospecific tadpoles to the frogs that either did or did not belong to the same family as the focal species and were either phytotelm or non-phytotelm breeders. It was then analyzed which species were avoided for tadpole respectively clutch depositions in order to distinguish if taxonomic or ecological reasons led the frogs' decisions. The results of this study are published online first in *Animal Cognition*, doi 10.1007/s10071-013-0659-2.

After identifying which tadpoles (besides conspecifics) were avoided by *R. variabilis* for both clutch and tadpole depositions, **Chapter II** aims to identify what kind of chemical compounds were produced by those tadpoles (i.e. con- versus heterospecifics), in order to classify them. After finding a way to achieve the active compounds from the tadpole used water (i.e. when frogs did not respond to the water in the biotests anymore, compounds were successfully extracted), extraction was conducted with captive-bred tadpoles in the laboratory. Using HPLC-MS and chemical fractionation, the extracted substances were divided into defined compound mixtures. Those fractions were thereafter used for in-situ biotests. The cycle was repeated using those fractions that turned out to contain the active chemical compounds and smaller mixtures respectively single compounds were tested in-situ again. Compounds of the different species were compared at the end. This way, conclusions about the accurate definition of chemical communication (i.e. communication by chemical cues or signals, see above) between *R. variabilis* and heterospecific (non-predatory) tadpoles in comparison to conspecifics could be drawn. The results of this study are summarized in an unpublished manuscript.

After finding that not only conspecific but also heterospecific tadpoles were recognized chemically, **Chapter III** covers the question if kairomones of predatory invertebrate larvae (occurring in the same types of phytotelmata) are recognized and avoided by *R. variabilis* as well. Further, it was investigated if, besides chemical communication, visual tadpole cues are also of importance during parental care. For this purpose the results of in-situ pool-choice experiments using predator kairomones and tadpole models were compared with the results of experiments conducted with chemical tadpole cues and present predators. The results of this

study are published as a book chapter in *Chemical Signals in Vertebrates 12*, p. 309-321, Springer (2013).

Since it could be shown that chemical communication between larval and adult *R. variabilis* was based on species-specific chemical compounds, in **Chapter IV** the possibility of interspecific chemical signaling between adult frogs was studied. Males of *R. variabilis* are known to call in response of the appearance of a female (Brown et al., 2008b). In order to test if this reaction might be triggered by chemical signals sent by females (that show no visual sexual dimorphism to the males), frogs were confronted with either hetero- or conspecific chemical substances and their reaction towards them (i.e. preference or avoidance) was tested. The study is published in *Behavioural Processes* 100, 32-35 (2013).

The following chapters try to bring more clarity into the extent of parental care conducted by *R. variabilis* using the knowledge about chemical communication in this context. For its sister species, *R. amazonica*, Poelman and Dicke (2007) found that, even if conspecific tadpoles are usually avoided for clutch or tadpole depositions, in the dry season clutches were preferentially deposited with tadpoles. They interpreted this behavioural change as a way to provide their tadpoles with food (i.e. eggs) in order to accelerate their development when facing desiccation risk. To see if *R. variabilis* conducts a similar behaviour, the study presented in **Chapter V** treats the possibility of behavioural changes triggered by seasonal shifts. In-situ pool-choice experiments using chemical cues of conspecific tadpoles were carried out throughout the change from rainy to dry season. Using a change-point analysis, the exact seasonal change was defined and differences between frogs' choices were analysed for both clutch and tadpole depositions. The results of this study are published in *Evolutionary Ecology* 27, 711-723 (2013).

To further see if the avoidance behaviour of *R. variabilis* towards conspecific chemical cues for clutch or tadpole depositions is less rigorous when phytotelmata are occupied by offspring (or its chemical cues), the final two studies were conducted. In **Chapter VI** results of pool-choice experiments executed with chemical cues blended together from several unfamiliar tadpoles were compared with results of experiments where frogs were able to reuse the same phytotelmata, i.e. deposit clutches with their own tadpoles. The parenthood was defined by a mark-recapture study. Using ArcGIS, the home ranges of male frogs were calculated and multiple used phytotelmata plotted inside. Furthermore the ability of tadpoles to feed on

conspecific eggs was retested, because results of Summers (1999) and Brown et al. (2009) were inconsistent. The study has been accepted for publication in *Ethology Ecology & Evolution*.

In **Chapter VII** a study is presented where frogs were confronted with chemical cues of single tadpoles that were found in their home range previously. After choosing between a phytotelm with or without the cues of these tadpoles, genetic samples of both the tadpoles emitting the chemical cues and those deposited together with or next to them were collected. Genetic samples were then genotyped and relatedness between those tadpole pairs was calculated using the program KINSHIP. A possible relation between deposition site and kinship was analysed. The results of this study are summarized in an unpublished manuscript.

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## Chapter I

# **A danger foreseen is a danger avoided: how chemical cues of different tadpoles influence parental decisions of a Neotropical poison frog**

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## Abstract

The protection of offspring against predators and competitors is especially important in organisms using spatially separated breeding resources, impeding the offspring's chances to escape. One example of such isolated reproductive resources are phytotelmata (small water bodies in plant axils), exploited by the Neotropical poison frog *Ranitomeya variabilis* (Dendrobatidae) for both clutch and tadpole deposition. Because poison frog tadpoles are often cannibalistic, parents tend to avoid deposition with conspecifics. Previous studies have shown that this avoidance is based on chemical cues produced by conspecific tadpoles. Further, cues produced by phylogenetically less-related tadpoles (Bufonidae) were avoided for clutch but not tadpole depositions. We analyzed how the different responses to tadpole cues are triggered. We tested the reactions of parental *R. variabilis* to tadpole cues of species differing in two aspects: whether or not they are dendrobatids, and whether or not they reproduce in phytotelmata. We found that for clutch deposition, tadpole cues were always avoided, i.e. all tadpoles were treated by the frogs as if they pose a danger to the eggs. However, responses varied for tadpole depositions: while dendrobatid larvae living in phytotelmata were avoided, those breeding in streams were not. Non-poison frog tadpoles were ignored when associated with habitat other than phytotelmata, but they were preferred when living in phytotelmata. This suggests that both phylogeny and tadpole habitat are important triggers for the decisions made by *R. variabilis*. Only tadpoles using the same breeding resources are considered as relevant for the frog's own larvae (i.e. as a potential danger or food resource), while further decisions are related to evolutionary relationship.

**Keywords:** *Ranitomeya variabilis*, phytotelmata, parental care, predation

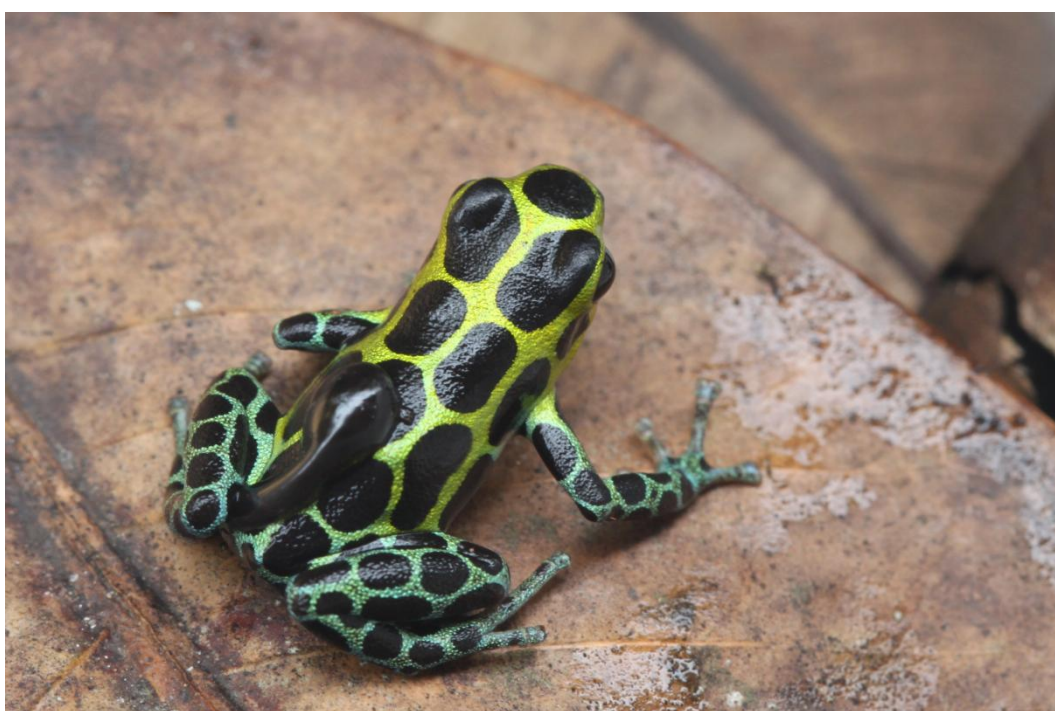
## Introduction

Prey organisms show a variety of behavioral adaptations to protect themselves or their offspring from predators (Caro, 2005; Lima, 2009; Lima and Dill, 1990). A timely detection of approaching danger is therefore essential. Animals use different cues (unintentionally emitted by the predator) or signals (alarm signals emitted by conspecifics; defined by Steiger et al., 2011) for the detection of predators (e.g. Kappler, 2006; Munoz and Blumstein, 2012). Besides visual (e.g. Lima and Bednekoff, 1999) or acoustic vigilance (e.g. Acharya and McNeil, 1998), chemical stimuli play a significant role in predator detection and are known from all vertebrate groups (fish: Hartman and Abrahams, 2000; amphibians: Murray et al., 2004; reptiles: Miller and Gutzke, 1999; birds: Amo et al., 2008; mammals: Caine and Weldon, 1989). In aquatic media particularly, both chemical cues and signals are of great importance because a large number of compounds can dissolve and disperse, whereas vision can often be impaired, especially in turbid or flowing waters (Hara, 1994; Wisenden, 2000).

While anuran amphibians are mainly vulnerable to terrestrial predators when adult, in embryonic and larval life stages they mostly face threats related to the aquatic environment (Wells, 2007). As escape behavior is limited in early life stages, parent frogs and toads are responsible for choosing adequate habitats for their offspring: that is, the avoidance of water bodies containing potential predators to the brood (Binckley and Resetarits, 2002, 2003; Hopey and Petranka, 1994; Resetarits and Wilbur, 1989; Spieler and Linsenmair, 1997).

In some anurans, parental care even goes a step beyond. Poison frogs in the Neotropical family Dendrobatidae do not only choose a safe location for egg deposition but also make decisions of where to transport their tadpoles on their back after hatching (e.g. Caldwell and de Oliveira, 1999a; Lötters et al., 2007; Roithmair, 1994; Summers and McKeon, 2004; von May et al., 2009). The Amazonian poison frog *Ranitomeya variabilis* (Zimmermann and Zimmermann, 1988) deposits its clutches at the water surface of small phytotelmata, such as small water bodies in plant axils (Varga, 1928). After the tadpoles hatch, the male parent returns and transports them individually into different phytotelmata (Brown et al., 2008b; Fig. 1). Because larvae of this species are cannibalistic (as are those of many other poison frog species: Brust, 1993; Poelman and Dicke, 2007; Pramuk and Hiler, 1999; Summers, 1999), parent *R. variabilis* need to locate unoccupied phytotelmata for each tadpole to increase their individual reproductive success (Brown et al., 2008a). In field experiments, we have shown

that these amphibians recognize and avoid phytotelmata that contain chemical cues of conspecific tadpoles, i.e. physical presence of other larvae is not necessary (Schulte et al., 2011). This preventive behavior was observed for both tadpole and egg deposition, suggesting that the conspecific tadpoles might pose a danger for both developmental stages. Interestingly, chemical cues of the omnivorous – but not live tadpole-consuming – larvae from the toad *Rhinella poeppigii* (Bufonidae) were avoided for clutch but not for tadpole depositions. This is remarkable, as these ‘generalized’ (Altig and McDiarmid, 1999) toad larvae are common in ponds at the edge of the forest and should be able to consume frog eggs but not live tadpoles. Our observations suggest that *R. variabilis* recognizes *R. poeppigii* tadpoles as a danger for its eggs but not for the already hatched larvae (Schulte et al., 2011). However, because *R. poeppigii* larvae should be unfamiliar to *R. variabilis*, the question arises how parent frogs could make the distinction here.



**Fig. 1** The Neotropical poison frog *Ranitomeya variabilis* performs parental care; males are responsible for carrying the freshly hatched tadpoles to phytotelmata. The transportation to these little pools usually takes place one-by-one, so males have to return up to six times to their clutches.

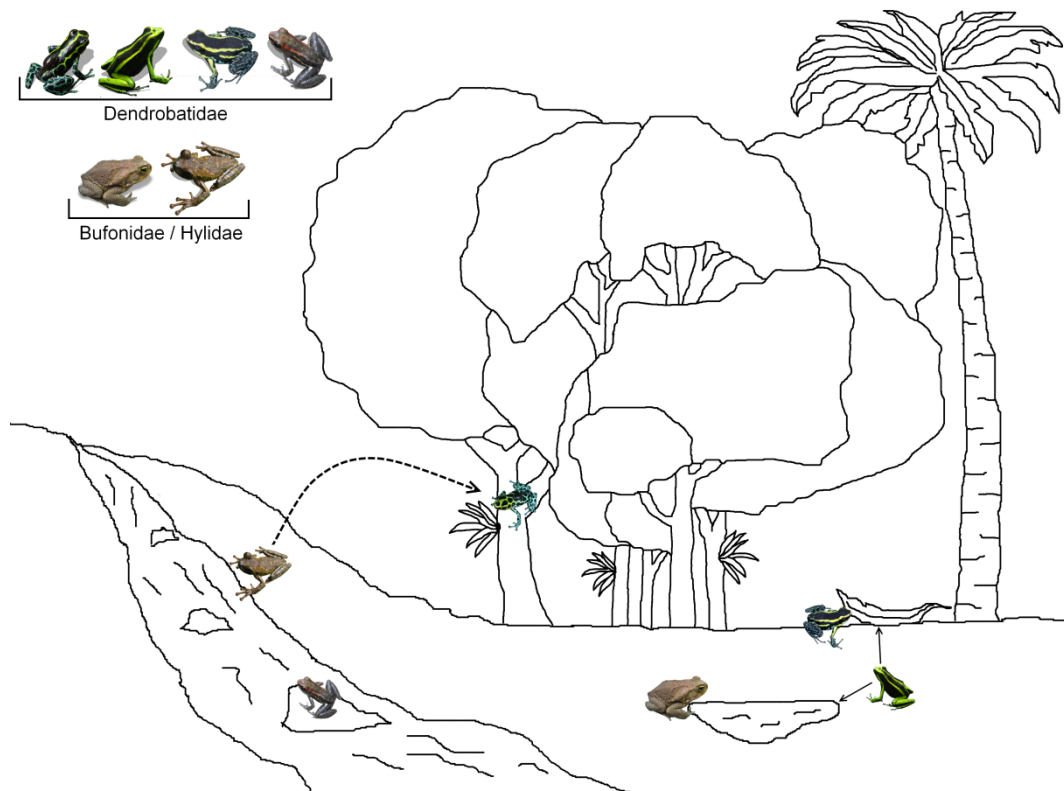


In this paper, we study the ability of *R. variabilis* to respond to chemical cues of generalized (i.e. non-cannibalistic) tadpoles of anuran species, other than those tested so far. We hypothesize (i) that this species avoids cues of all generalized anuran larvae for egg deposition, as many generalized tadpoles potentially prey on amphibian eggs (Wells, 2007). With regard to tadpole depositions we hypothesize (ii) that *R. variabilis* avoids the chemical cues of larvae of species in the same family (Dendrobatidae), while those of larvae from non-dendrobatid species are not avoided. This hypothesis is warranted, because *R. variabilis* is not the only poison frog species that avoids already occupied phytotelmata for tadpole deposition (Brown et al., 2008a; Brown et al., 2008b; Brust, 1993; Caldwell and de Araújo, 1998; Caldwell and de Oliveira, 1999b; Poelman and Dicke, 2007; Summers, 1990, 1999; Weygoldt, 1980; Zimmermann and Zimmermann, 1984). Hence, it is conceivable that the use of chemical cues for this behavior is present in other dendrobatid frogs as well (perhaps as a synapomorphy). Alternatively, we hypothesize (iii) that cues of larvae of non-dendrobatid frog species but that still develop in phytotelmata are recognized by *R. variabilis* for tadpole depositions, while larval cues from anurans that do not exploit phytotelmata (including dendrobatid species) are not. This might imply that *R. variabilis* is able to learn and discriminate the tadpole cues of species it shares its larval habitat with against those of other species.

## Materials and methods

### Study site

Data were obtained *in situ* 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. Three study sites were established on the western side of the river at an altitude between 540 and 580 m above sea level. Between these sites, a minimum distance of 30 m was maintained. This exceeds the known home range size of *Ranitomeya variabilis* (Brown et al., 2009a) in order to avoid pseudo-replication, i.e. repeated measures on the same specimens. A wooden hut in the forest served as a field laboratory.



**Fig. 2** The frog species used in our experiments (top left) and the sites they breed or transport their tadpoles to, respectively (within the drawing): Dendrobatidae (from left to right) – *Ranitomeya variabilis* (small tree phytotelmata, like bromeliads), *Ameerega trivittata* (large ground phytotelmata, puddles), *Hyloxalus azureiventris* (large ground phytotelmata, especially water-filled palm leaves), *H. nexipus* (streams); Bufonidae – *Rhinella poeppigii* (ponds and puddles); Hylidae – *Osteocephalus mimeticus* (streams), a species here used as an ‘alternative’ to *O. leoniae* (small tree phytotelmata, like bromeliads; see arrow).

## Experimental trials

Tadpoles of different species (see below) were collected from their natural habitats (Fig. 2) and kept in captivity during the trials. Each specimen was housed individually in 50 ml of rainwater in a standardized polypropylene cup at ambient temperature. Every two days, we took the water of the tadpoles and mixed it (separated by species), refilled the cups with fresh rain water and fed the larvae *ad libitum* with flaked fish food (Tetra®). After finishing the trials or when tadpoles reached developmental stage 41 (Gosner, 1960), they were released back into the wild.

For the setup in the three forest sites, we established artificial phytotelmata. Polypropylene plastic cups (200 ml volume, 10 cm height, 7 cm in diameter), wrapped in dark plastic

membranes and with two-thirds of the opening covered, were fixed to trees at 0.5-1.5 m above ground. They were installed in pairs, directly next to each other, and filled with 25 ml water each. One cup per pair received clean rainwater, while the other cup received water used for two days by tadpoles of one of the different species held in captivity (compare Schulte et al., 2011). To avoid diluting the tadpole-treated water, we put a non-rusting metal roof 10 cm above each cup pair to protect it against rainfall. After generating the used tadpole water in the field laboratory, cups in the forest were checked for newly deposited tadpoles and clutches of *Ranitomeya variabilis* and both clean and treated water was changed if no depositions were found. That way water in the cups in the forest was renewed every other day. To minimize pseudo-replication, we kept a minimum of 4 m between cup pairs belonging to the same sub-trial and scored only one randomly chosen deposition for any same-day deposition in neighbouring cup pairs (Brown et al., 2008a; Schulte et al., 2011).

### **Species with generalized tadpoles used for comparisons**

#### *Ameerega trivittata*

Between 12 June and 4 September 2010, 43 tadpoles of *Ameerega trivittata* (Dendrobatidae) from different clutches were kept in captivity and their water was taken every other day as described above. Seventy-five cup pairs (25 per site) were used for testing the reaction of *Ranitomeya variabilis* to the chemical cues of the tadpoles of this species. The sympatric *A. trivittata* is more than double the size of *R. variabilis* and transports its omnivorous (but not live tadpole-consuming) larvae all at once to relatively large ground phytotelmata (e.g. water-filled holes in fallen tree trunks), puddles or little rivulet basins (Lötters et al., 2007). Cups were never utilized by *A. trivittata* in our study, perhaps due to their small size (as pools used by this species generally contain several liters of water; Twomey et al., 2008). However, we occasionally observed *R. variabilis* deposit its larvae in larger ground phytotelmata. Therefore, we conclude that *R. variabilis* might be familiar with the cues of *A. trivittata*.

#### *Hyloxalus azureiventris*

Thirty-six tadpoles of *Hyloxalus azureiventris* (Dendrobatidae) from different clutches were kept in captivity from 5 June to 4 September 2010. Their water was distributed over 60 cup

pairs (20 per site). Like *Ameerega trivittata*, *H. azureiventris* has omnivorous larvae (which likely do not feed on living tadpoles) that are transported all at once to ground phytotelmata (Lötters et al., 2007); in the Cainarachi Valley we noticed that these are fallen palm tree leaves. On some occasions, *H. azureiventris* tadpoles were found in the cups hung up for *R. variabilis* and *R. variabilis* larvae were found in water-filled palm tree leaves. Hence, it can be concluded that *R. variabilis* might be familiar with the cues of *H. azureiventris*.

### *Hyloxalus nexipus*

Between 6 April and 12 June 2011, 15 tadpoles of *Hyloxalus nexipus* (Dendrobatidae) from different clutches were kept in captivity and their used water was distributed over 30 cup pairs (10 per site). This species has omnivorous larvae that are unlikely to feed on live tadpoles. It never exploits phytotelmata but rather deposits its larvae into running water (Frost, 1986), so that *R. variabilis* should not be familiar with *H. nexipus* cues.

### *Osteocephalus mimeticus*

*Osteocephalus leoniae* (Hylidae; Jungfer and Lehr, 2001) exploits the same phytotelmata for its offspring as *R. variabilis* (Jungfer et al., 2013). Although *O. leoniae* occurs within our study area, we failed to find sufficient tadpoles for our experiments. As an alternative, we used tadpoles of the sympatric and closely related *O. mimeticus*, which uses little streams for the deposition of its eggs. Its tadpoles hatch and live within these streams (Henle, 1992; Jungfer, 2010). Thirty-six tadpoles from different clutches were kept in captivity from 9 June to 4 September 2010 and their water was distributed over 60 cup pairs (20 per site) every other day. *Ranitomeya variabilis* might not be familiar with the cues of this species. However, we will not rule out that, due to the phytotelmata-breeding *O. leoniae*, cues of *Osteocephalus* in general are recognized.

## **Data analysis**

For each treatment type we compared the frequencies of tadpole and clutch deposition, respectively, in each water type (clean or treated) using a G test (Woolf, 1957). This is

suggested to be of superior exactness for limited observations (Gotelli and Ellison, 2004; Sachs, 1974). Since each dataset had less than 200 observations, we adjusted the G test in the manner of Williams (1976). As a null hypothesis, we assumed that the frequency of deposition events was random (0.5 in either pool). Thereupon, deposition frequencies within the same treatment (i.e. clutches versus tadpoles) were compared calculating a Fisher's exact test (Fisher, 1922; Townend, 2002). In order to examine differences between responses towards chemical cues of different species we conducted a non-parametric Kruskal-Wallis one-way ANOVA (Kruskal and Wallis, 1952) for both tadpole and clutch depositions. Statistical tests were carried out using PASW 18 (SPSS Inc. 2009).

## Results

As shown in Table 1 and Figure 3, of the three dendrobatids tested all were either significantly or at least nearly significantly avoided by male parent *Ranitomeya variabilis* for clutch and two for tadpole depositions. Only *Hyloxalus nexipus* was not avoided for tadpoles, i.e. the only dendrobatid not utilizing phytotelmata. However, in turn, we also found no particular preference for cues of this species' tadpoles. As suggested by the Fisher's exact test, observations for clutch and tadpole depositions were shown to be almost significantly different with respect to *H. nexipus* cues. In contrast, for cues from larvae of *Ameerega trivittata* and *H. azureiventris*, results of the Fisher's exact test confirmed that *R. variabilis* shows the same avoidance for both tadpole and clutch depositions. In the only non-dendrobatid studied here (which might either be understood as exploiting phytotelmata or not at all, see above), tadpole cues were largely avoided for clutch depositions but not so for tadpoles (Table 1; Fig. 3). The opposite was the case in response to *Osteocephalus*: we found that *R. variabilis* significantly preferred cues of *Osteocephalus* for tadpole depositions. Eggs, in contrast, were mainly deposited in clean water. Fisher's exact test suggested that deposition preferences for tadpoles and clutches were significantly different.

An overall comparison between deposition choices in different treatments revealed significant differences for tadpole depositions (Kruskal-Wallis,  $\chi^2 = 35.366$ ,  $p < 0.001$ ) but not for clutch depositions (Kruskal-Wallis,  $\chi^2 = 1.950$ ,  $p = 0.856$ ).

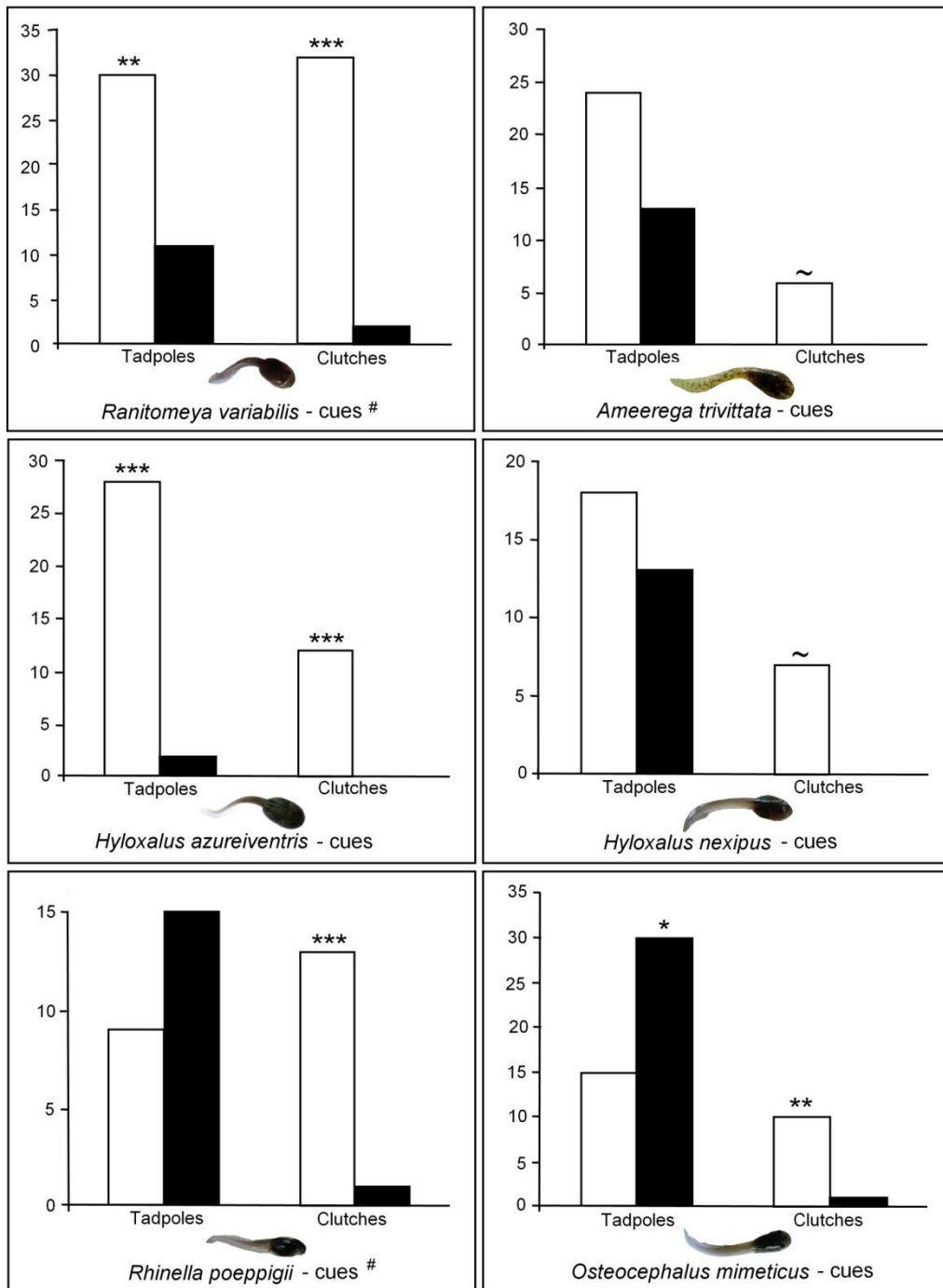
**Table 1** Total egg and tadpole depositions and those placed in clean water by male parent *Ranitomeya variabilis* when cup pairs (see text) were offered, with one cup containing clean water and the other containing chemical cues of four different anuran species that do or do not utilize phytotelmata, followed by results of statistical test (compare Fig. 3).

	<i>Ameerega</i> <i>trivittata</i>	<i>Hyloxalus</i> <i>azureiventris</i>	<i>Hyloxalus</i> <i>nexipus</i>	<i>Osteocephalus</i> <i>mimeticus</i>
Family	Dendrobatidae	Dendrobatidae	Dendrobatidae	Hylidae
Phytotelmata utilized	Yes	Yes	No	No*
<i>Ranitomeya variabilis</i> :				
Clutch depositions total/in clean water	6/6	12/12	7/7	11/10
G test (clutches)	Sample size too low	$G_1 = 15.970$ $P < 0.001$	Sample size too low	$G_1 = 8.176$ $P = 0.004$
Tadpole depositions total/in clean water	37/24	30/28	31/18	45/15
G test (tadpoles)	$G_1 = 3.276$ $P = 0.070$	$G_1 = 26.452$ $P < 0.001$	$G_1 = 0.797$ $P = 0.372$	$G_1 = 5.041$ $P < 0.025$
Fisher exact test (tadpoles vs. clutches)	$P = 0.155$	$P = 1.000$	$P = 0.072$	$P = 0.001$

\* Phytotelmata are utilized in the sympatric related species *Osteocephalus leoniae* (see text).

## Discussion

Our results demonstrate that wild *Ranitomeya variabilis* are able to discriminate chemical cues of tadpoles belonging to different anuran species characterized by larval habitat (i.e. phytotelmata vs. non-phytotelmata) and/or evolutionary relatedness (i.e. dendrobatid vs. non-dendrobatid). As expected by our hypothesis (i), *R. variabilis* avoids chemical cues of tadpoles of all species for egg deposition, no matter to which family they belong or if their larvae occur in phytotelmata or not (i.e. if familiar or not). This goes hand in hand with the observations by Schulte et al. (2011) on a non-phytotelm breeding bufonid toad. Apparently, this mechanism is highly advantageous to *R. variabilis*, which lays up to six eggs only (Brown et al., 2008b).



**Fig. 3** Number of tadpoles and clutches deposited by *Ranitomeya variabilis* in clean water (white bars) or water containing cues of tadpoles of different anuran species (black bars), legend: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , ~ data size too small for statistical tests, # data already published in Schulte et al. (2011) .

Our results reject our hypothesis (ii), however. Male parent *R. variabilis* do not always avoid tadpole depositions when chemical cues of larvae of species in the family Dendrobatidae were present. They only avoided those species in the same family that, at different degrees, exploit phytotelmata. This tentatively proposes that the evolution of chemical cue recognition is not related to phylogenetic relationship, i.e. member of the same family, and that perhaps the chemical compounds involved (still unknown) are not a synapomorphy to the Dendrobatidae. This is furthermore supported by the relationship between the different genera studied here: although the genus *Ranitomeya* is more closely related to *Hyloxalus* than to *Ameerega* (Grant et al., 2006), chemical cues of *H. azureiventris* and *A. trivittata* were avoided while those of *H. nexipus* were not.

These findings so far support the alternative hypothesis (iii), predicting the recognition of properties of phytotelmata-breeding species only. Beyond this, the observed non-avoidance of a bufonid toad (Schulte et al., 2011), which does not utilize phytotelmata, is also in concert with this hypothesis. However, the explicit preference of *Osteocephalus mimeticus* larvae for tadpole depositions is puzzling here, as it neither is a dendrobatid nor exploits phytotelmata as its larval habitat. Despite the reaction to this species by male parent *R. variabilis* being contrary to any expectation, our data advocate that it is able to recognize *O. mimeticus*. This does contradict hypothesis (iii) because *O. mimeticus* is a stream-breeding species and should be unfamiliar to *R. variabilis*. A hypothetical ‘solution’ to this contradiction might be that in this case chemical recognition by *R. variabilis* has an ecological ‘sense’ and takes place at a higher taxonomic level here, a phenomenon known in other animal species (Ferrari et al., 2007; Ferrari et al., 2008; Ferrari and Chivers, 2009; Griffin et al., 2001; Stankowich and Coss, 2007). Under these assumptions, *O. mimeticus* larvae that are unfamiliar to *R. variabilis* are mistaken for the non-cannibalistic phytotelmata-breeding *Osteocephalus leoniae*, whose geographic distribution overlaps with that of *R. variabilis* (compare Jungfer et al., 2013). If so, the recognition of this ‘species’ is well in agreement with hypothesis (iii), as a particular preference of *O. leoniae* larvae for tadpole deposition might be interpreted as the recognition of a potential food source for the carnivorous *R. variabilis* tadpoles. That this poison frog tends to deposit its larval offspring with potential prey was shown by Brown et al. (2009b), as well (tadpoles were preferentially deposited with other males’ embryos, which were eaten by the cannibalistic larvae right after hatching).



As *Osteocephalus* tadpoles are expected to cause no risk to *Ranitomeya* larvae, one may propose that not only the mechanism to recognize potential predators to eggs (see above) but perhaps also the mechanism to recognize potential food to larvae is an advantageous trait evolved in *R. variabilis*. Interestingly, *Ranitomeya reticulata* larvae occur in the same phytotelmata and prey on *O. deridens* tadpoles (unpublished observations K-H. Jungfer). As the two mentioned species of *Ranitomeya* belong to distinct clades (Brown et al., 2011), it may be worth studying whether this proposed trait has evolved once or even in parallel.

The ability to discriminate and respond only to cues of species known to a population due to prior experience is widespread in the animal kingdom (Chivers and Smith, 1994; Griffin, 2004; Maloney and McLean, 1995; Mathis et al., 1993; Wisenden et al., 1997). Kats et al. (1988), for example, have shown that tadpoles frequently encountering a common fish predator increased refuge use when exposed to chemical predator cues. Tadpoles from populations that rarely encounter this predator, however, did not respond to its chemical properties. Other studies provide comparable results with anurans and introduced predators. While populations that are already accustomed to these predators are able to respond with avoidance to their chemical cues, animals that have never been in touch with the exotics do not show the appropriate responsive behavior (Chivers et al., 2001; Kiesecker and Blaustein, 1997; Polo-Cavia et al., 2010). These examples show some parallels with the tadpole deposition decisions of *R. variabilis*. While all syntopic poison frog tadpoles were avoided, the allotopic ones were not even noticed.

In contrast, in terms of *R. variabilis* egg depositions, this assumption seems to be refuted. Frogs did not discriminate between different species but rather avoided all of them for their clutches. There are three possible explanations for this behavior: (1) The avoidance of water used by tadpoles prior to clutch deposition is not primarily triggered by chemical cues, but by a lack of oxygen due to the uptake rate of the tadpoles. Oxygen is an important parameter for breeding water quality (e.g. Seymour, 1999). However, this factor mainly influences species that deposit their eggs underneath the water surface. *Ranitomeya variabilis* in contrast does not only deposit its eggs in phytotelmata known for a generally low oxygen concentration (Wells, 2007), it furthermore attaches its eggs right at the water surface, so they are usually in contact with the air (Brown et al., 2008a). Hence, this explanation might be refuted. (2) Different chemical cues trigger the avoidance behavior during egg and tadpole deposition.

While a more general cue that all tadpoles have in common is involved in egg depositions, the cue(s) involved in tadpole depositions are more specific and differ between species. The positive reaction towards *Osteocephalus* tadpoles especially favors this hypothesis, since it differed from the responses towards all other tadpoles. (3) There is one cue that all tadpoles produce, but at different concentrations. While a potential concentration has to be reasonably high to trigger avoidance during tadpole deposition, the sensitivity to this cue is much higher during egg deposition (possibly due to the females involved). Under such a scenario, *Osteocephalus* tadpoles may produce an additional cue, hypothetically triggering *R. variabilis* to deposit its tadpoles with this species.

Such a generalization, as suggested by hypothesis (3), may include that the ability to discriminate and respond to tadpole cues is less affected by prior experience but rather genetically determined, i.e. innate. There are several studies showing that the recognition of predators has a genetic basis (Blumstein, 2006; Hawkins et al., 2004; Magurran, 1990 ; Mueller and Parker, 1980; Riechert and Hedrick, 1990; Veen et al., 2000). On the other hand, if different cues for egg and tadpole depositions play a role, as suggested by hypothesis (2), an innate avoidance behavior towards all tadpoles in relation to egg depositions and a target-orientated, i.e. learned avoidance (likewise preference) for tadpole depositions might be considered. We consider this to be a promising research field in need of more satisfactory answers.

There are several studies that refer to parental care in anurans in the sense of habitat choice for the offspring (i.e. for eggs or tadpoles). Some show that water inhabited by predators like fish, shrimp or cannibalistic conspecifics are avoided by adult anurans (e.g. Downie et al., 2001; Schulte et al., 2011; Spieler and Linsenmair, 1997; Takahashi, 2007). Others confirm the avoidance of pools containing conspecific competitors (e.g. Lin et al., 2008; Murphy, 2003; von May et al., 2009), and yet others illustrate that pools with conspecific tadpoles in contrast are preferred, since their presence may function as an indication of resource quality (e.g. Downie et al., 2001; Rudolf and Rödel, 2005). To the best of our knowledge, we are not aware of any study demonstrating the reaction of parental anurans towards heterospecific tadpoles; and in particular, the preference instead of avoidance of another anuran species has not yet been shown. These novelties, so far exclusively performed by the here-studied

*Ranitomeya* species, open an interesting field in cognitive, physiological, ecological and evolutionary research.

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## Chapter II

### **The chemistry of parental care: the characterization of chemical communication between poison frogs and their tadpoles**

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## Abstract

When defining communicative systems, one has to distinguish between evolved functions (i.e. signals) and incidental effects (i.e. cues). The accurate categorical classification of inter- and intraspecific chemical communication is often puzzling and sometimes only a closer look at the chemical compounds involved brings clarification. The Neotropical poison frog *Ranitomeya variabilis* (Dendrobatidae) conducts parental care and deposits its tadpoles in phytotelmata (small water bodies in plant axils). It chemically recognises and avoids both, predatory conspecific tadpoles as well as non-predatory tadpoles of the dendrobatid species *Hyloxalus azureiventris* for offspring deposition. The chemical structures of both tadpole substances triggering this behaviour are unknown. While those emitted by the conspecific larvae might be defined as chemical cues (only advantageous for the receiving individuals), the substances of the heterospecific tadpoles might be either an unintentionally released family specific byproduct or a chemical signal, independently evolved in order to prevent the deposition of predatory *R. variabilis* tadpoles. Here, we extracted the active chemical compounds from the water of both tadpole species, and after conducting a LC-HRMS analysis, we separated them in order to determine which fractions triggered the avoidance behaviour of the frogs in an in-situ bioassay. We found that the two species released different biologically active compounds, triggering the same behaviour in the adult *R. variabilis*. We suggest that the chemicals released by *H. azureiventris* tadpoles are chemical signals that are advantageous for both, the tadpoles and the heterospecific frogs (avoiding a competition situation for their offspring). Hence they can be classified as synomones, allelochemicals that are advantageous for both emitter and receiver. This is the first time that communication by synomones has been shown to occur between two vertebrate species.

**Keywords:** chemical communication, Dendrobatidae, parental care, synomones

## Introduction

Communication in the biological sense is defined by Wilson (1970) as the action of one organism that alters the probability pattern of behaviour in another one. The major difficulty when defining communicative systems is to distinguish between evolved functions and incidental effects (Otte, 1974). Chemical communication is generally regarded as the oldest and most widespread form of communication that occurs at all levels of biological organization (Agosta, 1992; Bradbury and Vehrencamp, 2011; Wyatt, 2003). Beside chemical signals, intentionally released by the sender organism, receiving organisms also react towards unintentionally released chemicals, which are defined as chemical cues (Steiger et al., 2011). While chemical signalling is usually advantageous to the sender, releasing chemical cues is either neutral or damaging to it. However, if the reaction of the receiver towards chemical cues is beneficial to the sender, evolution towards signalling is expected (Wyatt, 2010). The evolution of chemical communication is therefore described as ‘chemical ritualization’ towards chemical cues (Steiger et al., 2011). Both chemical cues and signals are used intra- and interspecifically. Intraspecific chemical cues can indicate for example the (previous) presence of conspecifics (Saleh et al., 2007; Wilms and Eltz, 2008), interspecific cues can reveal the presence of a predator (also referred as kairomones, e.g. Hartman and Abrahams, 2000; Takahara et al., 2012). Signals in the intraspecific context are defined as pheromones (Wyatt, 2003; e.g. sex or mammary pheromones Mason et al., 1989; Schaal et al., 2003), while signals between different species can be divided into synomones (benefiting sender and receiver) and allomones (benefiting the sender only) (Nordlund and Lewis, 1976).

The proper categorical classification of a chemical substance that triggers a special behaviour in another species is often puzzling and sometimes only a closer look at the chemical compounds involved brings clarification. One example for this can be found in bumblebees that refuse flowers with scent marks from recent visitors. Several authors specified those scent marks as chemical signals (e.g. Cameron, 1981; Stout and Goulson, 2002), but Saleh et al. (2007) demonstrated, by using a comparative chemical analysis, that in fact they are chemical cues that bumblebees leave behind everywhere they walk. While there are many studies that concentrate on the decoding of chemical signals in insects (e.g. Butenandt and Hecker, 1961; Hölldobler et al., 2001; Lagoutte et al., 2013) and mammals (e.g. Hurst et al., 2001; Müller-Schwarze et al., 1974; Rasmussen et al., 2002), in amphibians this branch has mainly

focussed on salamanders and newts (e.g. Eom et al., 2009; Feldhoff et al., 1999; Kikuyama et al., 1995). Only in recent years this field also has opened up for anuran amphibians (frogs and toads), although the influence of chemicals from con- and heterospecifics on their behaviour is known for many years (e.g. Eibl-Eibesfeldt, 1949; Holomuzki, 1995; Pfeiffer, 1966; Takahara et al., 2008a).

Few studies specified pheromones emitted from glands of adult male frogs, acting as attractants to conspecific males or females (King et al., 2005; Poth et al., 2012; Wabnitz et al., 1999, 2000). Of those contributions, treating chemical communication in tadpoles, so far only two examined the chemicals more closely (besides some preliminary approaches by Hrbáček, 1950; Mirza et al., 2013; Takahara et al., 2013; Takahara et al., 2008b) and both of these studies significantly improved the understanding of the communication systems examined. An alarm pheromone acting between tadpoles was described by Fraker et al. (2009). The existence of tadpole-released chemicals affecting behavioural responses in conspecifics was known for many years, but until their further examination they were all handled as chemical cues rather than actively secreted pheromones (e.g. Adams and Claeson, 1998; Ferrari et al., 2007; Hews and Blaustein, 1985). Ferland-Raymond et al. (2010) described another chemical of importance for tadpoles. Predatory damselfly larvae excrete a kairomone that the authors could only isolate when the predators were fed with tadpoles before. A further analysis might clarify if it is consistent with an alarm pheromone excreted by the tadpole while being eaten.

Behavioural studies of the Neotropical poison frog *Ranitomeya variabilis* (Zimmermann and Zimmermann, 1988) provide another case where the definition of cues and signals is puzzling. This dendrobatid frog displays advanced levels of parental care, using phytotelmata (such as small water bodies in plant axils, Varga, 1928) for both clutch and tadpole deposition. It attaches its clutches just above the water surface and when the larvae hatch the male parent returns and transports them individually into different phytotelmata (Brown et al., 2008b). Because its tadpoles are cannibalistic, feeding on both conspecific tadpoles and eggs (Summers, 1999), parent *R. variabilis* need to locate unoccupied phytotelmata for their offspring. Schulte et al. (2011) showed that the avoidance of occupied water bodies was triggered by chemicals released by conspecific tadpoles. An identical or even stronger reaction was shown towards chemicals released by tadpoles of a species of the same family, *Hyloxalus azureiventris* (Schulte and Lötters, in press). It occupies big ground phytotelmata,

which sporadically are also used by *R. variabilis* for tadpole depositions. The avoidance of predatory conspecific tadpoles (i.e. the sender) increases the frogs' (i.e. the receiver) reproductive success, but is not advantageous to the tadpoles occupying the phytotelmata (losing a possible food resource). In this case we might define the chemicals that trigger the avoidance behaviour as chemical cues, released unintentionally by the tadpoles. However, this might not be the case for the chemicals released by *H. azureiventris*. The heterospecific tadpoles are not predatory and should therefore not pose a threat (but maybe a competition situation) to the offspring of *R. variabilis*. But since *H. azureiventris* tadpoles evade the threat of both predation and competition, the avoidance by *R. variabilis* is supposed to be advantageous for them. This would suggest that the chemicals released by *H. azureiventris* are signals. But the taxonomic relation between the two poison frog species on the other hand raises the question if the chemicals released by both species might not be the same, i.e. the avoidance of *H. azureiventris* could be a reaction of *R. variabilis* not being able to distinguish the con- and heterospecific tadpoles.

Using a combination of bioassays in the field and comparative chemical analyses of both species, we tested the following hypothesis: (i) the chemical compound(s) avoided by *R. variabilis* are produced by tadpoles of both *R. variabilis* and *H. azureiventris* as a non-specific byproduct and might therefore be defined as chemical cue(s). Alternatively, we hypothesise that (ii) the biologically active compounds differ between the two species so that those released by *H. azureiventris* can be defined as signals (i.e. allomones or synomones).

## Material and methods

### Chemicals and analytical instruments

Methanol (gradient grade), dichloromethane (HPLC grade) and formic acid (analytical reagent grade, 98 %) were supplied by Merck. For detection of chemical cues, we used a Agilent 1200 Liquid chromatography (LC) system consisting of an Autosampler, a binary pump, a column oven and a diode-array detector. The LC system was coupled to a high resolution-high mass accuracy mass spectrometer (HRMS; LTQ Orbitrap XL, Thermo Scientific) using an electrospray ionization (ESI) interface. This system was also used to define the fraction boundaries in the preparative fractionation procedure based on the

occurrence of prominent peaks in the HRMS chromatograms. For fractionation, a semipreparative LC system was used consisting of a Rheodyne manual valve, a Varian ProStar 210 Binary Pump System with 25 mL stainless steel pump heads (Varian), an UVD340U diode-array-detector (Dionex) and a Foxy 2000 fraction collector (Teledyne Isco Inc.) controlled by the Chromeleon 6.7 software (Dionex).

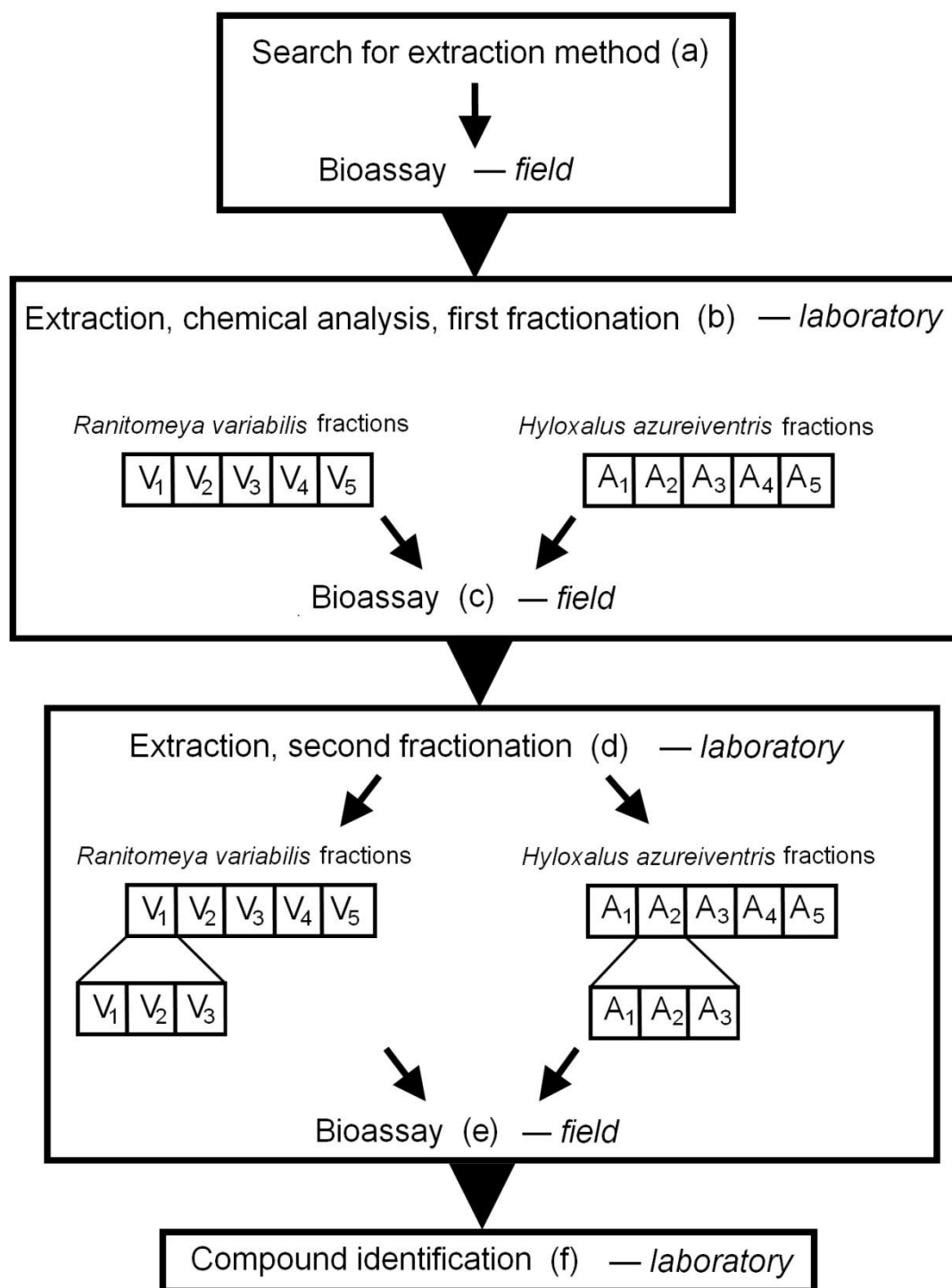
An overview of the succession of the methodical steps is given in figure 1.

### **(a) Search for a procedure to extract the chemical cues**

To find a suitable solid-phase extraction (SPE) sorbent for concentrating the cues from water samples, at first experiments were conducted to eliminate the active cues from the tadpole water by sorption to a SPE material and test these purified water samples for their activity. Successful sorption to the SPE material should cause that parental *R. variabilis* do not avoid the water previously used by tadpoles anymore. These experiments were conducted *in situ* 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.

#### *(i) Activated carbon*

Between 23 March and 8 May 2011 27 tadpoles of each *Ranitomeya variabilis* and *Hyloxalus azureiventris* were collected from their natural habitat and raised in captivity in a wooden hut in the forest serving as a field laboratory during the trials. Each specimen was housed individually in 50 mL of rainwater in a standardized polypropylene cup. Every other day, we mixed the water of the tadpoles (separated by species), refilled the cups with fresh rain water and fed the larvae ad libitum with flaked fish food (Tetra®). After finishing the trials or when tadpoles reached Gosner's developmental stage 41 (Gosner, 1960), they were released back into the wild. The water obtained from the tadpoles was thereafter filtered through approximately 30 g of granular activated carbon (Merck Millipore, 1.5 mm diameter) in order to absorb the odour producing substances (Greenberg, 1987).



**Fig. 1** Systematic methods overview. Methodical steps accomplished between 2011 and 2013 in order to find the chemical tadpole cue, are numbered in accordance with the text (a - f).

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(ii) *Chromabond and DSC-18*

Twenty-five *Ranitomeya variabilis* tadpoles were collected and kept in the field laboratory from 25 March to 8 May 2011 together in a container in 1250 mL of rainwater. Because tadpoles of this species are cannibalistic, each individual was enclosed in a little bag made of net lace. The meshes were big enough to let excrements pass, but small enough to prevent physical contact between the tadpoles. A wide opening towards the water surface facilitated the feeding procedure and enabled the animals to take up oxygen. At the bottom of each container a passive sampler was placed (Sartorius membrane filter, 7cm diameter, 1.2 µm pore size) containing either 1.7 g of Chromabond HR-X (Macherey-Nagel) or 4 g of Discovery DSC-18 (Supelco) to sorb the compounds. Differences in filter content weights were given due to particle size of the absorbents. The water of the trials was collected and replaced every second day (followed by feeding of the tadpoles, see (i)). The passive samplers were changed with every third water change. Trials were not conducted with *Hyloxalus azureiventris* because we could not find a sufficient amount of tadpoles of this species.

(iii) *Bioassays in the forest*

We established three study sites on the western side of the Cainarachi River at an altitude between 540 and 580 m above sea level. In order to avoid pseudo-replication, i.e. repeated measures on the same specimens, we maintained a minimum distance of 30 m between the sites, which exceeds the known home range size of *Ranitomeya variabilis* (Brown et al., 2009). For the setup in the three forest sites, we established artificial phytotelmata. Polypropylene plastic cups (200 mL volume, 10 cm height, 7 cm in diameter), wrapped in dark plastic membranes and with two-thirds of the opening covered, were fixed in pairs to trees at 0.5-1.5 m above ground. Each cup was filled with 25 ml water, one cup per pair with clean rainwater and the other cup with activated carbon, HR-X or C18 treated water as explained in (i) and (ii). For each treatment we spread 45 cup pairs through the three forest sites. They were checked every second day for newly deposited tadpoles and clutches of *R. variabilis* and both clean and treated water was changed if no depositions were found. Pairs that received a deposition were not used again but another pair was hung up at another tree instead. In order to minimize pseudo-replication, we kept a minimum of 4 m between cup



pairs belonging to the same trial and scored only one randomly chosen deposition for any same-day deposition in neighbouring cup pairs (Brown et al., 2008a; Schulte et al., 2011).

### **(b) Directed chemical extraction and primary fractionation of the chemical cues**

Based on the results of (a) we used Discovery DSC-18 (Supelco) for the extraction and collection of the active chemical cues from the tadpole water. Although we had no data related to the extraction of chemical cues produced by *Hyloxalus azureiventris*, we applied our methods in the laboratory to both *Ranitomeya variabilis* and *H. azureiventris*.

We used tadpoles from captive breeding colonies at Trier University and kept them from 12 November 2011 to 24 January 2012 at the Department of Effect-Directed Analysis, UFZ Leipzig, where fractionation and chemical analysis were conducted. During this time we had between six and nine *R. variabilis* tadpoles and eleven to 23 *H. azureiventris* tadpoles. They were kept individually and their water was changed twice a day: six hours (+/-1) a day they were kept in approximately 50 mL of tap water with abundant food supply (see (a)) and 18 hours (+/-1) a day in 10 mL without food. The water without food, containing excretions produced by the tadpoles only, was collected over two days (separated by species) and vacuum filtered for further analysis (Whatman glass microfibre filters, pore size 0.7 µm). Thereupon a solid phase extraction was conducted, using Discovery DSC-18 (Supelco) as sorbent. The sorbent was preconditioned using dichloromethane (DCM), methanol (MeOH) and bidistilled water before the water sample was passed through the cartridge at a flow rate of about 10.0 mL/min. Once the entire sample had passed through, cartridges were dried under nitrogen and eluted two times with 6 mL of MeOH and DCM each. Eluates were collected and reduced in volume to dryness in a rotary evaporator. The dried cues were collected at 4 °C and while 214 *R. variabilis* and 652 *H. azureiventris* samples were kept in the dry form as total samples ( $V_{\text{total}}$  and  $A_{\text{total}}$ ) for later use in the biotests, 238 *R. variabilis* and 406 *H. azureiventris* samples were reconstituted in MeOH (10 µL per tadpole sample) for fractionation.

As a control sample to check for background contamination that derived from the fish food, a water sample containing only fish food but no tadpoles was processed by SPE as described above. The LC fractionation was carried out using a Lichrospher 100 RP-18 column (Merck,

250 x 4 mm, 5 µm particle size). A gradient elution was carried out using bidistilled water:methanol 95:5 (eluent A) and water:methanol 5:95 (eluent B) at a flow rate of 1.0 mL/min. The gradient program was 0-4 min 100 % A, 4-22 min 0-100 % B, 22-25.5 min 100 % B, and re-equilibration to 100 % A for 5 min. Fractions of samples A and V were collected every minute. Based on LC-HRMS analyses of the sample the one-minute fractions were combined as follows: Fraction A<sub>1-12</sub>/V<sub>1-12</sub> 10-18 min, A<sub>2-12</sub>/V<sub>2-12</sub> 18-21 min, A<sub>3-12</sub>/V<sub>3-12</sub> 21-25 min, A<sub>4-12</sub>/V<sub>4-12</sub> 25-30 min, A<sub>5-12</sub>/V<sub>5-12</sub> 30-40 min.

### **(c) Bioassays to test which fraction(s) contain the avoided chemical cue(s)**

Between 10 February and 18 April 2012 we conducted bioassays in the field to find out which fraction(s) contained the cue(s) avoided by the frogs. We set up 20 pairs of artificial phytotelmata for each trial as described in (a) and conducted water changes every other day with either clean rainwater or with water containing either the total samples (V<sub>total</sub> or A<sub>total</sub>) or one of the fractions (V<sub>1-12</sub> - V<sub>5-12</sub> or A<sub>1-12</sub> - A<sub>5-12</sub>) produced in the lab. To mix the dried samples into water, we acted in accordance with the concentrations used in trials with fresh tadpole water before (compare (a) and Schulte et al., 2011). We first dissolved our dried samples in 15 µl rainwater per tadpole sample and deep-froze them in measured portions. Those portions were then mixed with rainwater in the field, filling 15 µl each (i.e. one tadpole that used water in the lab for one day) in 25 ml water. Due to a lack of chemical cues, we had to use a smaller amount of water in our cups than before. In trials with samples from *R. variabilis* we filled 10 ml in each cup and in trials with samples from *H. azureiventris* we used 15 ml per cup.

### **(d) Second fractionation of the chemical cues from the tadpole water**

From 18 May to 19 June 2012 and 23 August 2012 to 4 January 2013 water samples of tadpoles from Trier University were collected daily as described in (b). The number of tadpoles varied between five and 23 in *Ranitomeya variabilis* and two and 15 in *Hyloxalus azureiventris*. Chemical cues were extracted at the Soil Science Department, Trier University, in the way described in (b). Of *R. variabilis* 1089 tadpole samples were thereafter not further processed and kept in dried form for a second test of the total sample (V<sub>total</sub>) in the field. The

remaining samples (1197 of *R. variabilis* and 978 of *H. azureiventris*) were further fractionated at UFZ Leipzig. We thereby focused on the fractions that turned out to be avoided in the previous bioassays.

Fractionation of the *R. variabilis* samples was conducted in the same manner as described in (b), but the fractions collected every minute were pooled differently and those with no effects in 2012 were abolished. Fractions were combined as follows: Fraction  $V_{1-12}$  13-16 min,  $V_{2-13}$  16-20 min,  $V_{3-13}$  20-23 min. The *H. azureiventris* samples, however, were at first fractionated and pooled together as in 2012. For a finer fractionation with different selectivity we fractionated fraction  $A_{2-12}$  using a Polaris 5 Amide C18 column (Varian, 150 x 4.6 mm, 5  $\mu$ m particle size). A gradient elution was carried out using bidistilled water:methanol 50:50 (eluent A) and water:methanol 5:95 (eluent B) at a flow rate of 1.0 mL/min. The gradient program was 0-10 min 100 % A, 10-15 min 0-100 % B, 15-16 min 100 % B, and re-equilibration to 100 % A for 3.5 min. Fractions of  $A_{2-12}$  were collected every minute and combined as follows: Fraction  $A_{1-13}$  0-7 min,  $A_{2-13}$  7-15 min,  $A_{3-13}$  15-20 min.

**(e) Bioassays to test which fraction(s) contain the avoided chemical cue(s)**

Bioassays to test which fractions/cues were avoided by the frogs were conducted in the field, same Peruvian sites as mentioned above, with artificial phytotelmata again (compare (a) and (c)). Between 5 February 2013 and 24 May 2013 we offered the fractions and mixtures of fractions to the frogs. Fractions of *H. azureiventris* were mixed for the case that the compounds were active only in mixtures. The mix of  $A_{1-13}$  and  $A_{2-13}$  is hereafter called  $A_a$ ,  $A_b$  is the single fraction  $A_{3-13}$ ,  $A_c$  is a mix of  $A_{1-13}$  and  $A_{3-13}$  and  $A_d$  equals  $A_{2-13}$ . Due to the combination of single and mixed fractions, we were able to find out if a single cue was avoided by the frogs as well. Dried samples were diluted in the same manner as described in (c) with one difference: due to the non- or only nearly significant results for *R. variabilis* in 2012 we doubled the concentration of both the total sample as well as the fractions of this species used in 2013. The amount of water used in the cups in the forest was 10 ml each.

**(f) Instrumental analysis and LC-HRMS data analysis**

To determine the chemical cues by LC-HRMS, we used the Lichrospher 100 RP-18 column (250 x 4 mm, 5  $\mu$ m particle size) and gradient described above. The LC was connected to an ESI source and an LTQ Orbitrap XL mass spectrometer. Samples were analysed in positive and negative full scan mode at a nominal resolving power of 100,000 with data-dependent acquisition of MS/MS spectra at a resolving power of 15,000 triggered by the most and second-most intense peaks in the chromatograms.

The compound identification procedure followed the nontarget screening approach described by Hug et al. (2014). Briefly, fractions were finally analysed by LC-HRMS using a Kinetex Core-Shell C18 column (100 mm  $\times$  3.0 mm; 2.6  $\mu$ m; Phenomenex) and a gradient elution with water (A) and methanol (B) both containing 0.1 % formic acid at a flow rate of 0.2 mL/min. Deconvolution of the full scan chromatograms and peak detection was carried out using the software MZmine 2.9. From the obtained peak lists, those peaks occurring in blank and fish food control samples were removed. For the peaks remaining in active fractions, molecular formulas were calculated based on accurate masses and isotope patterns, and the Chempidder and KEGG databases were searched for all candidate structures of the particular molecular formula. To exclude unlikely candidate structures, we employed MS2 fragmentation prediction and retention time prediction as detailed in Hug et al. (2014).

**(g) Statistical analysis**

For each treatment tested in (a), (c) and (e) we pooled clutch and tadpole depositions since chemical substances of both species are avoided for both deposition types (Schulte and Lötters, in press). We compared the deposition frequencies in each water type (clean or treated) using a G test (Woolf, 1957), that is suggested to be of superior exactness for limited observations (Gotelli and Ellison, 2004; Sachs, 1974). Since each dataset had less than 200 observations, we adjusted the G test in the manner of Williams (1976). As a null hypothesis, we assumed that the frequency of deposition events was random (0.5 in either pool).

Because the change from rainy to dry season is known to have an effect on the depositions decisions of parental *Ranitomeya variabilis* (Schulte and Lötters, 2013b), we conducted a changepoint analysis in R (R Development Core Team, 2011) with the rainfall measurements

taken during each field season, using the package ‘changepoint’ (Killick and Eckley, 2011a, b). While there was a significant seasonal change in 2011 (compare Schulte and Lötters, 2013b), no change could be measured in 2012 and 2013 (bootstrap  $p < 0.05$ ). We therefore did not have to split our data before analysis.

## Results

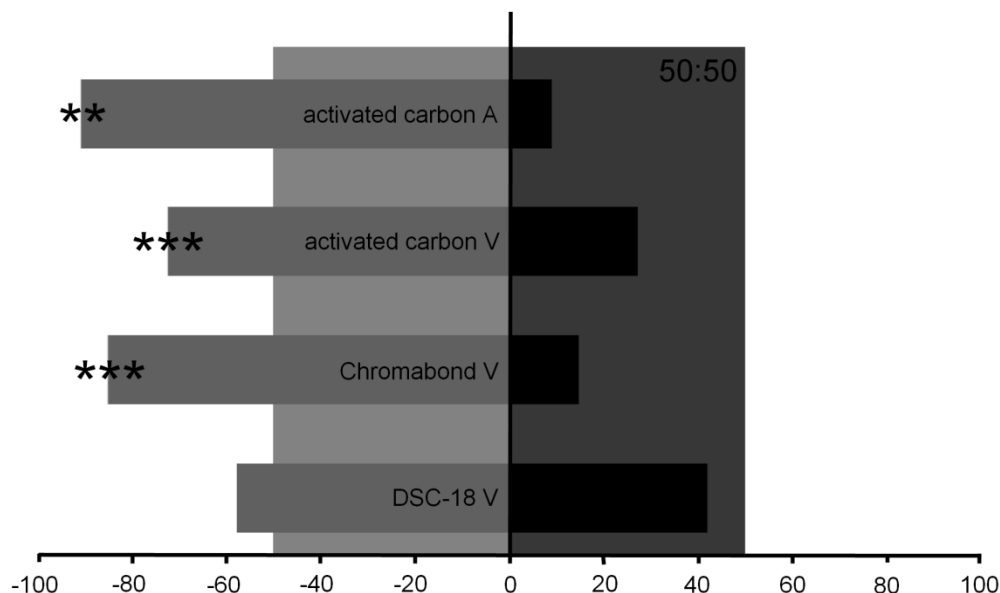
### (a) Absorbent to extract the chemical cues

The water used by *Ranitomeya variabilis* or *Hyloxalus azureiventris* was still avoided by parental *R. variabilis* for egg and clutch deposition after filtering it through activated carbon (24 in clean and 9 in filtered *R. variabilis* water,  $G = 6.97$ ,  $p < 0.01$ ; 31 in clean and 3 in filtered *H. azureiventris* water,  $G = 26.45$ ,  $p < 0.001$ ; figure 2). Furthermore the passive sampler filled with Chromabond HR-X could not sufficiently bind the avoided chemical cues. Parental *R. variabilis* still preferred clean over treated water significantly (24 in clean and 4 in passively filtered *R. variabilis* water,  $G = 14.51$ ,  $p < 0.001$ ). However, water used by *R. variabilis* tadpoles containing a passive sampler filled with Discovery DSC-18 was not avoided anymore (18 in clean and 13 in passively filtered *R. variabilis*-water,  $G = 0.08$ ,  $p = 0.37$ ; see figure 2). An influence of the beginning dry season on this result as shown by Schulte and Lötters (2013b) can be ruled out here. Experiments were stopped a few weeks after the seasonal change (compare changepoint in Schulte and Lötters, 2013b) and treatments that involved Discovery DSC-18 were not only distributed uniformly after, but also before the changepoint (before changepoint: 4 depositions in clean and 4 in treated water, after changepoint: 11 in clean and 9 in treated water).

### (b) *Ranitomeya variabilis* fractions

The results of the bioassays conducted in 2012 and 2013, testing the frogs’ reactions towards the different chemical fractions obtained from *Ranitomeya variabilis* tadpole-water, are shown in table 1 and figure 3. Of the fractions tested in 2012 none was avoided significantly, but frogs showed a strong tendency towards the clean water over fraction 1 ( $V_{1-12}$ ). When splitting  $V_1$  into smaller fractions in 2013, we found a significant avoidance of fraction 2

(V<sub>2-13</sub>). Of the compounds found in V<sub>2-13</sub> (see table 2), one was found in the water of *Hyloxalus azureiventris* as well.



**Fig. 2** Preliminary trials. Percentaged ratio of offspring depositions in clean and treated water (grey and black bars, respectively). The anticipated distribution (50:50) is shown with paler colours in the background. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

### (c) *Hyloxalus azureiventris* fractions

In 2012 parental *Ranitomeya variabilis* strongly avoided both the total sample of the chemically processed *Hyloxalus azureiventris* tadpole-water ( $A_{\text{total}}$ ) as well as fractions 2 ( $A_{2-12}$ ) and 4 ( $A_{4-12}$ ) (table 2, figure 3). Fraction 4 was not avoided in 2013 anymore (tested again because compounds differed between 2012 and 2013). However, the mix of the fractions  $A_{1-13}$  and  $A_{3-13}$  ( $= A_c$ ) was avoided significantly. Other fractions or mixes of fractions did not show significant results. One of the compounds found in  $A_c$  (see table 2) was found in the water of *R. variabilis*, too. Nonetheless, this compound was not active independently, because it could also be found in  $A_a$ , which was not avoided by the frogs.

**Table 1** Pooled egg and tadpole depositions placed by parental *Ranitomeya variabilis* in cups with clean or treated water, i.e. water containing fractions of chemical substances from *R. variabilis* (V) or *Hyloxalus azureiventris* (A), followed by the results of the G-test.

		Depositions in experiments with <i>R. variabilis</i> -fractions				Depositions in experiments with <i>H. azureiventris</i> -fractions				
		clean water	treated water	G-Test	p		clean water	treated water	G-Test	p
2012 + 2013	V <sub>total</sub>	29	16	3.77	0.05	A <sub>total</sub> *	18	3	11.61	< 0.001
2012	V <sub>1-12</sub>	16	7	3.54	0.06	A <sub>1-12</sub>	11	8	0.46	0.50
	V <sub>2-12</sub>	11	11	0.00	1.00	A <sub>2-12</sub>	21	1	21.87	< 0.001
	V <sub>3-12</sub>	7	8	0.06	0.80	A <sub>3-12</sub>	10	12	0.18	0.67
	V <sub>4-12</sub>	9	12	0.42	0.52	A <sub>4-12</sub>	17	7	4.21	0.04
	V <sub>5-12</sub>	11	8	0.46	0.50	A <sub>5-12</sub>	12	10	0.18	0.67
2013	V <sub>1-13</sub>	17	15	0.12	0.73	A <sub>a (=1+2-13)</sub>	18	12	1.19	0.28
	V <sub>2-13</sub>	22	6	8.46	< 0.01	A <sub>b (=3-13)</sub>	11	20	2.61	0.11
	V <sub>3-13</sub>	20	10	3.34	0.07	A <sub>c (=1+3-13)</sub>	21	8	5.94	0.01
	-	-	-	-	-	A <sub>d (=2-13)</sub>	21	11	3.13	0.08
	-	-	-	-	-	A <sub>4-13</sub> **	12	16	0.56	0.45

\* A<sub>total</sub> was only tested in 2012, \*\* A<sub>4-13</sub> in 2013 is the same fraction as in 2012, but missing some cues that could not be found again in 2013

#### (d) Identification of the chemical substances

In the final active fractions of 2013 a low number of compounds could be detected which were present in the active fractions of 2012 as well (table 2). In fraction V<sub>2-13</sub> these were two compounds showing ions in positive ion mode at m/z 134.0594 (compound V1) and 171.1488 (compound V2), respectively. The latter was also found in fraction A<sub>1-13</sub> of fraction-mix A<sub>c</sub> (accordingly compound V/A2). Since this compound could not be shown to be active in fraction mix A<sub>a</sub>, we further analysed A<sub>3-13</sub> (the second fraction in A<sub>c</sub>). Compound A1 found in this fraction showed an ion at m/z 228.1962, but it could not be found in A<sub>2-12</sub>.

Based on the accurate mass and isotope patterns and the underlying assumption that protonation in ESI+ occurred, we determined molecular formulas for these compounds (table 2) and searched the Chemspider database for corresponding structures. For compound V1

(C<sub>8</sub>H<sub>7</sub>NO) 186 candidate structures were found, for compound V/A2 (C<sub>9</sub>H<sub>18</sub>N<sub>2</sub>O) 1653 and for compound A1 (C<sub>13</sub>H<sub>25</sub>NO<sub>2</sub>) 582.

Based on the approach by Hug et al. (2014), we reduced the number of candidate structures by comparing predicted and measured MS2 spectra using the software Metfrag (Wolf et al., 2010). Candidate structures with a score < 0.8 were excluded. Subsequently, measured and predicted LC retention (as expressed by the chromatographic hydrophobicity index, CHI) was compared based on the method of Ulrich et al. (2011) and all candidates removed which were not within  $\pm 6$  units of CHI.

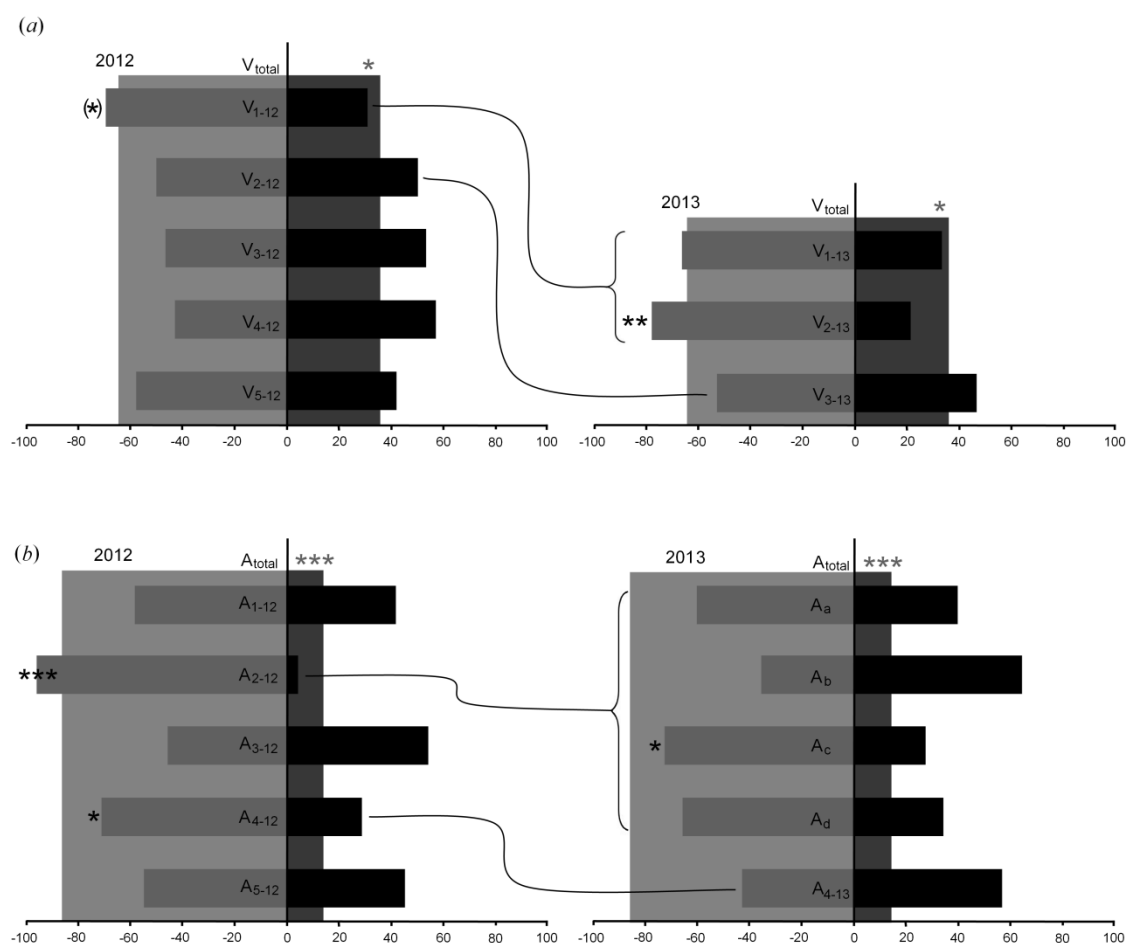
This candidate reduction procedure resulted in 11 candidates for compound V1, of which two were considered unlikely and for compound V/A2 14 candidates were left, of which two were considered unlikely. However, for compound A1 the candidate reduction procedure was not possible.

**Table 2** Overview of compounds found in fractions avoided in the biotests by *Ranitomeya variabilis*.

Ionisation	<i>m/z</i>	Retention time	Fractions 2012	Fractions 2013	Retention time of standard method	Molecular formula	Compound naming
<i>Ranitomeya variabilis</i> compounds							
positive	<b>134.0594</b>	<b>14.0</b>	<b>V<sub>1-12</sub></b>	<b>V<sub>2-13</sub></b>	<b>17.6</b>	<b>C<sub>8</sub>H<sub>7</sub>NO</b>	<b>V1</b>
	<b>171.1488</b>	<b>16.3</b>			<b>20.2</b>	<b>C<sub>9</sub>H<sub>18</sub>N<sub>2</sub>O</b>	<b>V2*</b>
negative	151.0400	15.5			nd	nd	
	297.1522	16.2			nd	nd	
	311.1679	16.9			nd	nd	
<i>Hyloxalus azureiventris</i> compounds							
positive	<b>228.1962</b>	<b>nd</b>		<b>A<sub>3-13</sub></b>	<b>24.1</b>	<b>C<sub>13</sub>H<sub>25</sub>NO<sub>2</sub></b>	<b>A1</b>
positive	<b>171.1487</b>	<b>16.3</b>	<b>A<sub>2-12</sub></b>	<b>A<sub>1-13</sub></b>	<b>20.2</b>	<b>C<sub>9</sub>H<sub>18</sub>N<sub>2</sub>O</b>	<b>A2*</b>
	478.2970	17.7			nd	nd	
negative	515.3034	16.1			nd	nd	
	527.3033	16.3			nd	nd	

\* compounds found in fractions of both species; nd = no detection





**Fig. 3** Biotest results. Percentaged ratio of offspring depositions in clean and treated water (grey and black bars, respectively) in water treated with chemically processed *Ranitomeya variabilis* (a) and *Hyloxalus azureiventris* substances (b). The distribution of the total samples (V<sub>total</sub> and A<sub>total</sub>) is shown with brighter colours in the background. Connecting lines leading from results from 2012 to 2013 show which fractions were further processed in 2013 and contain identical compounds. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

## Discussion

Our comparative chemical analysis of the fractions that triggered avoidance behaviour in adult *Ranitomeya variabilis* revealed that one compound (compound V/A 2) was produced by tadpoles of both *R. variabilis* and *Hyloxalus azureiventris*. This finding would approve our hypothesis (i). However, for *H. azureiventris* the fraction containing this compound could only be shown to be active (i.e. triggering avoidance in the frogs) in combination with one other fraction, but not in another combination. Hence, we suggest that the shared compound is only biologically active in combination with a second compound that differs between *R.*

*variabilis* and *H. azureiventris*. This leads us to reject hypothesis (i) and approve the alternative hypothesis (ii), since the unique compounds found in each species (compounds V1 and A1) are actually of major interest for the interpretation of our results.

With regard to the intraspecific chemical communication between adult *R. variabilis* (the receiver) and their tadpoles (the sender), we defined the chemical compounds as chemical cues, because the avoidance is advantageous to the frogs (i.e. their offspring), but not to the tadpoles (Schulte et al., 2011). However, this definition might be context dependent, since during the dry season the conspecific tadpoles were not avoided anymore but fed with younger tadpoles (Schulte and Lötters, 2013b). Since in our study we focussed on the rainy season, we cannot tell if this feeding behaviour is triggered by the same chemical compounds as the avoidance behaviour, possibly initiating the evolution towards chemical signalling (as suggested by Wyatt, 2010), or if it is triggered by another compound that might be defined as a chemical signal (i.e. pheromone).

Regarding the communication between adult *R. variabilis* and *H. azureiventris* tadpoles, the achieved knowledge that the composition of the active chemical compounds differs between the species, brings us a step further towards the definition of the interspecifically operating chemicals. Being able to exclude the possibility that those compounds are mere byproducts due to taxonomic relation between the two poison frog species, we suggest that this interspecific chemical communication is based on allelochemicals, respectively chemical signals. For the sender (*H. azureiventris*) the avoidance triggered through the chemical compounds is definitively beneficial. Not only are the predatory tadpoles of *R. variabilis* dangerous to the omnivorous, non-predatory *H. azureiventris* tadpoles, but also competition in the relatively small water bodies might be a relevant factor. Even though, the eggs of *R. variabilis* are not dangerous to *H. azureiventris* tadpoles, this might be negligible here. The pools that house *H. azureiventris* tadpoles and that are used regularly for *R. variabilis* tadpole depositions (Schulte and Lötters, 2013a) are too large for egg depositions by *R. variabilis* (Brown et al., 2008a). Unfortunately we cannot show at this point how *H. azureiventris* produces the chemical compounds illustrated here, nor how they are excreted (e.g. with faeces or through epidermis). Nonetheless we are positive about their signalling function. Besides their beneficial characteristics for the sender and their delimitation from the confamilial cues, the intensity of avoidance by parental *R. variabilis* is remarkably higher than the avoidance of

conspecific tadpoles (Schulte and Lötters, 2013a), possibly due to a stronger acting chemical compound.

Assuming that we are dealing with an interspecific signal here, there is still the issue to categorise it into an ecological context. Due to its advantage for the sender, it can be either classified as an allomone or a synomone. Accordingly, the question to be answered is if the avoidance of the signal emitted by larval *H. azureiventris* is advantageous to *R. variabilis* (i.e. its offspring) or not. Since *Ranitomeya* tadpoles are able to feed on *H. azureiventris* tadpoles (pers. comm., J.L. Brown) the avoidance of this potential prey does not seem to be beneficial to *R. variabilis*. This would suggest that the signals are allomones, i.e. beneficial only to the sender. The use of allomones is primarily known from predator-prey systems. On one hand in the way that the predators allure prey animals with imitations of prey specific pheromones (e.g. Haynes et al., 2002), on the other hand by defensive allomones used by the prey to repel predators (for review see Whitman et al., 1990). If those repellents that indicate the toxicity of a prey animal are as well defined as allomones or rather as synomones is controversial. While Nordlund and Lewis (1976) originally defined them as allomones, Dicke and Sabelis (1988) reclassified them as synomones because the avoidance of toxic prey is supposed to be advantageous for the predator, too. Since then both definitions were used in studies dealing with avoidance due to poison that signals the inedibility of the prey (as allomones: Ruther et al., 2002; Schlee, 1999; Whitman et al., 1990; as synomones: Augner, 1994; Sbarbati and Osculati, 2006).

Adult poison frogs are an example of signalling toxicity. While they use visual signals (aposematic coloration) to warn predators such as birds and mammals (Amézquita et al., 2013; Richards-Zawacki et al., 2013; Saporito et al., 2007), there is evidence that predators such as spiders, ants and snakes receive chemical signals from the frogs as indicator for poisonousness (Fritz et al., 1981; Maan and Cummings, 2012; Szelistowski, 1985). Due to the fact that toxins (skin alkaloids) are assimilated through food uptake by adult dendrobatid frogs (for review see Saporito et al., 2012), they do not yet exist in their tadpoles, except for those in the genus *Oophaga* fed with eggs by their parents (pers. comm. J. L. Stynosky, R.A. Saporito). We therefore do not believe that toxins are involved in the predator-prey relationship between larval *R. variabilis* and *H. azureiventris*.

Since *H. azureiventris* tadpoles grow bigger than *R. variabilis* tadpoles and *R. variabilis* tadpoles are very small when deposited (Gosner's stage 25; Gosner, 1960), we assume that the avoidance by parental *R. variabilis* is correlated with competition. Single small- or medium-sized *R. variabilis* larvae are apparently not capable of preying on larger *H. azureiventris* tadpoles (N = 14, attempted for two weeks; unpublished data, J. L. Brown). We therefore conclude that the signal released by larval *H. azureiventris* and avoided by parental *R. variabilis* is a synomone. The avoidance is for both species advantageous in terms of competition prevention. There are many examples for synomones between plants and animals, for instance between flowers and pollinators (e.g. Harrewijn et al., 1994; Tan and Nishida, 2000) or between parasite-hosting plants and natural enemies of those parasites (e.g. Boevé et al., 1996; Sabelis and De Jong, 1988). Synomones between different animal species are less common (aside from predator-warning toxins, depending on the definition, see above). The probably best known examples are synomones released by sea anemones that initialize their mutualistic relationship with clown fishes (Konno et al., 1990; Murata et al., 1986). To the best of our knowledge, chemical communication based on synomones between two species of vertebrates (aside from predator-warning toxins) has never been described before.

We did not only for the first time analyse intraspecific chemical cues used between parental frogs and their offspring, we furthermore described an interspecific signal that might be the first synomone shown in vertebrates, that is used to prevent competition between the offspring of two species.

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## Chapter III

### **Avoiding predation: the importance of chemical and visual cues in poison frog reproductive behaviour**

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## Abstract

The detection of biological signals is especially important in predator-prey systems. Anuran amphibians have evolved a remarkable diversity of defense strategies against predators, but the most risk-free is the prevention of a possible danger. This is valid for the protection of offspring as well. The neotropical poison frog *Ranitomeya variabilis* deposits both eggs and tadpoles in phytotelmata. The exploitation of these small pools is advantageous as it lowers the risk of offspring predation compared to larger water bodies. None the less, there are potential predators in these pools as well. We analysed how the parent frogs avoid conspecific cannibalistic tadpoles and damselfly larvae of the species *Microstigma rotundatum*. We compared the use of chemical and visual cues and show that *R. variabilis* avoids conspecific tadpoles for the deposition of its offspring using chemical cues, while visual tadpole models alone were not avoided by the frogs. Damselfly larvae in contrast were avoided when present, but could not be detected by chemical cues alone. We suggest that the invertebrate predators mask their chemical cues, forcing the frogs to use other senses to detect them.

## Introduction

Biological communication is defined as an action on the part of one organism that alters the probability pattern of behaviour on another (Wilson, 1970), i.e. sending and receiving honest or manipulative signals (Hart, 1996). Animals use a variety of means to communicate with both conspecifics and heterospecifics (Bradbury and Vehrencamp, 2011). Communication between heterospecifics includes predator-prey systems: the danger emanating from the predator organism alters the prey's behaviour, as the behaviour of the prey alters the hunting strategy and the associated success of the predator (Hasson, 1991).

The different life-stages of anuran amphibians, i.e. frogs and toads, run the risk of being preyed by a great variety of predators. Toledo et al. (2011) recently found that adult anurans alone showed more than 30 different defensive strategies against potential attacks. The performance of those behaviours is influenced by the stage of predation (i.e. from localisation to digestion) and can be divided into primary and secondary defence strategies. While the former are independent of the presence of a predator, secondary defence strategies are directly elicited by the predator (Edmunds, 1974). Examples include the 'unken' reflex (e.g. Haberl and Wilkinson, 1997; Williams et al., 2000), puffing up the body (e.g. Duellman and Trueb, 1986; Prates et al., 2011), defensive vocalisation (Toledo et al., 2009), biting or different ways of escaping (e.g. McDiarmid and Gorzula, 1989).

Primary defence mechanisms can be understood as an avoidance of contact with the predator and can contain both honest and manipulative signals. There are numerous examples of such mechanisms including cryptic colour patterns (Osorio and Srinivasan, 1991; Stevens and Merilaita, 2009), aposematic colours (e.g. Darst and Cummings, 2006; Ruxton et al., 2004) or false eyes (Lenzi-Mattos et al., 2005; Martins, 1989; Toledo and Jared, 1995), as well as nocturnal activity (Duellman and Trueb, 1986) or hiding behaviour (Marchisin and Anderson, 1978; Pröhl and Ostrowski, 2011). In addition, abilities to recognise honest signals from the predator are considered anuran primary defence mechanisms. These may be conducted via recognition of visual or chemical cues associated with the predator.

With regard to visual predator recognition, frogs and toads tend to avoid all moving objects, larger than themselves (Cooper et al., 2009; Ewert, 1974; Ingle, 1990). More is known about precautions based on chemical predator cues. Especially in aquatic predator-prey systems, chemical cues are the primary vehicle for information about predation risk (Brönmark and

Hansson, 2000). Accordingly, much is known about tadpoles avoiding either predator cues, cues from predators fed with conspecifics or alarm cues deriving from injured individuals (Eklöv, 2000; Ferrari et al., 2007; Hetttyey et al., 2011; Hews, 1988; Smith et al., 2008; Stauffer and Semlitsch, 1993). Already embryos are able to recognise such cues and either change the time remaining until hatching or their behavioural response to predators after hatching (Ferrari and Chivers, 2010; Saenz et al., 2003). Compared to tadpoles and juveniles, there are relatively few studies on the use of chemical cues in adult frogs and toads (Hamer et al., 2011). Avoidance of chemical predator cues by adult anurans is reported by Murray et al. (2004) and Hamer et al. (2011) and indirectly by Wirsing et al. (2005), who noted an avoidance of injured conspecifics by adult frogs.

Besides terrestrial predators such as birds or snakes, aquatic predators represent an indirect danger to frogs and toads as these may prey upon their offspring. It has been shown for several anuran species that they avoid, for tadpole or egg depositions, water containing predators, but it remains mostly unknown how the frogs recognise them (Petranka et al., 1994; Resetarits Jr and Wilbur, 1989; Rieger et al., 2004). Some authors suggest that chemical



**Fig. 1** A male *Ranitomeya variabilis* carrying one of its larvae to a phytotelm. This species typically uses water-filled leaf axils (phytotelmata) of bromeliad plants. Tadpoles are usually transported one-by-one, but occasionally two or three can be found on the back of the male parent.

cues may be the main source for predator recognition (Binckley and Resetarits Jr, 2003; Hopey and Petranks, 1994; Spieler and Linsenmair, 1997). This has been demonstrated for predatory fish by Takahashi (2007), showing that chemical cues were avoided for egg deposition by tree frogs. Recently, Schulte et al. (2011) have demonstrated the avoidance of chemical cues of conspecific cannibalistic tadpoles for both egg and tadpole deposition in the Neotropical poison frog *Ranitomeya variabilis* (Dendrobatidae).

To the best of our knowledge, there are no studies on the influence of visual cues of aquatic tadpole or egg predators on parental decisions in anurans. The present study fills this gap and compares the recognition of different chemical and visual tadpole predator cues by *R. variabilis*. Like other poison frogs, this species has evolved a complex reproductive strategy including parental care (Lötters et al., 2007). It deposits its clutches at the water surface of a phytotelm (such as small water bodies in plant axils; Varga, 1928) and after hatching the male transports the larvae singly into different phytotelmata (Brown et al., 2008a; Brown et al., 2008b; Schulte et al., 2011; Fig. 1). Because of their small size, phytotelmata have nutritive limitations and commonly cannot sustain more than one tadpole at the same time (Brust, 1993; Caldwell and de Araújo, 1998; Lannoo et al., 1987; Lehtinen et al., 2004; McDiamond and Altig, 1999; Summers, 1999; Teixeira et al., 2006). As a consequence, tadpoles within these phytotelmata are competitive and often perform cannibalism (Brust, 1993; Poelman and Dicke, 2007; Pramuk and Hiler, 1999; Summers, 1999). Accordingly, parent frogs need to locate unoccupied pools for each tadpole to increase their individual reproductive success.

In pool choice experiments, we showed that wild *R. variabilis* are able to exploit chemical cues produced by conspecific tadpoles and so avoid phytotelmata already occupied (Schulte et al., 2011). Brown et al. (2008a), found evidence that predatory damselfly larvae (Odonata: *Microstigma rotundatum*) were recognised and avoided by *R. variabilis*, when present (both visually and chemically). In the present study, we tested if these invertebrate predators can be recognised by chemical cues alone. Furthermore, we asked whether the recognition of the cannibalistic tadpoles is possible by visual cues alone, using artificial tadpole models. We formulated the following hypotheses: (i) both con- and heterospecific predators are recognised and avoided by *R. variabilis* for egg and tadpole deposition via chemical cues; (ii) visual cues do not play a role in this system, as predators are mainly hidden within the phytotelmata.

## Methods and Materials

### Study Sites

Field work was conducted in a late-stage secondary rain forest close to the upper Cainarachi River, Región San Martín, Peru. Three study sites were established between 540 and 580 m above sea level on the western side of the river. Minimum distance between study sites was 30 m, thus exceeding the known home range size of *Ranitomeya variabilis* (Brown et al., 2009) and avoiding pseudo-replication i.e. repeated measures.

### Chemical Cues from Damselfly Larvae

From July to November 2008, we performed pool choice experiments using pairs of plastic cups (200 ml volume, 10 cm height, 7 cm in diameter, made of polypropylene) as artificial phytotelmata (Schulte et al., 2011). These cups were non-transparent and two thirds of the openings were covered to impair visual inspection by *Ranitomeya variabilis*. The pairwise setup consisted of one cup with 25 ml clean water and one with the same amount of tadpole-treated water. Water was treated by conspecific (i.e. cannibalistic *R. variabilis*) tadpoles collected in the forest nearby. For this purpose, they were housed individually in standard 50 ml plastic tanks and the used water of all tadpoles was taken and mixed for use in the field every other day (for details see Schulte et al., 2011).

From June to August 2010 and April to June 2011, we conducted similar experiments, but with damselfly larvae (Odonata: *Microstigma rotundatum*) as the water treatment. Brown et al. (2008a) demonstrated that these larvae can be recognised and avoided for tadpole depositions by *R. variabilis* when both visually and chemically present (using pool choice experiments with damselfly larvae with mandibles removed, as described in Wissinger and McGrady, 1993). To test the effect of only chemical cues produced by these invertebrate tadpole predators, they were collected from artificial phytotelmata in the field and were kept individually in 50 ml rain water. Water change was conducted every other day and each damselfly larva was fed with one to two mosquito larvae afterwards. The used water was mixed and offered to the frogs in the field, side by side with clean water (as in the 2008 experiments, see above). A water change of both treated and clean water in the field was done every other day, to avoid a potential degradation of damselfly larvae cues and to prevent



assimilations of drowned insects and detritus in the cups. At this occasion, cup pairs were controlled for new deposited clutches and tadpoles of *R. variabilis*. Occupied cups were not included in the following water changes. Instead a new pair of cups was fixed on another randomly chosen tree. If there was more than one tadpole deposition in adjacent cup pairs on the same day, just one was chosen randomly for analysis. This was done to minimise pseudoreplication from repeated depositions by the same male, carrying all tadpoles of the same clutch within a short period (Brown et al., 2008a). To ensure that the experimental treatment and no previous depositions had an influence on the deposition choice of the frogs, only single deposition events within each cup pair were recorded.

### **Visual Cues from Tadpoles Models**

In 2008, we conducted an experiment similar to Brown et al. (2008a), but using cannibalistic *Ranitomeya variabilis* tadpoles instead of damselfly larvae as predator stimuli. We gave the frogs the choice between empty pools and pools containing a tadpole within a little cage (to ensure they could not feed on newly deposited tadpoles). In this way, tadpoles were present both visually and chemically.

In 2010, we tested how the frogs reacted if only visual tadpole cues were available. For this purpose, we scattered pairs of artificial phytotelmata within our study plots (as described above), which were transparent and the top completely open. Both cups were filled with 25 ml rain water and in one of them we hung a tadpole model made of heat hardened polymer clay (FIMO®). It was fixed to a transparent fibre hanging down from a wire installed above the water surface. The fragile setup ensured a movement of the model with every vibration of the cup caused by an arriving frog. To exclude the potential influence of chemical cues caused by the clay, we put a flat piece of clay on the bottom of the other cup. Furthermore, we equipped this cup with a horizontal wire, so both cups were exactly the same except for the swimming tadpole model. The cups were checked every other day for egg or tadpole depositions and were cleaned of detritus and drowned insects when necessary.

## Statistical Analyses

Egg and tadpole deposition frequencies in each artificial pool type were compared using a G-test (Woolf, 1957), which is considered similar to but more exact than a  $\chi^2$ -test when small sample sizes are used (Gotelli and Ellison, 2004; Sachs, 1974). To further increase exactness, we adjusted the G-test in the manner of Williams (1976). Our null hypothesis assumed that the frequency of deposition events was random (0.5 in either pool). For comparisons of depositions between the different treatments, contingency tables according to Pearson (1956) were calculated testing the null hypothesis that all distributions were the same. When some of the expected values were too small to use Pearson's  $\chi^2$ -Test, Fisher's exact test (Fisher, 1922) was calculated instead, as suggested by Townend (2002).

## Results

### Chemical Cues from Damselfly Larvae

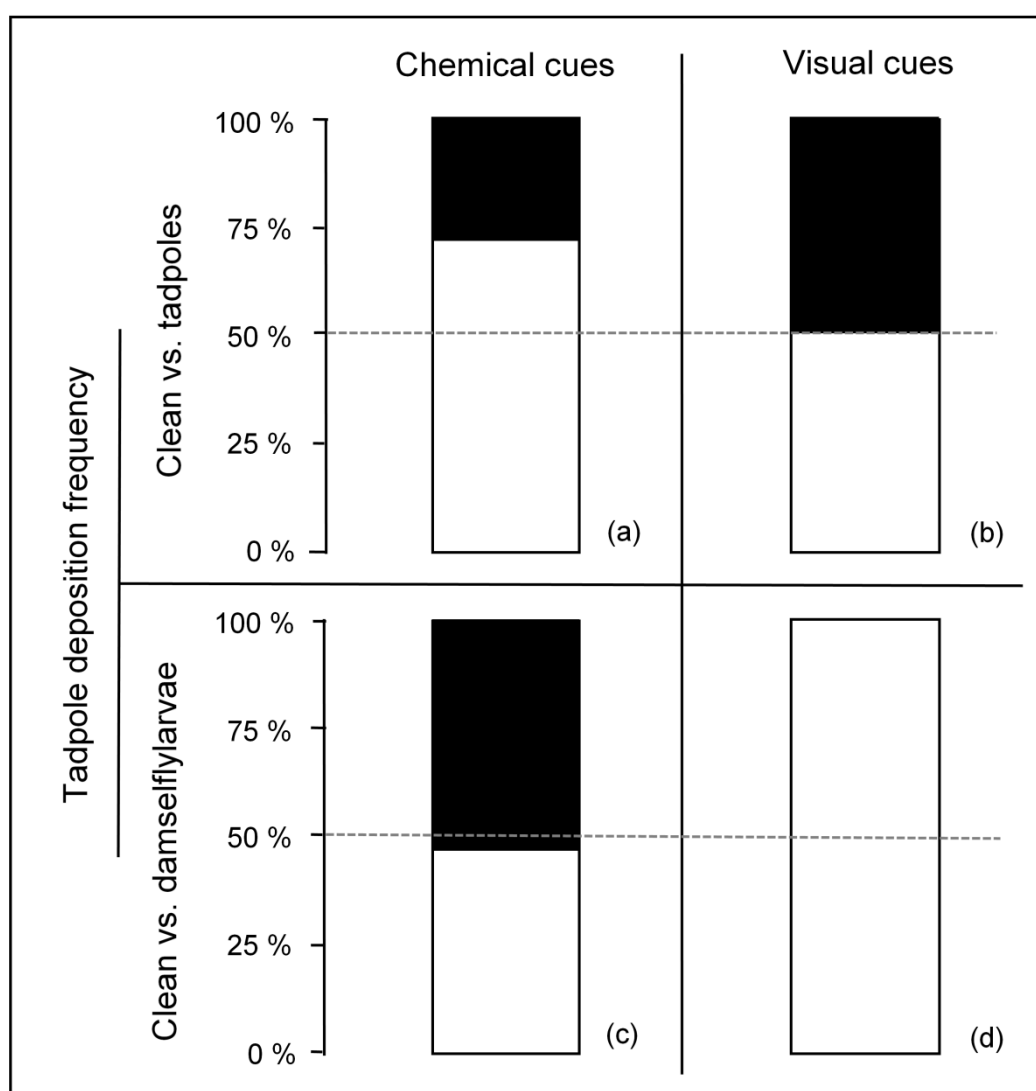
Pooling the results from 2010 and 2011, 36 tadpole and 23 clutch depositions of *Ranitomeya variabilis* were counted in setups where water treated by damselfly larvae versus clean water was offered to the frogs. Nineteen tadpoles were deposited in treated and 17 in clean water, while 9 clutches were deposited in clean versus 14 in treated water. Calculating a G-test with both results, there is no significant avoidance of the damselfly cue, neither for tadpole depositions ( $G_1 = 0.107$ ,  $P > 0.05$ ) nor for clutch depositions ( $G_1 = 1.072$ ,  $P > 0.05$ ).

Comparing the reactions of the frogs to the different chemical signals (from cannibalistic tadpoles versus predatory damselfly larvae), the difference between them is highly significant for clutch depositions ( $\chi^2 = 20.545$ ,  $P < 0.001$ ) and nearly significant for tadpole depositions ( $\chi^2 = 3.445$ ,  $P = 0.06$ ).

### Visual Cues from Tadpole Models

For tadpole depositions, there was no significant preference between empty pools versus pools containing a tadpole model (18 larvae each in both cup types,  $G_1 < 0.001$ ,  $P = 1.00$ ). For clutch depositions, in total we recorded six depositions only, five in the empty cup and one with the clay model. This was insufficient for calculating a G-test.

For a comparison of our results with those of Brown et al. (2008a) for the presence of damselfly larvae, we used Fisher's exact test to calculate a contingency table, as the quantity of data in Brown et al. (2008a) was too small for Pearson's  $\chi^2$ -test. The difference between the reactions on purely visual tadpole cues and visual (but also chemical) damselfly cues was significant ( $\chi^2 = 5.250$ ,  $P < 0.05$ ).

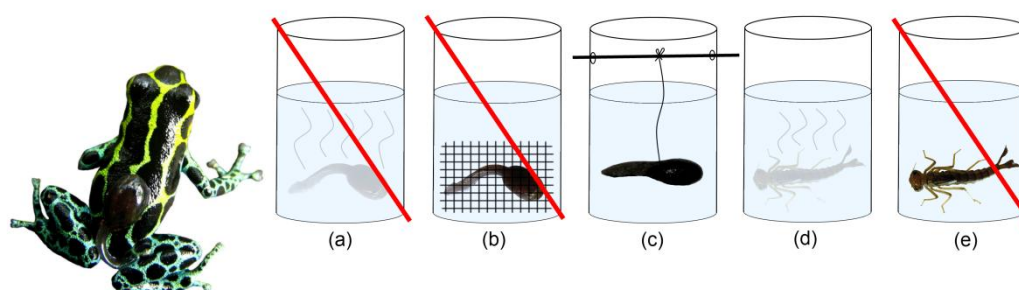


**Fig. 2** Percentage tadpole deposition distribution of *Ranitomeya variabilis* in different pool choice experiments. White shows depositions in clean water, black in treated water. Top: water treatment by chemical cues (a) vs. visual cues (clay models) of tadpoles (b). Bottom: Water treatment by chemical cues of damselfly larvae (c) vs. physical presence of damselfly larvae (d).

### Comparisons between Visual and Chemical Detection

Calculating a contingency table to compare the results of chemical tadpole recognition (Schulte et al., 2011) with those of visual tadpole recognition, the frequencies between both detection strategies differ significantly from each other ( $\chi^2 = 4.383$ ,  $P < 0.05$ ; Fig. 2a, b). This suggests that *Ranitomeya variabilis* is able to recognise cannibalistic tadpoles using only chemical cues but not when only visual cues are given.

Additionally, tadpole depositions in clean versus damselfly treated water were compared to the results of Brown et al. (2008a), where damselflies were visually (but also chemically) present. The significant difference (Fisher's exact test:  $\chi^2 = 4.383$ ,  $P < 0.05$ ; Fig. 2c, d) revealed that the frogs were able to recognise damselfly larvae only when visual cues were involved but not when chemical cues alone were available.



**Fig. 3** Different cues offered to *Ranitomeya variabilis* for tadpole depositions. Frogs avoided conspecific cannibalistic tadpoles when only their chemical cues (a) or a tadpole individual (in a cage) was present (b). Tadpole models (visually recognisable) were not avoided (c). Contrary to the tadpole cues, chemical cues of damselfly larvae (*Microstigma rotundatum*) were not avoided (d) while visually (and chemically) present damselfly larvae were avoided (e).

### Discussion

Our experimental studies demonstrated that adult *Ranitomeya variabilis* use different cues and accordingly different sensory systems to recognise distinct aquatic predators of their tadpoles. While conspecific cannibalistic tadpoles are detected only by chemical but not visual cues, the presence of predatory damselfly larvae in phytotelmata cannot be detected by chemical cues alone (Fig. 3). This contradicts our hypothesis that (i) all predators are

recognised chemically by *R. variabilis* and that (ii) in this species visual cues do not play a role in the recognition of predators inhabiting phytotelmata.

Though, due to their diurnality, vision is an important sense in poison frogs (e.g. Forester and Wisnieski, 1991; Richards-Zawacki and Cummings, 2010; Summers et al., 1999), one could assume that chemical recognition of phytotelmata-inhabiting predators is more important than visual recognition. The rationale for this might be that predators in phytotelmata are hidden on the ground and therefore can be easily overseen (authors' unpubl. pers. observation). A possible explanation for the inability to perceive chemical cues from predatory damselfly larvae might be that larval odonate predators mask or break down chemical signals to be cryptic in the phytotelmata. Thus, the invertebrate predator might be involved in an evolutionary predator-prey arms race with *Ranitomeya* species that use the same phytotelmata for breeding (e.g. Brodie and Brodie Jr, 1999; Wirsing et al., 2005).

There are several studies documenting the ability of anuran larvae to recognise and avoid chemical cues of predatory odonate larvae (Petranka and Hayes, 1998; Takahara et al., 2008). However, some species show only weak or no reactions at all to cues of starved odonate larvae or larvae fed with non-anuran prey compared to those fed with tadpoles before (e.g. Chivers and Mirzal, 2001; Ferland-Raymond and Murray, 2008; Laurila et al., 1997; Laurila et al., 1998). Ferland-Raymond et al. (2010) tried to encode the cue avoided by anuran tadpoles and found that dragonfly larvae only produced this cue when previously fed with tadpoles. This might support our suggestion of predator-prey arms races between anurans and odonates. While some predators can be recognised and avoided by their prey, others are able to mask their own cues – but tend to fail after an uptake of alarm cues. However, we never tested if parent *R. variabilis* do react in a different way to *Microstigma rotundatum* after feeding on *R. variabilis* tadpoles. We exclusively fed the invertebrate predators with mosquito larvae in our trials, whilst in the experiments of Brown et al. (2008a) larvae were not fed at all for one week before presented to the frogs (pers. comm., J. L. Brown).

As we are lacking a comparative test with artificial damselfly larvae we cannot determine if visual cues alone are sufficient to recognise damselflies or if a combination of both visual and chemical cues is essential. Another possible source of predator detection is water vibration as a result of moving damselfly larvae. Despite these remaining questions, our study shows that *R. variabilis* is able to react to different cues, depending on the circumstance and/or the

transmitter. Such a situation depending on a switchover between (or combination of) different sensory stimuli is already known in anurans, especially for acoustic and visual cues associated with mate choice or territorial behaviour (e.g. Candolin, 2003; Gomez et al., 2009; Hödl and Amézquita, 2001; Narins et al., 2005; Narins et al., 2003). A switchover between chemical and acoustic cues is also documented in frogs. Pearl et al. (2000), for instance, indicates that the perception of sexual signals changes according to distance in African dwarf frogs (*Hymenochirus*): while distant males are recognised by females on the basis of calls, close males are chosen by chemical cues. A similar combination of different cues was confirmed by I. Starnberger (pers. comm.): females of African reed frogs (*Hyperolius*) have the option to choose between visual, chemical and acoustical cues to select their mates.

Especially when trying to perceive signals from deadly predators, the ability to recognise the ‘right’ cue(s) is essential. The avoidance of chemical cues of predators has been demonstrated across a wide range of prey taxa (see Kats and Dill, 1998 for review) and is especially important in aquatic systems (Brönmark and Hansson, 2000). But if the predator is able to mask such cues, the prey has to adapt by switching to other signals (e.g. from chemical to visual, mechanical or tactile). In our study, we found that chemical communication is an advantageous tool for the detection of some but not all predators of *R. variabilis* and show how (indirect) prey alters its behaviour depending on the signals available from the predator.

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## Chapter IV

### **Do poison frogs recognize chemical cues of the other sex or do they react to cues of stressed conspecifics?**

Lisa M. Schulte • Daniela C. Rössler

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## Abstract

Although anuran communication primarily takes place acoustically, chemical cues are also often used for intra- and intersexual communication in frogs. In the present study we analyzed the behavior of the poison frog *Ranitomeya variabilis* when presented chemical cues of same-sex or opposite-sex conspecifics. Chemical cues were obtained by keeping a single frog on a moist paper towel for about 47 hours. Afterwards two paper towels were offered to a test animal, one containing the chemical cues, the other containing rainwater only. We ran trials presenting female cues to males, males cues to males as well as male cues to females. The results of the trials were not significant in terms of intersexual communication. The overall response revealed a clear avoidance strategy which leads us to the assumption that disturbance cues unintentionally occurred during the experiment. The rather small size of the containers used to obtain chemical cues prior to the trials probably lead to confinement stress which consequently caused increased urination containing stress hormones that were detected by the test animals. This is the first proof of disturbance cues and their effects in adult anurans. The results of this study do not allow conclusions about inter- or intrasexual chemical communication of *R. variabilis*, but they allow implications and revisions for future experiments on this topic.

**Keywords:** chemical communication, disturbance cues, mate recognition, *Ranitomeya variabilis*

## Introduction

Chemical communication is the evolutionary oldest way of intra- and interspecific interactions in the animal kingdom (Wilson, 1970; Wyatt, 2003). Vertebrates use chemosignals to recognize conspecifics (Graves et al., 1993; Heller and Halpern, 1982; Ibáñez et al., 2013), mark and recognize territories as well as home ranges (Gosling and Roberts, 2001; Mathis, 1990; Sillero-Zubiri and Macdonald, 1998) or to identify potential sexual mates (Bonadonna and Mardon, 2013; Martín and López, 2013; Sorensen and Stacey, 1999). In amphibians, chemical sex recognition is especially well documented in salamanders and newts (Caspers and Steinfartz, 2011; Dawley, 1984; Houck, 1986; Kikuyama et al., 1997; Rollmann et al., 2000), while for anurans the primary mode of communication is generally assumed to be acoustic (Gerhardt and Huber, 2002; Narins et al., 2006). However, the knowledge about intersexual chemosignaling in anurans is increasing. Many studies consider that male skin glands may release chemical signals involved in sexual interactions (e.g. Smith et al., 2003; Starnberger, pers. comm.; Thomas et al., 1993) and several behavioral studies show that intersexual attraction is based on chemical cues. Forester and Thompson (1998) for example, showed that males of the American toad (*Bufo americanus*) approach toward the smell of females, while a similar reaction in females toward male chemical cues was demonstrated for the magnificent tree frog *Litoria splendida* (Wabnitz et al., 1999) and the dwarf African clawed frog *Hymenochirus* sp. (Pearl et al., 2000). In the Australian toadlet *Pseudophryne bibronii* males preferred substrate marked by females but avoided substrate marked by other males, while females showed a preference toward substrate marked by either sex over unmarked substrate (Byrne and Keogh, 2007). Korbeck and McRobert (2005) conducted similar experiments with the poison dart frog *Dendrobates auratus* (Dendrobatidae). While chemical cues of the same sex did not elicit a reaction in the focal frogs, both males and females showed a clear attraction toward chemical cues of the opposite sex.

Another member of the same family, the poison dart frog *Ranitomeya variabilis*, has been shown to be able to respond to chemical cues of conspecific tadpoles (Schulte and Lötters, 2013; Schulte et al., 2011), but a possible influence of chemical cues on intersexual communication has not been documented so far. *Ranitomeya variabilis* has a highly promiscuous mating system. Reproduction is initiated by the male, calling in response to the

presence of a female, while the presence of another male during mating leads to aggressive responses (Brown et al., 2008). Since sexual dimorphism rarely exists in this species (there is no difference in coloration and females are usually only slightly bigger than males, with a strong overlap existing), the question arises how the frogs recognize individuals of the other sex. One possibility would be a behavioral attribute that the males (but not human observers) recognize. An alternative to this is tested in the current study: Experiments were designed in order to test the possibility of intersexual recognition based on chemical cues in *R. variabilis*. The following hypotheses were proposed: (i) male *R. variabilis* recognize and approach chemical cues of conspecific females; (ii) males recognize and avoid chemical cues of conspecific males; (iii) females do not show a strong reaction toward male chemical cues since males call during the entire courtship (Brown et al., 2008).

## Materials and Methods

### Experimental trials

The study was carried out from 21 February to 29 March 2013. During this time, individuals of *Ranitomeya variabilis* were collected in a premontane, late-stage secondary forest along the Tarapoto-Yurimaguas road (km 30) in the upper Cainarachi Valley, region San Martín, Peru. The sex of each frog was determined (if possible based on reproductive activity or using very big/small animals) and it was kept separately for 47 (+/- 2) h in a 150 ml polypropylene plastic cup closed with tulle and the inner surface covered with a moist paper towel. The paper towels were then used as the source of conspecific chemical cues. Visible fecal matter and eggs were removed from the paper towels prior to use. For each trial half the bottom of a rectangular plastic box (23.04 x 8.73 x 6.10 cm) was lined with a wet paper towel containing conspecific cues while the other half was lined with a paper towel containing rainwater only. The two paper towels were separated by 4 cm. The adjustment of the paper towels in the box (left or right side) was chosen randomly.

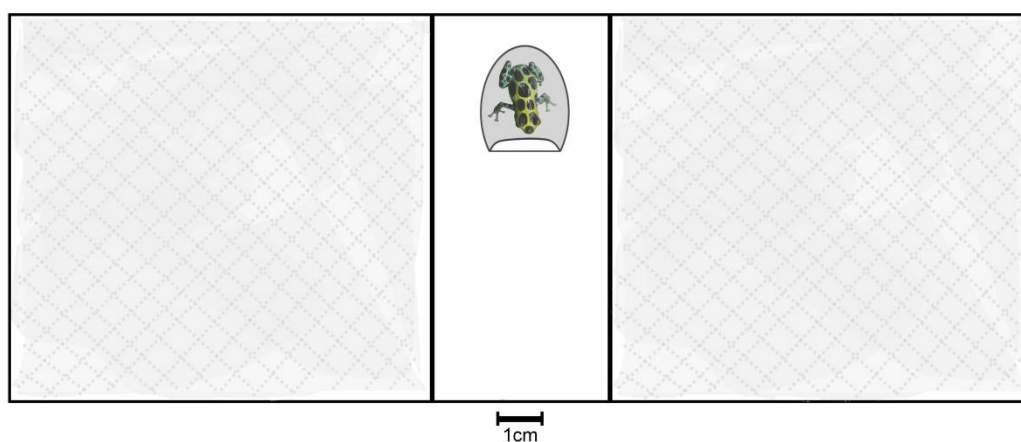
A single test animal, caught in the forest shortly before the start of the trial, was placed in the middle of the box, covered by a small transparent capsule that opens in one direction, to prevent that the frog starts jumping before the box is closed (Fig. 1). Through the transparent lid of the box the activities of the frog were filmed for 30 minutes. Afterwards the frog was



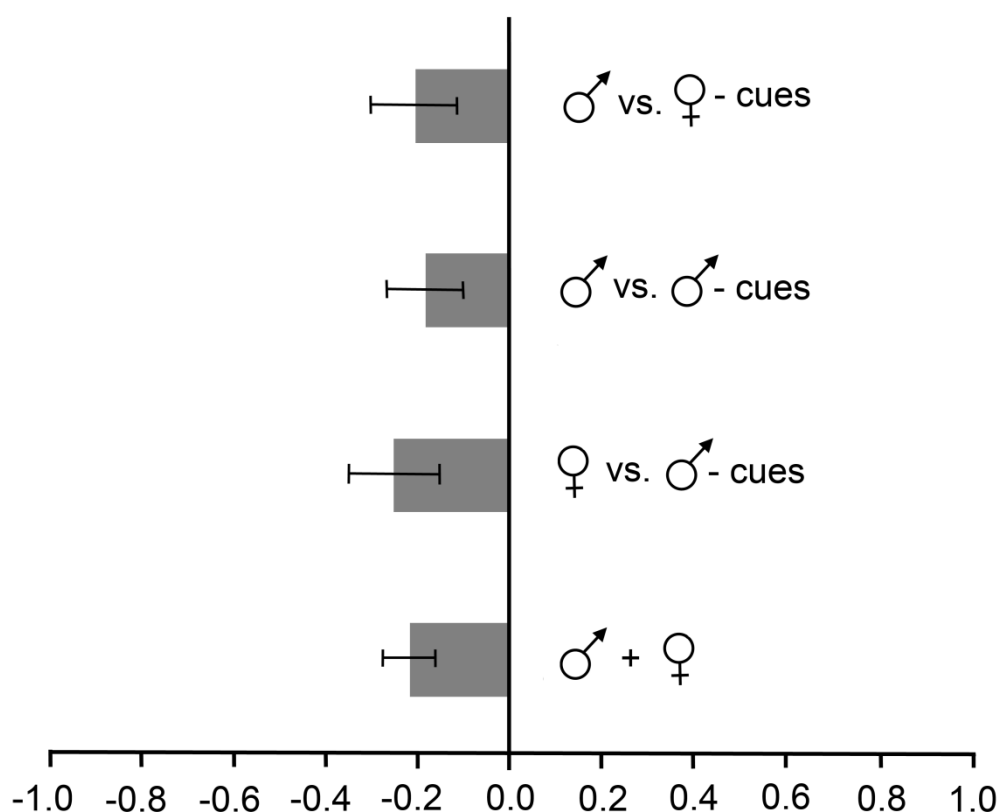
kept for approximately 47 h in captivity in order to collect further chemical cues on a new paper towel for following trials. Between trials the box was rinsed thoroughly with rainwater. In total 55 frogs were tested: 21 males with female conspecific cues, 13 males with male conspecific cues and 21 females with male conspecific cues.

### Data analysis

Videos were analyzed blind by counting the time to the exact second that each frog spent on either of the two towels in the box. To compare the responses toward the conspecific cues to those toward the blank towels, a response index (R) was calculated, defined as:  $R = (A1 - B1)/(A1 + B1)$ , A1 being the time spent on the towel with conspecific cues and B1 being the time spent on the blank towel. R can range from -1 to +1, with 0 indicating no difference in response, +1 maximal response for the conspecific towel, and -1 maximal response for the blank towel (compare Johnson and Basolo, 2003). The null-hypothesis that *Ranitomeya variabilis* shows no preference or avoidance towards the cues of conspecifics (thus the mean of the response indices equals 0) was tested using a One-sample t-Test. Beforehand a Kolmogorov-Smirnov-Test was carried out to confirm normal distribution. T-Tests were conducted with responses of males tested with cues of females, males tested with cues of males and females tested with cues of males as well as with all individuals pooled together. Data for all combinations were normally distributed ( $p > 0.05$ ). All statistical tests were carried out in PASW 18.0; all tests were two-tailed with  $\alpha = 0.05$ .



**Fig. 1** Experimental setup. Both sides of the box are lined with a wet paper towel. Which side of the container held the paper towel with chemical cues of a conspecific frog was randomly chosen by throwing a coin.



**Fig. 2** Mean response indices. Positive responses represent a preference, negative responses an avoidance toward conspecific cues.

## Results

The mean response index of each of the combinations was negative (see Fig. 2). *Ranitomeya variabilis* males significantly avoided chemical cues of both conspecific females ( $T = -2.219$ ,  $p = 0.038$ ) and males ( $T = -2.186$ ,  $p = 0.049$ ). Females showed a significant avoidance of the male cues as well ( $T = -2.626$ ,  $p = 0.016$ ). This leads to an overall avoidance of conspecifics when all individuals are pooled together ( $T = -4.208$ ,  $p < 0.001$ ).

## Discussion

The overall negative responses toward paper towels used by conspecifics, despite their sex, disprove hypotheses (i) and (iii). Contrary to an expected preference of the males and a neutral reaction of the females toward cues of the other sex, males and females avoided the

chemical cues of each other. These results, as well as the avoidance of male chemical cues by other males (hypothesis ii), might be interpreted as a general avoidance of conspecific chemical cues instead of intersexual communication.

This is at least one conclusion that can be drawn from our findings. Another possibility might be that frogs were able to detect slight differences in moisture between the paper towels (not detectable to the experimental executor) or that the pH was different due to the storage of the paper towels in the animal containers. However, we assume that those effects, although possible, might not trigger such a strong avoidance behavior as shown in our results and that the avoidance of chemical cues released by conspecifics is more likely.

In other amphibians chemical cues could be shown to trigger avoidance between males as part of intrasexual mating competition (Byrne and Keogh, 2007; Jaeger and Gergits, 1979; Park and Propper, 2001). However an overall avoidance of conspecific cues usually has other reasons. One possibility are chemical alarm cues or “Schreckstoffe” (for review see Chivers and Smith, 1998). They are released from damaged tissue after predator attacks and are very common in aquatic vertebrates (fish: Brown and Smith, 1997; Smith, 1979; newts: Marvin and Hutchison, 1995; Woody and Mathis, 1997; tadpoles: Eibl-Eibesfeldt, 1949; Ferrari et al., 2007; Hagman and Shine, 2008;), but may be important in terrestrial environments as well (salamanders: Chivers et al., 1996; Chivers et al., 1997; Lutterschmidt et al., 1994; frogs: Chivers et al., 1999; Chivers and Mirzal, 2001). However, even if the release of alarm cues is an active process (Fraker et al., 2009), it still requires a skin lesion.

A more probable reason for the results of the present study therefore is the possibility that *Ranitomeya variabilis* avoided conspecific cues due to disturbance cues, i.e. stress hormones. Disturbance cues are chemicals released by individuals that have been disturbed or stressed, but not captured and injured by a predator (Gonzalo et al., 2010). They can be evoked for example by chasing (Wisenden et al., 1995), handling (Watson et al., 2004) or confining (Giaquinto and Hoffmann, 2012) the cue producing individual. When conspecifics perceive those cues, they show similar reactions as toward alarm cues: they reduce their activity (e.g. Bryer et al., 2001), their feeding behavior (e.g. Giaquinto and Hoffmann, 2012) or try to avoid or leave the area containing the cues (e.g. Jordão and Volpato, 2000). Many authors assume that those stress pheromones are released via urine, especially since urination increases in stressful situations (e.g. Giaquinto and Hoffmann, 2012; Mackay-Sim and Laing, 1980).

Kiesecker et al. (1999) could show for juvenile red-legged frogs (*Rana aurora*) that ammonium ( $\text{NH}_4^+$ ), the main metabolic waste of tadpoles, was not only excreted more when tadpoles were stressed, it also led to reduced activity in conspecifics.

A similar effect might have unintentionally occurred during the trials with *R. variabilis*. Since chasing and catching the sender frogs usually occurred about 1 h before they were placed into the cups and the handling in between was very short, it can be assumed that frogs might have experienced confinement stress. The cups used to keep the sender individuals for about two days in order to collect their chemical cues were rather small. They were chosen due to the ability of *R. variabilis* to climb up the side walls of a container. To make sure the frogs did not leave the paper towels while climbing up, the towels had to be lined at both the bottom and the sides, which is only possible in a relatively small container. After the two days of confinement the paper towels usually contained urine and feces of the sender animals. These contaminations might have contained disturbance cues and reflected the stressful situation of the sender animals toward their conspecifics. The avoidance behavior of the receiver animals might be interpreted as an antipredator behavior, since avoidance is a typical behavior to avoid encounters with predators (for review see Kats and Dill, 1998).

These findings do not exclude that *R. variabilis* produces and recognizes intersexual chemical cues as well, however due to the possible danger that is being signaled to the test animal via chemical disturbance cues, the reaction toward cues of the other sex might be of secondary importance (compare Watson et al., 2004 for feeding behavior during disturbance cue presence).

## Conclusions

In summary, the results do not enable us to draw conclusions concerning inter- or intrasexual communication of *Ranitomeya variabilis* on the basis of chemical cues. However a first proof of responses toward conspecific same-sex or opposite-sex disturbance cues in adult frogs can be provided by this study. Regardless of the sex of both the test and the sender animal, the overall negative responses can be explained by the presence of disturbance cues most likely in the form of stress hormones, which lead to a general sex-independent avoidance strategy.

In this study we did not only gain insight into the effects of disturbance cues on the behavior of adult anurans, but also were able to extract implications for future experiments studying inter- and intrasexual chemosignaling in this species. In future experiments, the method must be optimized in terms of the container the animal is kept in (more space) to avoid confinement stress. With the help of a revised experimental setup it will be interesting to once more analyze the proposed hypotheses (i – iii) to reveal the concept of chemical communication between individuals of the poison frog *R. variabilis*.

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## Chapter V

### **The power of the seasons: rainfall triggers parental care in poison frogs**

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## Abstract

The quality of breeding sites is of great importance for the reproductive success and accordingly the fitness of many animal species. Hence, individuals should decide carefully where to rear their offspring. Often parents have to account for multiple characteristics of habitat quality at once, which in turn might change over time. Specimens confronted with such variability may evolve the ability to display context-dependant decision plasticity. Anuran amphibians breeding in ephemeral pools largely face two risks for their offspring: desiccation and predation. The Neotropical poison frog *Ranitomeya variabilis* deposits both eggs and tadpoles in phytotelmata. These small tadpole nurseries lower the risk of offspring predation. However, because most poison frog tadpoles are cannibalistic, even these pools need to be surveyed for predators, and parents tend to avoid deposition with conspecifics. We tested if this avoidance behaviour does change in parental *R. variabilis* depending on seasonal circumstances. Over several months we provided the frogs the option to deposit their eggs or tadpoles in pools that did and did not contain chemical cues of cannibalistic conspecifics, respectively. During the rainy season, frogs strongly avoided conspecific cues for both eggs and tadpoles. Anyway, with the change to the dry season, parental preferences changed such that parent frogs were more likely to deposit tadpoles (but not eggs) in pools containing cues of conspecific tadpoles. We suggest that *R. variabilis*, a species that typically isolates its cannibalistic offspring, has evolved a plastic feeding behaviour with regard to the risk of phytotelmata desiccation. We interpret that parents provide older tadpoles with younger ‘trophic’ tadpoles in order to accelerate their development and save them from impending desiccation.

**Keywords:** reproductive behaviour, *Ranitomeya variabilis*, seasonal change, Neotropics, offspring provisioning, trophic tadpoles

## Introduction

The reproductive success of many animals strongly depends on the quality of breeding sites. Since especially juvenile stages are vulnerable to a wide array of risks, parental site choice is of great importance for them (Refsnider and Janzen, 2010). This has been well investigated, but many studies focus on site selection in a single ecological context only (e.g. Thompson and Pellmyr, 1991; Shine and Harlow, 1996; Binckley and Resetarits, 2002; Mokany and Shine, 2003; Sanuy and Joly, 2009). However, quality characteristics of breeding sites can be highly instable and vary in space and time (Rudolf and Rödel, 2005). Therefore, it can be expected that parents confronted with such variability display plastic responses in their reproductive strategies (Gross, 1996) and as part of that may show variation in parental care performance (Webb et al., 1999).

In animals with reproductive strategies dependent on water, seasonal climate changes are particularly important, as desiccation might have a negative effect on their progeny. Edgerly et al. (1998) demonstrated that female mosquitoes (*Aedes triseriatus*) avoided water already containing conspecific larvae at the beginning of the breeding season. However, at the end of the breeding season, females were less selective and even showed a slight positive response towards conspecifics (as occupied pools might reflect pool stability).

Many anuran amphibians face similar risks related to their breeding strategies. Desiccation of water bodies, especially ephemeral pools, is considered one of the most important abiotic mortality risks to tadpoles (e.g. Smith, 1983; Newman, 1988; Murphy, 2003a; Muths et al., 2003; Rudolf and Rödel, 2005). This risk factor is obvious in more arid areas (e.g. savannahs), however it has also shown that in ecosystems with abundant annual rainfall, including tropical rainforests, organisms can be highly sensitive to climatic seasonality (Haddad and Pombal, 1998; Engelbrecht et al., 2006; Touchon and Warkentin, 2009). Seasonality in form of annual rainfall variability is among the top factors for reproductive success in numerous rainforest anurans (e.g. Aichinger, 1987; Donnelly and Guyer, 1994; Bevier, 1997; Bertoluci and Rodrigues, 2002; Gottsberger and Gruber, 2004; Abrunhosa et al., 2006). For that reason, many frog and toad species respond to water quantity of potential breeding pools (Spieler and Linsenmair, 1997; Rudolf and Rödel, 2005; Lin and Kam, 2008).

Apart from this, biotic factors also influence the reproductive success in anurans. The selection of pools that minimize tadpole predation (e.g. Petranka et al., 1994; Rieger et al., 2004), cannibalism (e.g. Spieler and Linsenmair, 1997; Halloy and Fiaño, 2000) and competition (e.g. Dillon and Fiaño, 2000; Matsushima and Kawata, 2005) is fundamentally important in many anurans (Refsnider and Janzen, 2010).

One of the smallest and probably most fragile types of anuran breeding pools are phytotelmata, small water bodies in plants such as leaf axils or tree holes (Varga, 1928; Kitching, 2000). They imply the advantage to minimize the risk of offspring predation associated with larger water bodies (Aspbury and Juliano, 1998), but they are vulnerable to desiccation (e.g. Summers, 1999). Because of their small size and limited food resources (Lehtinen et al., 2004), competition between tadpoles is significant and frogs often avoid phytotelmata already occupied by conspecifics (e.g. Kam et al., 2001; Lin et al., 2008; Schulte et al., 2011). However, at the end of the rainy season, when only few water holding phytotelmata remain, predator avoidance can diminish. For example, Lin et al. (2008) observed that in the Asian tree frog *Kurixalus eiffingeri* competition avoidance lost its relevance compared to pool stability.

Several species of the Neotropical poison frog family Dendrobatidae make use of phytotelmata for reproduction. Different to other phytotelm-breeding frogs, most dendrobatids do not use them for oviposition but transport their tadpoles to these small pools (e.g. Aichinger, 1991; Pröhl and Hödl, 1999; Lötters et al., 2007). Because many poison frog tadpoles are cannibalistic, parents deposit them singly in phytotelmata, thus minimizing the risk of predation by conspecifics and congeners (e.g. Caldwell and de Araújo, 1998; Summers, 1999; Schulte et al., 2011). In contrast to species that face the trade-off between desiccation and competition at the end of the rainy season (see above), poison frogs face the trade-off between desiccation and cannibalism. This leads to the assumption that, unlike other anurans, avoidance of conspecifics should not lose its intensity with seasonal changes. Nevertheless, Poelman and Dicke (2007) found that in *Ranitomeya amazonica*, an Amazonian dendrobatid that uses phytotelmata also for egg deposition, the opposite was the case. In the French Guiana population they studied, most of the time conspecifics were clearly avoided, however at the end of the rainy season clutches were preferential deposited together with conspecific tadpoles. The authors explained this behaviour as a plastic food provisioning

strategy and as an evolutionary step towards egg feeding which is performed in several poison frog species (Brust, 1993; Caldwell and de Oliveira, 1999; Brown et al., 2008b; Brown et al., 2010). Several questions arise from the results of Poelman & Dicke (2007): Expecting that *R. amazonica* always performs the same behavioural plasticity, do related species change behaviour in a similar way in response to seasonal changes? Is not only egg but also tadpole deposition influenced by seasonality? Based on which cues do frogs make their decision to avoid versus ‘feed’ their offspring? In order to contribute to these questions, we studied *R. variabilis* from the upper Amazon basin. Together with *R. amazonica*, it forms the *Variabilis* Clade of *Ranitomeya*, to which usage of phytotelmata for both egg and tadpole deposition, is obligatory (Brown et al., 2011). Interestingly, *R. variabilis* has been shown to recognize and avoid phytotelmata already occupied by conspecific tadpoles on the basis of chemical cues (Schulte et al., 2011). Due to this and the observations on the sister species *R. amazonica*, we hypothesize (i) that *R. variabilis* changes its egg deposition behaviour along with seasonal change; (ii) that also tadpole transportation shifts towards occupied pools in order to provision older tadpoles with food when desiccation risk rises and (iii) that frogs make their decision based on chemical cues of the tadpoles.

## Methods

### Study species

*Ranitomeya variabilis* (Zimmermann and Zimmermann, 1988) is a dendrobatid frog species that displays advanced levels of parental care. It uses phytotelmata for both clutch and tadpole deposition and attaches its clutches just above the water surface (Lötters et al., 2007; Brown et al., 2008a,b; Schulte et al., 2010; Brown et al., 2011). After hatching, larvae are transported singly on the back of the male parent into phytotelmata, avoiding depositions with previously deposited tadpoles by means of chemical cues (Brown et al., 2008b; Fig. 1; Schulte et al., 2011).



**Fig. 1** A male *Ranitomeya variabilis*, carrying its offspring. In this species tadpole transportation to phytotelmata usually takes place one-by-one, so males have to return repeatedly to their clutches.

### Study site

Our study area was located in a late-stage secondary forest in the upper Cainarachi Valley between Tarapoto and Yurimaguas (km 32), Región San Martín, Peru. An *in situ* laboratory was established in a hut within the forest and three nearby study sides were defined between 540 and 580 m above sea level. Site 1 was separated from the others by an old coffee plantation, while sites 2 and 3 were separated by a minimum distance of 30 m. This exceeds the known home range size in *Ranitomeya variabilis* (Brown et al., 2009), preventing pseudo-replication, particularly with regard to repeated measures. Precipitation was measured every day to the nearest 100 mm, collecting rainwater on an open spot next to the hut.

### Animal husbandry

About 60 *Ranitomeya variabilis* tadpoles were collected from artificial phytotelmata and kept in captivity using rain water collected in a rain barrel. Each specimen was housed singly in 50 ml water in a standardized polypropylene cup under ambient temperature. Possible toxic

chemicals influencing the mortality can be excluded (see Schulte et al., 2011). Every other day the water of the tadpoles was taken and mixed for the work conducted in the field. Fresh rain water was added and larvae were fed ad libitum with flaked fish food (Tetra®). Once captive tadpoles had reached developmental stage 40 (Gosner, 1960) they were released and replaced with new tadpoles from the wild (also to be released later).

### **Experimental trial**

Studies were conducted between 23 March and 12 June 2011. For the experimental setup we used polypropylene plastic cups (200 ml volume, 10 cm height, 7 cm in diameter) as artificial phytotelmata. These are quickly adopted by wild *Ranitomeya variabilis* (Brown et al., 2008a; Schulte et al., 2011). All cups were enwrapped in dark plastic membranes and two-thirds of the opening of each plastic cup was covered with a dark plastic membrane to impair possible visual inspections for tadpoles by the frogs (compare Schulte et al., 2011). Those artificial phytotelmata were installed in pairs, directly next to each other on trees at 0.5-1.5 m above ground. In each of the three study sites we placed 30 of these cup pairs randomly (180 cups total) and filled each cup with 25 ml water: one cup per pair with clean rainwater and the other one with water used for two days by the tadpoles held in captivity before (compare Schulte et al., 2011). To avoid diluting, especially of the tadpole treated water, we put a non-rusting metal roof 10 cm above each cup pair as a protection against rainfall. Every other day, after changing and collecting the used tadpole water in the hut, cups in the field were checked with a flashlight for newly deposited clutches and both clean and treated water was changed afterwards. To minimize pseudo-replication, we scored just one randomly chosen deposition for any same-day deposition in adjacent pools within the same site (Brown et al., 2008a; Schulte et al., 2011).

### **Data analysis**

#### *Tadpole depositions*

The frequencies of tadpole depositions in each artificial phytotelm type (with clean or treated water) were compared using a G test (Woolf, 1957). It underlies the same assumptions as the more common chi-square test, but is of superior exactness for limited observations (Sachs,

1974; Gotelli and Ellison, 2004). Since we had less than 200 observations for each dataset we further adjusted the G test in the manner of Williams (1976).

To determine thereupon if the decision of where to deposit a tadpole was influenced by the amount of precipitation, we calculated a binominal logistic regression with PASW 18.0 (SPSS Inc. 2009). The dependent variable was the deposition in clean or treated water, the independent one the amount of precipitation. For this purpose we summed the rain amount of three days previous to each deposition control to account for all precipitation events potentially involved in a frog's decision.

In addition, we divided our data into two parts: the end of the rainy season and the onset of the approximate dry season. For determination of the cut off we applied a changepoint analysis in R (R Development Core Team, 2011) to the rainfall measurements taken, using the package 'changepoint' (Killick and Eckley, 2011b; a). As data were not normally distributed (Kolmogorov-Smirnov test,  $p < 0.001$ ), a non-parametric changepoint test was accomplished, often based on changes in mean (Brodsky and Darkhovsky, 1993; Johnson et al., 2011). We calculated the most probable changepoint using the cumulative sums test of Page (1954), defined to find a single change in means for data that are not assumed to follow a specific distribution. After defining the most probable changepoint for data division, we split the results of the pool choice experiments after the next control following the changepoint. We compared deposition frequencies in different pool types for each of the so obtained two datasets calculating a G-Test (see above). A contingency table was calculated and deposition distributions before and after the changepoint (i.e. rainy versus dry season) were compared using a Fisher's exact test (Fisher, 1922; Townend, 2002).

### *Egg depositions*

With respect to egg depositions we also calculated a G-Test, comparing clutch frequencies in clean and treated water. We further compared our results before and after the changepoint with each other. To determine if there was a correlation between clutch deposition and rainfall in general, a Spearman's rank correlation was calculated (since precipitation data were not normally distributed), using again the sum of the rain of three days previous to each control.



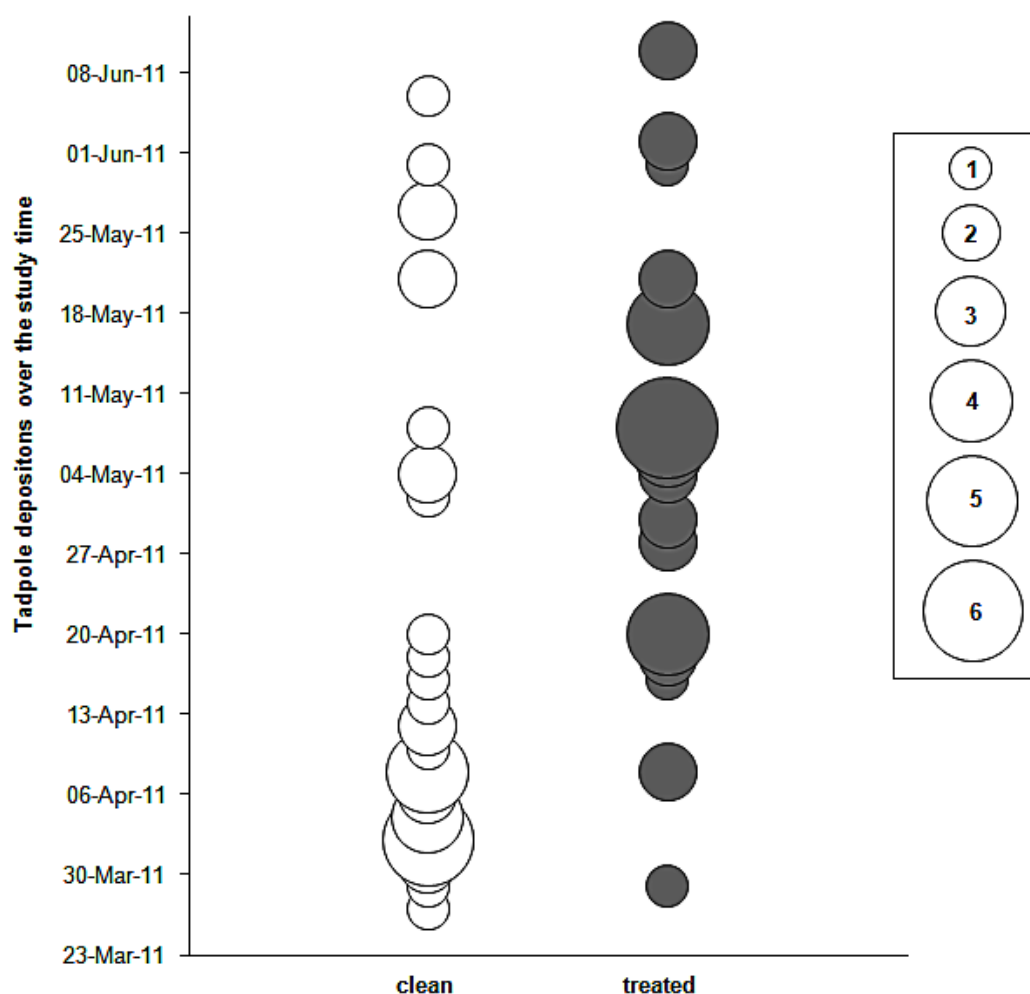
## Results

### Tadpole depositions

Counting all larval depositions by *Ranitomeya variabilis* during our study, 71 tadpoles were recorded, 35 of which in clean and 36 in treated water. Accordingly, there was no significant avoidance of one of the different pool types ( $G_1 = 0.014$ ,  $p = 0.906$ ). Having a look at the distribution of our data, a clear change can be noted (Fig. 2). Accounting for the amount of daily rainfall (binominal logistic regression), we found that tadpole deposition in clean versus treated water was significantly affected by foregoing precipitation ( $B_{[1]} = 0.033$ ,  $SE = 0.012$ ,  $P = 0.006$ ). According to the changepoint analysis, the most probable day for the partition between rainy and dry season in 2011 was 9 April (bootstrap  $p < 0.05$ ; Fig. 3). The tadpole deposition data were divided therefore after 10 April 2011, thus with a time lag of one deposition recording. For the separated analysis of the data of the end of the rainy season versus the onset of the dry season we received the following results: In the rainy season, tadpole depositions in clean water occurred significantly more often than in treated water ( $G_1 = 12.685$ ,  $p < 0.001$ ; Fig. 4). However, during the dry season it was the other way around: there were significantly more tadpoles deposited in treated than in clean water ( $G_1 = 5.962$ ,  $p = 0.015$ , Fig. 4). Comparing the distributions with each other, there is a highly significant difference between the decisions by the frogs in rainy versus dry season (Fisher's exact test,  $p < 0.001$ ).

### Egg depositions

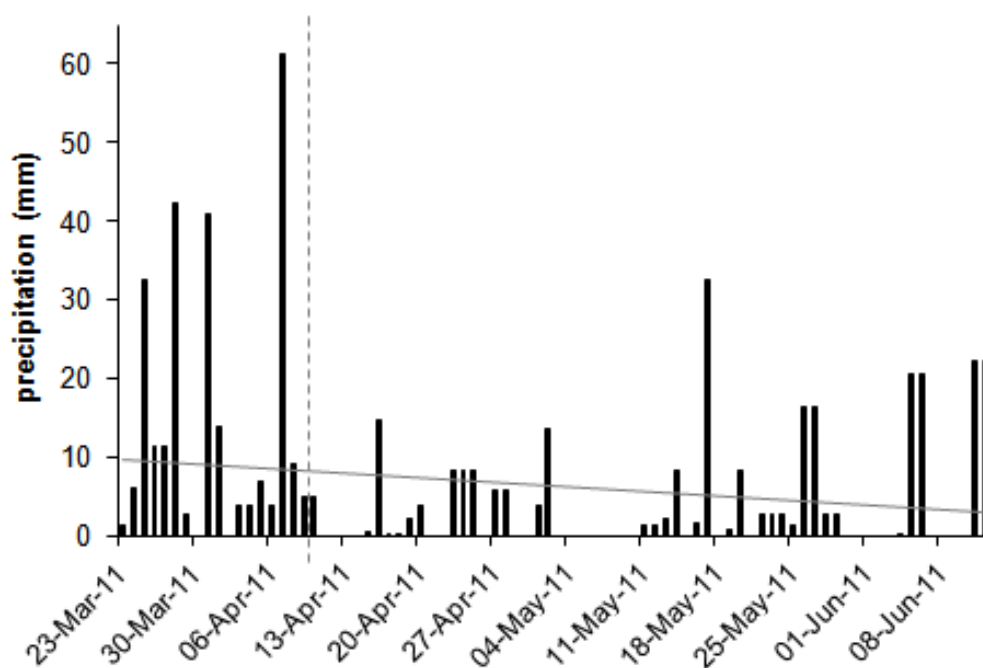
From March to June 2011, 15 *Ranitomeya variabilis* clutches were deposited in the artificial phytotelmata. All were found in clean water; hence, treated water was significantly avoided independent from season ( $G_1 = 20.124$ ,  $p < 0.001$ ). A non-significant trend towards a positive correlation between clutch depositions and precipitation could be found ( $r_s = 0.274$ ,  $p = 0.095$ ).



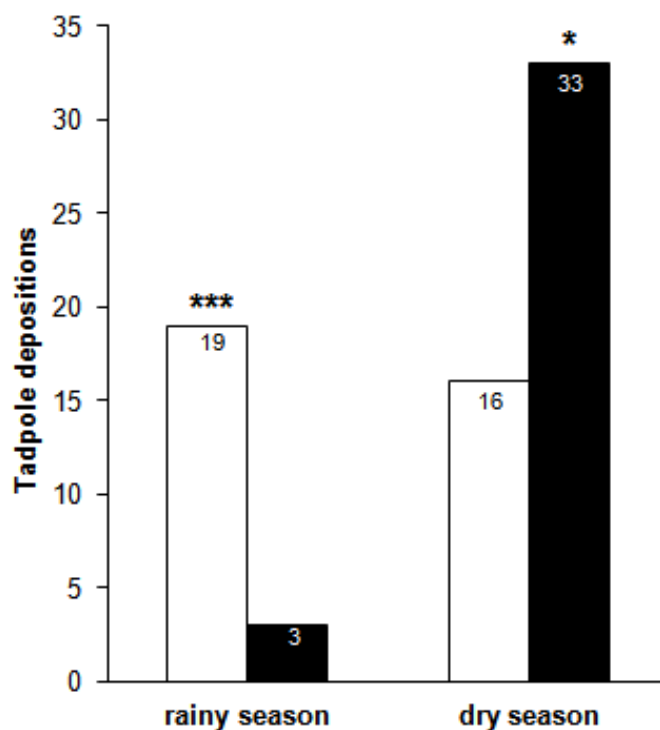
**Fig. 2** Distribution of tadpole deposition over time in clean and treated water: white and grey circles, respectively (see text for details). The size of each circle shows how many tadpoles were deposited a day.

## Discussion

Here we demonstrate that wild *Ranitomeya variabilis* show plasticity in parental care behaviour, triggered by climatic seasonality. With change in rainfall amount, chemical cues of cannibalistic conspecific tadpoles are responded differently. While relatively high precipitation causes avoidance, less rainfall, i.e. the dry season, causes preference of conspecific tadpole cues. This preference of cannibalistic conspecifics cannot be explained by a simple decrease of precaution towards predators or competitors when facing other, more severe risks (e.g. Edgerly, et al. 1998; Murphy, 2003a; Lin et al., 2008). Nor can it be interpreted as a preference towards competitors that serve as an indicator of site quality (e.g.



**Fig. 3** Amount of precipitation over the study period. The dashed vertical line shows the change-point calculated to discriminate rainy from dry season (see text). A general trend (grey line) shows the overall decrease of rainfall over time.



**Fig. 4** Number of tadpoles deposited in clean and in treated water (white and black bars, respectively) during the rainy and the dry season. Legend: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Heard, 1994; Edgerly et al., 1998; Downie et al., 2001; Rudolf and Rödel, 2005; Iwai et al., 2007), as the costs of choosing pools containing cannibalistic larvae overrules any possible abiotic benefit (compare Poelman and Dicke, 2007).

We assume that male *R. variabilis* treat all tadpoles within their territories as their own offspring. This was shown for clutches in the closely related *R. amazonica* (Poelman and Dicke, 2007) and may be underlined by the behaviour observed by Summers et al. (1997) showing that male *R. variabilis* from an Ecuadorian population chase other tadpole transporting males out of their area in order to prevent them from depositing tadpoles in pools within their territory. This assumption leads us to the following conclusion: Males, which on the one hand strictly separate their offspring (e.g. Brown et al., 2008b; Schulte et al., 2011), on the other hand display plastic feeding behaviour when facing the risk of phytotelmata desiccation. Like in *R. amazonica* with eggs (compare Poelman and Dicke, 2007), *R. variabilis* apparently provides older tadpoles (herein represented by their chemical cues only) with younger ‘trophic’ tadpoles. This way, parent frogs may increase the developmental rate and consequently the chance of metamorphosis of their supplied offspring. We consider this conclusion to be well supported by the fact that an increase in larval growth consuming conspecifics has been demonstrated in various anurans including *R. variabilis* (Crump, 1990; Summers and Amos, 1997; Caldwell and de Araújo, 1998; Heinen and Abdella, 2005).

Plasticity in parental care as a function of environmental impacts is not rare in the animal kingdom. For instance, in some passerine bird species parental care may vary depending on the climate or altitude of their breeding location: the harsher the conditions, the higher the investment in offspring care (Badyaev, 1993; 1994; Johnson et al., 2007). This supports the view of Clutton-Brock (1991) who suggested that the increase of parental investment is often associated with rough climatic conditions. Crump (1991) combined both biotic and abiotic aspects in her study, demonstrating that the Neotropical tree frog *Isthmohyla pseudopuma* avoided pools with cannibalistic conspecifics likewise to those posing the risk of desiccation. If confronted with the decision between deep pools containing conspecifics and shallow pools without conspecifics, frogs did not show a preference anymore.

But parental behaviour can also change time-dependently within a single breeding season, as trade-offs between current offspring and remaining breeding opportunities might change as a function of time (Webb et al., 1999; Székely and Cuthill, 2000). Apocrit wasps for example

have to choose adequate hosts as breeding sites for their offspring, as the larvae are not able to change to better hosts after hatching. Anyways, the choice between quality and quantity hosts depends on the remaining opportunities an individual has. This means that females with many eggs in their ovaries late in the year become less selective for good hosts, while those carrying only few eggs take their decisions more carefully (Mangel, 1989; Roitberg et al., 1992; Bouskila et al., 1995; Heimpel et al., 1996). Another study ordered to time-dependent decision changes was conducted by Murphy (2003b). He studied breeding site choice of the Amazonian leaf litter frog *Edalorhina perezii* over different seasons. Specimens preferred ephemeral pools in the middle of the rainy season, apparently to avoid high predator densities associated with permanent pools. In contrast, frogs started to exploit permanent pools as soon as ephemeral pools became less stable.

Not only can the choice of an adequate breeding site change. There are also several animal species displaying multiple patterns of parental care, changing for example from no care to uniparental care or from uniparental to biparental care (Webb et al., 1999). This might be caused by reproductive resource availability (respectively competition) or the divergence of habitats (e.g. Badyaev, 1993; 1994; Martins et al., 1998; Marsh and Borrell, 2001; Tarutis et al., 2005) as well as by individual characteristics such as body size or age, as animals in different age or size classes may have different optimal strategies (Balshine-Earn and Earn, 1997; Webb et al., 1999). Furthermore, changes in parental care decisions may arise from time-dependent shifts in environmental conditions during a single breeding season and can be seen as adaptive traits to seasonal changes in order to maximize the individual's fitness (Yamamura and Tsuji, 1993; Webb et al., 1999). Females of the Kentish plover (*Charadrius alexandrinus*), a widely distributed wader bird, for instance, often abandon their brood with the male parent to find a new mate, thus increasing their offspring quantity. However, when late in the breeding season, food availability decreases and parents have to move farther in search of food, while leaving the offspring unprotected, females usually stay and help their mates to care for offspring. That way Kentish plovers change their strategy from uniparental to biparental care (Székely and Cuthill, 1999; Székely and Cuthill, 2000). A change from no care to uniparental care could be shown by van den Berghe (1990) for Atlantic labrid fish (*Symphodus tinca*), where the increase of temperature and predators over the breeding season triggers parental care behaviour of the male.

Comparing data from this study with those of Poelman & Dicke (2007), the sister taxa *R. amazonica* and *R. variabilis* show similar patterns of parental care. Both are able to change from no care (regarding to the time after transportation to phytotelmata) in the rainy season to uni- or biparental care in the dry season. While in *R. amazonica* there are no data for tadpole depositions with the change of seasons, a switch to egg feeding behaviour could be shown. *Ranitomeya variabilis* in contrast did not change its egg deposition strategy with the beginning of the dry season even if there was evidence (first author's unpublished data) that egg deposition with older tadpoles occurs in this sister species to *R. amazonica*. Perhaps, egg feeding behaviour in *R. variabilis* does also exist, but starts later in the dry season, when chances of successful hatching are less likely. At the current state of knowledge, we can neither exclude interspecific (within one clade) nor intraspecific variation, e.g. among different populations in both these relatively wide-spread species (Brown et al., 2011). At least, we can conclude that feeding with tadpoles might be more advantageous at the early stage of the dry season, as hatchlings of one clutch can be divided among several tadpoles. Furthermore clutch deposition decisions might be influenced by the females, too. They only gain fitness when feeding their own tadpoles, avoiding depositions with tadpoles of other females. While Poelman and Dicke (2007) found frequent mating with the same male in *R. amazonica*, *R. variabilis* is known to have a more promiscuous mating system with little mate fidelity (Brown et al., 2008b). Discrimination of the own offspring via chemical cues by females could be an alternative or additional explanation for the strict avoidance of the cups with non-kin cues (i.e. cues provided by the authors). But this is unlikely, as the *R. variabilis* male feeding behaviour (i.e. the closest associates of tadpoles) does not show such recognition. For a better comparability between the behavioural changes triggered by season in *R. variabilis* and *R. amazonica* further studies drawn to the tadpole deposition of *R. amazonica* during the change of seasons, as well as the role of the females related to the clutch deposition decisions in *R. variabilis* are required.

To our knowledge multiple patterns of parental care triggered by seasonal changes has not been shown for any other anuran genus. So far, this is even unique to the two species of the *Variabilis* Clade of Brown et al. (2011) within *Ranitomeya*. In concordance with Poelman and Dicke (2007), we suggest that a plastic food provisioning strategy might not only be seen as an adaption to a changing environment but also as an evolutionary step towards regular egg feeding. This is well known in other *Ranitomeya* species, e.g. *R. imitator* and *R. vanzolinii*

(Caldwell and de Oliveira, 1999; Brown et al., 2008b). Poison frogs providing their larvae regularly with trophic eggs are seen as evolutionary most advanced (e.g. Summers and McKeon, 2004).

Plasticity in parental care triggered by climatic seasonality and so far exclusively performed in two *Ranitomeya* species opens an interesting field in physiological, ecological and evolutionary research. Despite possible intraspecific variation, future studies might address the functioning of the trigger itself as well as the emergence of plasticity in a larger evolutionary framework. Beyond this, we will not rule out convergent behavioural patterns in other frogs and toads, as anurans exhibit a remarkable diversity of reproductive modes, many of which associated with parental care (Duellman and Trueb, 1986; Haddad and Prado, 2005; Summers and McKeon, 2006).

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## Chapter VI

### **Feeding or avoiding? Facultative egg-feeding in a Peruvian poison frog (*Ranitomeya variabilis*)**

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## Abstract

The choice of breeding resources can be very important for the reproductive success and accordingly the fitness of an individual. Often, however, advantageous breeding sites also include disadvantageous characteristics. Neotropical poison frogs (Dendrobatidae) for example use phytotelmata for the deposition of their tadpoles. The exploitation of these small pools is advantageous as it lowers the risk of predation, but it is also costly because of limited resource availability. Several species evolved regular provision with unfertilized trophic eggs to compensate for the resource limitations for their tadpoles. Fertilized eggs in contrast are usually deposited outside of the phytotelmata in both egg-feeding and non-feeding species, to avoid consumption by cannibalistic conspecific tadpoles. The Peruvian species *Ranitomeya variabilis* is an exception: Clutches are deposited within the phytotelmata. Several studies show a strong avoidance for egg depositions within phytotelmata already containing a tadpole. Here the hypothesis is tested, that this avoidance behaviour is mainly directed towards unrelated offspring, while *R. variabilis* is less concerned and therefore shows less avoidance when faced with its own offspring. Therefore two setups were compared, one in which frogs had to face newly erected phytotelmata containing cues of non-familiar tadpoles and one where phytotelmata remained at the same place, so frogs could deposit and recognize their own tadpoles. While non-familiar tadpoles were completely avoided for egg deposition, phytotelmata with familiar tadpoles received several clutches, suggesting a form of parental care in *R. variabilis* that exceeds the transportation of tadpoles and might be a step towards egg-feeding.

**Keywords:** core area, Dendrobatidae, offspring provisioning, phytotelmata, reproductive strategy.

## Introduction

The decision where to breed can strongly influence the reproductive success of an animal, for which reason the choice of oviposition sites is usually nonrandom (e.g. Crump, 1991; Mariette and Griffith, 2012; Refsnider and Janzen, 2010). This is especially important in species that utilize spatially or temporally separated breeding resources for the development of their offspring, limiting its chances to move to new resources (e.g. Fincke, 2006; Harvey et al., 1994; Mayhew, 1997).

One example of isolated reproductive resources is phytotelmata (small water bodies in plants, e.g. bromeliads) used by various anuran species for either egg or tadpole deposition or both. Within the 39 anuran reproductive modes (Haddad and Prado, 2005), those including the use of phytotelmata belong to the derived ones, minimizing the risk of predation associated with larger water bodies (Aspbury and Juliano, 1998; Duellman and Trueb, 1986; Lehtinen et al., 2004a; Magnusson and Hero, 1991). Many phytotelmata-breeding species furthermore evolved parental care in various degrees, such as egg attendance, tadpole transport or provision of larvae with eggs (Brown et al., 2010; Jungfer and Weygoldt, 1999; Lehtinen et al., 2004b). Egg-feeding with either fertilized or unfertilized eggs repeatedly evolved in response to low food availability within small pools (Lannoo et al., 1987). A rudimentary mode of parental care involving egg-feeding is shown for example by Jungfer and Weygoldt (1999): parental *Osteocephalus oophagus* (Hylidae) lay a clutch of fertilized eggs in the same phytotelm every 5–7 seven days that either develops into tadpoles or gets eaten by tadpoles already present from a previous clutch. The advantage of this mode is that eggs not eaten by conspecifics are not ‘wasted’, since the costs for sperm production is relatively low. Most derived are species in the genus *Oophaga*, showing uniparental care, with only the female caring for the offspring, feeding it with unfertilized trophic eggs (e.g. Summers and McKeon, 2004). They belong to the Neotropical poison frogs (Dendrobatidae), which are known to use phytotelmata as a reproductive resource, carrying their tadpoles to these little pools (e.g. Brown et al., 2008a; Summers and McKeon, 2004; Weygoldt, 1980). Feeding with trophic eggs after tadpole deposition evolved in some but not all poison frog species (e.g. Lötters et al., 2007). Both feeding and non-feeding species deposit their fertilized eggs in the leaf litter or above the waterline in a phytotelm, to avoid their consumption by tadpoles (Caldwell and de Araújo, 2004; Magnusson and Hero, 1991; Summers and McKeon, 2004). While most



poison frogs of the behaviourally very diverse genus *Ranitomeya* lay their eggs in the leaf litter (Brown et al., 2011), some species attach their eggs above the waterline in a phytotelm, not allowing them to drop into the water below (e.g. Brown et al., 2008b; Caldwell and de Oliveira, 1999). However, three species attach their eggs to the wall of phytotelmata at or just below the water surface. These are *R. amazonica*, *R. variabilis* and *R. uakarii*, but the latter is known to make facultative use of leaf litter for clutch deposition (Bechter and Lescure, 1982; Brown et al., 2008b; Poelman and Dicke, 2007).

Both *R. amazonica* and *R. variabilis* are known to be cannibalistic, feeding on conspecific tadpoles if given the chance (Bechter and Lescure, 1982; Brown et al., 2008a; Poelman and Dicke, 2007; Summers, 1999; Summers and Amos, 1997; Summers and Symula, 2001). In addition egg consumption has been shown in *R. amazonica* (Poelman and Dicke, 2007) as well as for some populations of *R. variabilis* (Summers, 1999). In other populations of *R. variabilis* however, it is known that tadpoles ‘wait’ for the embryos to hatch before they feed on them (Brown et al., 2008b). Either way, adult frogs generally avoid pools already containing conspecific tadpoles for both tadpole and clutch deposition (Brown et al., 2008a; Schulte et al., 2011; Summers and McKeon, 2004). However, Summers (Summers, 1999) showed for *R. variabilis* that tadpoles and eggs can sometimes be found together in the same pool. Brown et al. (2009b) explained this phenomenon with a form of ‘brood parasitism’ in which male frogs transport their tadpoles systematically to phytotelmata already containing conspecific eggs so that hatching embryos could be used as a food source for the larger tadpoles. Poelman and Dicke (2007), on the other hand, demonstrated that adults of the closely related *R. amazonica* avoid pools already containing tadpoles for egg deposition during the rainy season, but prefer them at the onset of the dry season. By doing so, tadpoles deposited at the end of the rainy season get fed by their parents with fertilized eggs and have a greater chance to reach metamorphosis before pools dry out, as the consumption of conspecifics (or conspecific eggs) significantly increases growth and development in tadpoles (Caldwell and de Araújo, 1998; Crossland et al., 2011; Crump, 1990; Heinen and Abdella, 2005; Summers, 1999; Summers and Amos, 1997).

While the data of Poelman and Dicke (2007) show that *R. amazonica* is able to provide food in form of eggs to its offspring if necessary, Brown et al. (2008a) discussed the possibility of food provisioning for *R. variabilis* via trophic tadpoles: commonly all tadpoles of one clutch

get transported to different phytotelmata (Fig. 1), but sometimes males allow them all to hatch within a single phytotelm. This way only one of the tadpoles survives at the end, but is provided by a good food resource consisting of its siblings. Schulte and Lötters (2013) could further show that with the end of the rainy season tadpoles were preferably deposited in phytotelmata containing chemical tadpole cues. This behaviour was interpreted as a plastic food provisioning strategy, similar to that shown by Poelman and Dicke (2007). However, this behaviour could only be shown for tadpole depositions in *R. variabilis*, while the chemical cues of unknown tadpoles were always avoided for clutch depositions.

In this study the hypothesis is tested, if *R. variabilis*, generally showing a strong avoidance against cannibalistic tadpoles, is less ‘concerned’ when faced with its own offspring in order to speed their growth by egg feeding. For this purpose, two setups were compared: one in which frogs had to face newly erected phytotelmata containing non-familiar tadpoles and one where phytotelmata remained fixed at the same place over several months, so that frogs could make use of their ability to recognize tadpoles placed by themselves.



**Fig. 1** A male *Ranitomeya variabilis*, carrying its offspring. In this species tadpole transportation to phytotelmata usually takes place one-by-one, so males have to return repeatedly to their clutches.

## Methods

### Study site

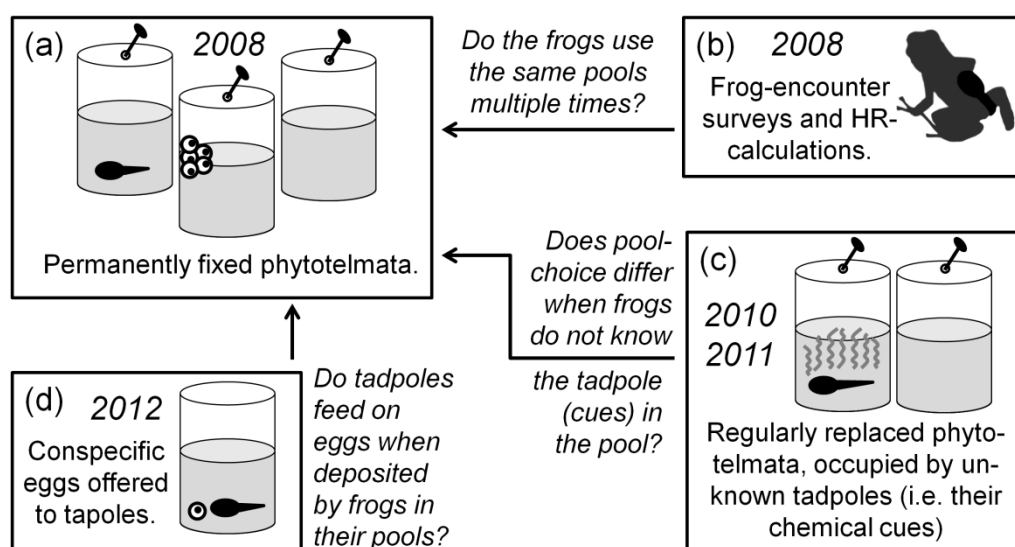
The study was carried out from 24 June to 17 November 2008 in a premontane late-stage secondary forest along the Tarapoto-Yurimaguas road (km 32) in the upper Cainarachi Valley, region San Martín, Peru. A study plot of 26 m by 34 m was established and divided into  $2 \times 2$  m grids, using numbered flags. Within the plot, 125 artificial phytotelmata (i.e. halves of transparent plastic bottles; compare Brown et al. 2008b) each containing 500 ml of water were nailed to trees. On each tree, five of these pools were fixed in a vertical line (see Schulte et al. 2010), so 25 spots with five artificial phytotelmata each in a distance of minimal 3 m and maximal 7 m were distributed throughout the study site.

### Clutch depositions

Every 7 days the phytotelmata were checked for clutch and tadpole depositions by *Ranitomeya variabilis*. While clutches can be seen, for tadpole detection, pool contents were poured through a mesh and the filtered matter was explored by hand. In addition, all natural phytotelmata within the site were searched and checked once a week for depositions.

After counting all depositions, a Shapiro-Wilk-Test was calculated to see if depositions were normally distributed over the trees equipped with artificial phytotelmata or if some spots were used more often than others. Subsequently, clutches deposited in unoccupied pools versus depositions into pools already containing a tadpole were counted, disregarding the first clutch depositions on each tree, when there had not been any tadpole by that time. These data were compared with clutch depositions from 2010 and 2011, where frogs had the choice between clean water and water used by *R. variabilis* tadpoles before (compare Schulte et al. 2011; Fig. 2a, c), using a Fisher's exact test (Fisher, 1922; Townend, 2002). While in 2008 the previously empty phytotelmata stayed on the same spots over several month and the frogs were able to use them several times, in 2010 and 2011 the cups were offered to the frogs already occupied and were replaced by a new setup at another place after depositions. In both cases, frogs had the choice between occupied and empty phytotelmata, either at the same tree or in a distance of at maximum 7 m.

If eggs were deposited in phytotelmata already containing a tadpole, their fate was checked in the following week. To test, if disappearance might be associated with egg feeding, 12 *R. variabilis* tadpoles between Gosner stage 28 and 36 (Gosner, 1960) were collected in the forest in 2012 and kept individually in 30 ml water. Each tadpole was fed with one conspecific egg (Gosner stage 1–18) and observed over several days (Fig. 2d).



**Fig. 2** Overview over the combined methods from several years (a–d) and relevant questions combining them with each other.

## Paternity

For conclusions about parenthood, adult frogs were searched through standardized visual encounter surveys crossing the plot 2 times the week for 5 hr during daytime, spending 1 min per  $2 \times 2$  m grid (Heyer et al., 1994; Fig. 2b). Individual frogs were identified using photo-identification cards showing the unique dorsal and ventral patterns, which were created after the first encounter with each individual. Every finding of an individual was recorded at 0.5 m resolution, subdividing each  $2 \times 2$  m grid with a measuring stick into eight squares (according to Brown et al., 2009a). Notes of any behavioural observations (e.g. courtship behaviour, calling, tadpole transport) were used for sex determination of each individual.

Spatial positions of males that were observed at minimum four times (Werner et al., 2011), were used in calculation of home ranges by the fixed-kernel density method (Worton, 1989).

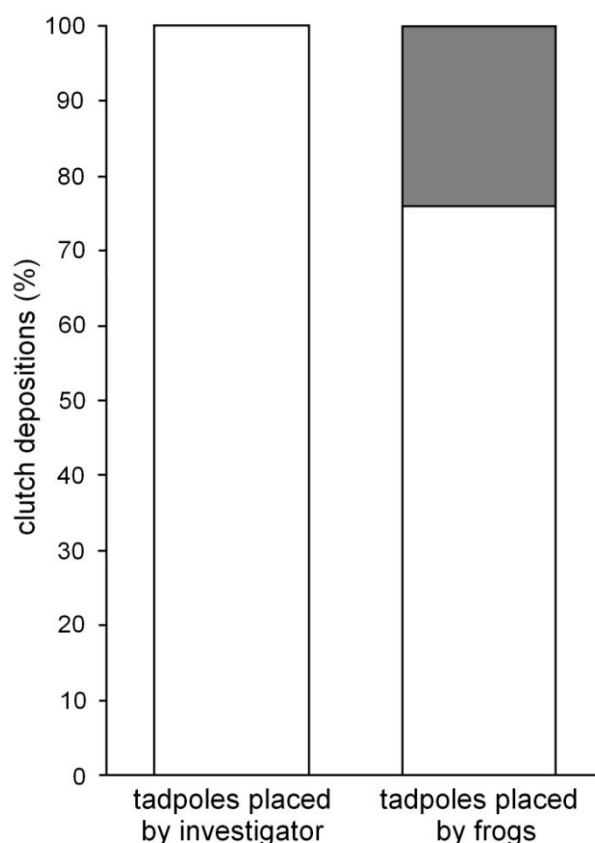
Therefore the Home Range Tools extension (HRT; Rodgers et al., 2007) was used in ArcGIS 9.3 (ESRI, Redlands, CA, USA), applying an ad hoc smoothing parameter ( $h$  ad hoc), choosing the smallest increment of the reference bandwidth ( $h$  ref) that produced a contiguous 95% kernel home range polygon and prevents over- or under-smoothing (Berger and Gese, 2007; Jacques et al., 2009; Kie et al., 2010; Rodgers and Kie, 2011). Furthermore, all deposition events were plotted on the kernel home ranges and manually scored under the aspect if located inside or outside the 50% kernels respectively core areas of the males (Poelman and Dicke, 2008). Results were compared using a G-Test (Woelf, 1957), adjusted in the manner of Williams (1976). This way it was possible to see, whether core areas could be used to assign individual males to phytotelmata repeatedly used for depositions.

## Results

### Clutch depositions

From 125 artificial phytotelmata 63 were used by *Ranitomeya variabilis*. None of the comparatively small natural phytotelmata was used by *R. variabilis*. There was a clear preference of some trees equipped with artificial phytotelmata (Shapiro-Wilk-Test  $W = 0.850$ ,  $P < 0.01$ ), suggesting a strong spatial structuring triggered by frequent use of few pools. Frogs showed significantly different reactions towards pools containing tadpoles if they could use them several times versus only one time, suggesting recognition of their own offspring. While in the experiments of 2010 and 2011, occupied pools were avoided for all 36 clutch depositions, in the experiments of 2008, 12 versus 38 clutches were deposited in pools already occupied by tadpoles (Fisher's exact test,  $P = 0.001$ ; Fig. 3).

Seven of 11 clutches deposited with tadpoles were gone after 1 week (one was found on the last day of the study period, so there are no data of its whereabouts), while clutches in empty pools usually could be found 2 weeks in a row (tadpoles usually hatch after 15 days, metamorphosis follows 2-3 month later; Lötters et al., 2007). In the test to determine whether tadpoles feed on eggs, nine out of 12 tadpoles were observed to consume the eggs within the first 5 days. After opening the egg capsule, first the embryo was eaten, before the egg remnants were consumed. The remaining three tadpoles first ate the egg capsule and then the embryo, which had taken them 10 to 13 days.

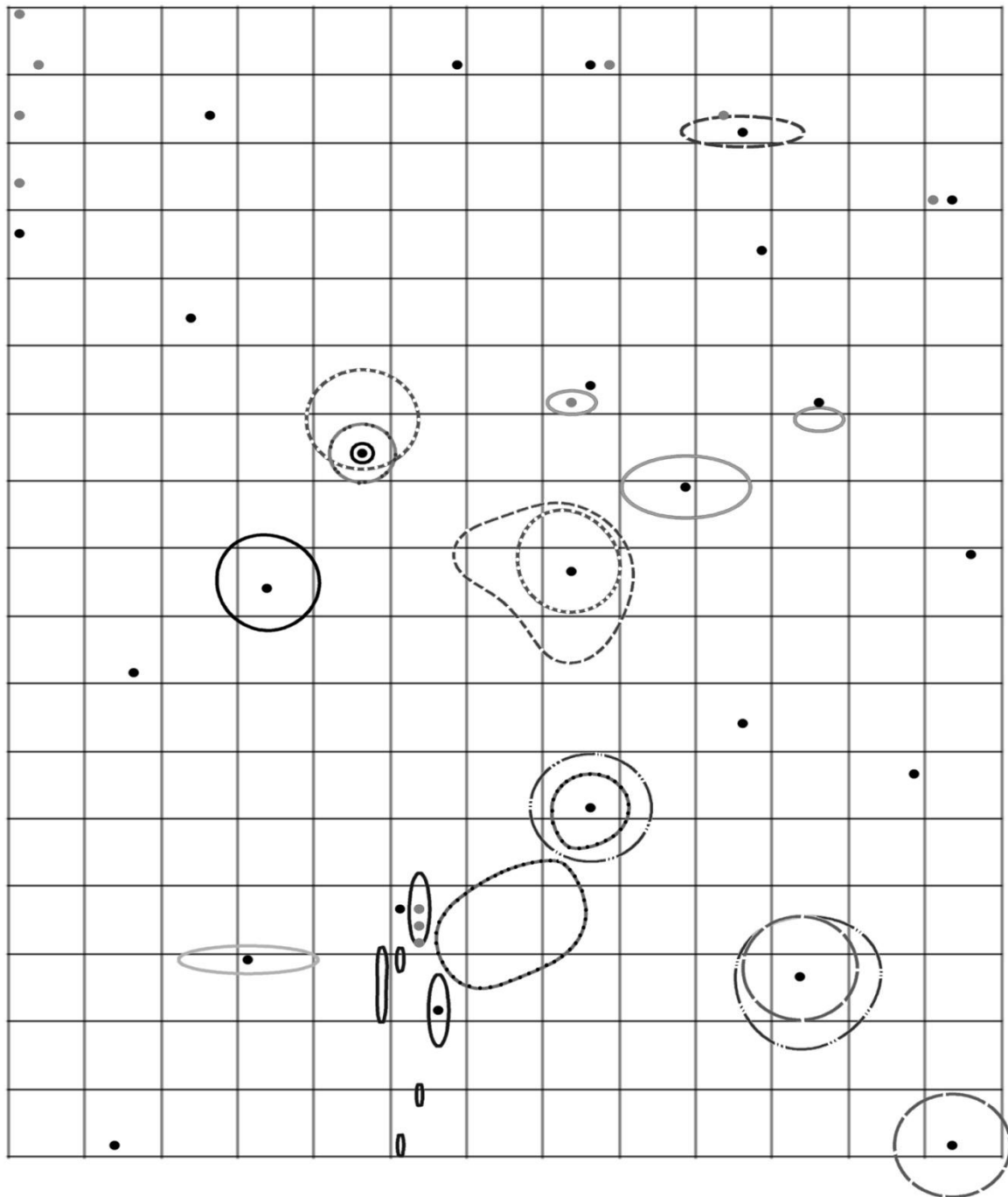


**Fig. 3** Percentage of clutches in empty pools (white) and in pools already occupied by tadpoles (grey) in the different experimental designs.

### Paternity

Forty-two adult *Ranitomeya variabilis* were found within the study plot. Nineteen of them were identified as males and 11 of these were found more than 3 times and their 50% kernels were calculated (see Fig. 4). Within these core areas significantly more depositions were found then outside: 59 versus 9 clutches (G-Test,  $G = 40.814$ ,  $P < 0.0001$ ) and 42 versus 10 tadpoles ( $G = 20.972$ ,  $P < 0.0001$ ).

Of the 12 clutches deposited together with tadpoles, six were in core areas occupied by one male only (4 times the same male and 2 times each another male), five clutches in core areas used by two males (three and two depositions) and one clutch was found in a phytotelm within the core area of three different males.



**Fig. 4** Core areas of the 11 males seen more than three times in the study plot, measuring 26 m by 34 m (different grey levels and patterns stand for different individuals). A clear distribution around certain phytotelmata trees (black dots) can be seen. Single dots stand for five artificial phytotelmata, fixed on one tree. Grey dots close to the black dots are shown, if trees were sloped, so not all five phytotelmata were in an exact vertical line.

## Discussion

Adult *Ranitomeya variabilis* avoided tadpole containing phytotelmata for clutch deposition in a clearly different way when occupied by tadpoles placed by the frogs themselves (i.e. being in a phytotelm frequently visited by a frog) than when occupied by tadpoles placed by the conductor of the experiment. While non-familiar tadpoles were avoided entirely, tadpoles placed by the frogs themselves were avoided less strongly, what may lead to the conclusion that frogs were occasionally feeding their own offspring with eggs. This assumption is supported by the typically careful and multiple check of each phytotelmata (Poelman and Dicke, 2008; Summers and McKeon, 2004) as well as the site fidelity the males showed during this study: egg deposition mainly occurred within the core areas and in half of the cases where eggs were deposited together with tadpoles, only one male frequently visited these phytotelmata. A possible preference due to better quality of those frequently visited phytotelmata cannot be excluded here, but not only should all the artificial pools be very similar in quality, the threat that the offspring could be eaten by cannibalistic (non-familiar) conspecifics might be considered the quality attribute most important to the frogs.

Contrary to the results of Brown et al. (2009b), regarding the same population, *R. variabilis* tadpoles feeding on conspecific eggs could be observed. A similar observation was made by Summers (1999) on *R. variabilis* tadpoles in an Ecuadorian population; however, only embryos but not the egg capsules were consumed. Also the closely related *R. amazonica* are known to feed on conspecific eggs in French Guiana (Poelman and Dicke 2007).

During this study, no tadpoles were deposited in pools containing clutches, even though Summers and Amos (1997), Summers (1999) and Brown et al. (2009b) did observe such behaviour. The opposed deposition of clutches in pools already occupied by tadpoles, shown in this study, was also observed in *R. amazonica* by Poelman and Dicke (2007). But while these authors observed this behaviour in dependence of the dry season, it was not possible to show such a switch between dry and rainy season here. Starting the study in the dry season, it took several weeks until *R. variabilis* used the artificial phytotelmata on a regular basis, so provisioning of tadpoles could be mainly observed in the second half of the field season.

Summers (1999) suggested three different explanations for the synchronous use of phytotelmata for eggs and tadpoles: (1) there are no empty pools available; (2) polygynous males parasitize the reproductive effort of females or other pairs that deposited a clutch before



the male added his tadpole; (3) the deposition of eggs in phytotelmata already containing a tadpole is a form of egg feeding. While none of the studies exhibited a lack of empty pools (Summers 1999; Poelman and Dicke 2007; Brown et al. 2009b; this study), the studies with *R. variabilis* of Summers (1999) and Brown et al. (2009b) suggested reproductive parasitism by the males placing tadpoles in pools already containing clutches. Poelman and Dicke (2007) in contrast indicated a kind of egg feeding in *R. amazonica* and interpreted it as a putative evolutionary link to feeding of unfertilized eggs in the genus *Ranitomeya*. The present study suggests a similar facultative feeding behaviour in *R. variabilis*, starting after pools were used by males over several months. This restriction to long known phytotelmata was in contrast not shown for the provisioning with tadpoles, a behaviour that could be only shown during the dry season, when the risk of phytotelmata desiccation rises (Schulte and Lötters, 2013).

Of the 16 species in the genus *Ranitomeya* (Brown et al., 2011), two species are known to perform biparental care and feed their tadpoles regularly with trophic eggs, *R. imitator* and *R. vanzolinii* (Brown et al., 2008b; Caldwell and de Oliveira, 1999). They only deposit trophic eggs within the phytotelmata, while fertilized eggs are deposited either in the leaf litter, or far above the water surface, so tadpoles cannot reach them. Terrestrial breeding is also common in most of their congeners and egg predation by tadpoles is considered to be the most important factor favouring terrestrial reproductive strategies, even beyond the genus *Ranitomeya* (Magnusson and Hero, 1991). In contrast both *R. amazonica* and *R. variabilis*, (together forming the *Ranitomeya variabilis* species group, see Brown et al. 2011), lay their eggs at or below the water level within a phytotelm. This could serve as a prevention of egg desiccation (Summers 1999), but might also be a first level of offspring provisioning (in concordance with Poelman and Dicke 2007) if deposited together with tadpoles.

Whether this behaviour represents a step towards biparental care (with the advantage of offspring replacement when not consumed; see introduction) and if females are also involved in the clutch deposition decision is unknown and could be clarified by genetic studies. While Brown et al. (2009b) showed that tadpoles deposited together with eggs were a mix of siblings, half-siblings and non-siblings, there are no data of the relationship when fertilized eggs were deposited with tadpoles. The fidelity to certain phytotelmata-containing trees by single males might indicate that tadpoles and embryos are probably at least half-siblings, leading to the assumption that *R. variabilis* males show (similar as *R. amazonica*) a form of

parental care that exceeds the transportation of tadpoles and might be a step towards egg feeding. Anyway, since there is evidence in other poison frog species that offspring is rather detected by phytotelmata location then by kinship (Poelman and Dicke, 2007; Stynoski, 2009), the possibility that between two deposition events other males ‘pirated’ a phytotelm (i.e. by depositing a bigger tadpole) in order to benefit from the feeding males effort, cannot be excluded here. Further studies, including the feeding behaviour of other usually non-feeding *Ranitomeya* species as well as a closer view on deposition site selection would bring a substantial advancement in our understanding of the evolution of biparental care in this genus.

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## Chapter VII

### **The response of a Neotropical poison frog (*Ranitomeya variabilis*) to larval chemical cues is not influenced by kinship**

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unpublished manuscript

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**Abstract**

Offspring recognition mediated by chemical cues is widely known among vertebrates. In order to test this ability in the poison frog *Ranitomeya variabilis*, we analyzed if parental frogs deposit their tadpoles rather with closely related than non-related tadpoles. We therefore conducted pool-choice experiments with one pool presenting chemical cues of a tadpole previously found at the same location. Tadpoles were mainly deposited in pools without tadpole cues and genetic analyses indicated that frogs did not choose to deposit their offspring with or without another tadpole due to relatedness. We conclude that frogs rather discriminate by location than by kin recognition.

**Keywords:** chemical recognition, Dendrobatidae, offspring identification, parental care, phytotelmata

In species that show parental care, offspring discrimination, i.e. the differentiation 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 high levels of parental care (e.g. Brown et al., 2008; Brust, 1993; Summers, 1990; Summers and McKeon, 2004; Summers and McKeon, 2006), 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, benefiting the tadpole both by eliminating competitors as well as by compensating for the limited food resources in phytotelmata (Caldwell and de Araújo, 1998; Summers, 1999). In the species *Ranitomeya variabilis* Schulte et al. (2011) showed that phytotelmata already occupied by cannibalistic larvae were recognized and avoided for tadpole deposition by parental frogs by means of chemical cues. However, Schulte and Lötters (2013) found that the frog's behaviour changed with the change of seasons. While chemical cues of the cannibalistic tadpoles led to the avoidance of phytotelmata in the rainy season, they were preferred for tadpole depositions during the dry season. This might be interpreted as the attempt to feed the older tadpoles within the own territory with younger conspecific larvae. Thus, the frogs only lose the younger hatchlings but might help the older ones to accelerate metamorphosis and escape the desiccating phytotelmata.

In this study we want to test, if this behaviour is especially developed when chemical cues of the own offspring are presented. Kin recognition mediated by chemical cues is widely known among vertebrates (Burger et al., 2011; Gustin and McCracken, 1987; Krause et al., 2012; Levy et al., 2004; e.g. Porter and Moore, 1981). In anurans for example it is known that larvae prefer grouping with siblings over non-siblings (Blaustein and O'Hara, 1982; Eluvathingal et al., 2009; Waldman, 1985). We hypothesize that adult *R. variabilis* have a similar ability and recognize their own tadpoles via chemical cues, depositing their younger tadpoles rather together with them 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 hung up a total of 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 non-transparent polypropylene plastic cups (200 ml volume, 10 cm



height, 7 cm in diameter), that were 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 or underneath a perforated second plastic floor (the two different ways were chosen in case one turned out not to work). The other cup was equipped with a tea bag or a second floor, too, but without a tadpole. The tadpoles used in our experiment were deposited by a male in a single artificial phytotelm hanging at the same spot before and were left in the cup during the whole experiment. They are called stationary tadpoles hereafter. Stationary tadpoles were fed once a week with flaked fish food (Tetra®), of which a little bit was placed in the cups not containing tadpoles as well.

Artificial phytotelmata were checked every 3-8 days for newly deposited tadpoles (called secondary tadpoles hereafter). They were removed from the cup pairs and a tissue sample was collected from each tadpole. Tissue samples of stationary tadpoles were taken at the end of the experiments. We further randomly collected embryos belonging to the same clutches, i.e. being full siblings (N = 7 clutches, 19 tadpoles) as well as toe clips from putatively not related adult frogs (five individuals from two forest sites each, being separated by a coffee plantation).

Genetic samples were stored in 99 % ethanol p.a. at room temperature until DNA was extracted with the Qiagen DNEasy blood and tissue kit. We then genotyped all specimens using seven polymorphic microsatellite markers specifically 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 the 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 used as 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 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 (ver. 2.2.3; van Oosterhout et al., 2004).

Pairwise relatedness between grouped individuals was analysed with the KINSHIP relatedness estimator (Goodnight and Queller, 1999; Queller and Goodnight, 1989) implemented in the program KINGROUP (ver. 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 for full siblings should be on average  $r_{QG} = 0.5$ , for half siblings  $r_{QG} = 0.25$ , and for unrelated individuals  $r_{QG} = 0.0$ . The significance level for each comparison was calculated with a simulation routine integrated in the program. For further clarity the average relatedness among the putatively unrelated individuals as well as among the embryos from the same clutches was calculated as well.

To determine if secondary tadpoles that were related respectively not related with the stationary tadpole were deposited in a different pattern (i.e. alone vs. with the stationary tadpole) we calculated a contingency table using the Fisher's exact test (Fisher, 1922).

Relatedness among putatively unrelated individuals was estimated as  $r_{QG} = -0.025$  (+/- 0.024) and among embryos (i.e. full siblings)  $r_{QG} = 0.391$  (+/- 0.040). Tadpoles from the same cup pairs estimated as significantly related had a range of  $r_{QG} = 0.285 - 0.495$ , those estimated as not related had a range of  $r_{QG} = -0.223 - 0.198$ .

Of all cup pairs hung up in 2010 and 2011 only nine received secondary tadpole depositions, but some were used more than once (after previously deposited tadpoles were removed). 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 one. This was also the case for 14 of those secondary tadpoles that were placed alone. Only three of the secondary tadpoles placed in the free cups were significantly related to the stationary tadpole (see table 1). Furthermore three pairs of the secondary tadpoles placed consecutively in the same cup pairs were related to each other (see table 1).

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

**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 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		

Although our study was conducted during the dry season, we mainly found tadpoles deposited alone rather than in the cups containing a hidden tadpole. While the only secondary tadpole deposited together with the stationary one turned out not to be its sibling, three of those that were not deposited together with the stationary tadpole were actually related with it. This leads us to the conclusion that either, contrary to our hypothesis, parental *R. variabilis* are not able to distinguish their own offspring from unrelated tadpoles by means of chemical cues or that this ability, if existing, was not used in the context of our study. However, the circumstance that several of the secondary tadpoles placed consecutively in the same cup were related to each other might indicate that frogs rather use location for discrimination. This was not possible for those secondary tadpoles deposited first, since we changed the

arrangement of the artificial phytotelmata at the location by replacing the originally chosen cup with a pair of new cups.

Stynosky (2009) and Poelman and Dicke (2007) showed for other poison frog species (*Oophaga pumilio* and *Ranitomeya amazonica*) that parental frogs could not discriminate between kin and non-kin, but rather used indirect recognition, i.e. discrimination by location. Since the breeding resources used by poison frogs are spatially separated and therefore precluding the chances of the offspring to move to new resources, the direct recognition of the own offspring is supposed to be less important in these species as it is for example in free-ranging species living in big flocks or colonies (Burger et al., 2011; Gustin and McCracken, 1987). Nevertheless, as *R. variabilis* was shown to deposit clutches regularly in the same phytotelm that already contains a tadpole (unpublished data) the recognition of the own offspring would be of high advantage for this species, too. When the tadpole in the preferred phytotelm dies and gets replaced by a new, non-familiar tadpole, parents should stop depositing eggs that are then likely to be eaten by the new tadpole. This way, frogs could prevent a form of reproductive parasitism, similar to that described by Brown et al. (2009b).

Our study cannot totally exclude that *R. variabilis* might be able to recognize its own offspring, even if we could not find any confirmation for this behaviour. An ex-situ choice test with cues of the own offspring versus cues of a non-related tadpole (excluding other deposition possibilities) could bring further clarification. However, the study of Summers and Symula (2001) also could not reveal a significant kinship recognition between *R. variabilis* tadpoles (even if there was a slight tendency that siblings were bitten less than non-siblings). In other anuran species in contrast recognition of siblings (often by means of chemical cues) is widely known (Blaustein and O'Hara, 1982; Cornell et al., 1989; Eluvathingal et al., 2009; Pfennig, 1999; Pfennig and Frankino, 1997; Waldman, 1985).

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## Conclusions

The results of this thesis broadly expanded the knowledge about chemical communication in anurans, particularly during parental care. It was shown that the focal species of the study, *Ranitomeya variabilis*, does not only recognize and avoid chemical substances of conspecifics, but also those of several heterospecific tadpoles. However, while chemicals of all tested species were avoided for clutch depositions, only some triggered the same avoidance behaviour for tadpole depositions. It turned out that only poison frog tadpoles that also occur in phytotelmata were recognized, while those living in rivers were not. Additionally, the chemical substances of a treefrog tadpole (Hylidae) were recognized. But they were not avoided but preferred for tadpole depositions, thus these tadpoles might be recognized as a potential prey for the predatory poison frog tadpoles. Generally speaking, the discrimination between tadpoles of different species does partly occur due to phylogenetic but mainly for ecological reasons. All tadpoles recognized by *R. variabilis* were phytotelmata breeders (or closely related to them), but only the phytotelm-breeding poison frog larvae were avoided for tadpole depositions while the others, i.e. the treefrog tadpoles, were preferred instead. Together with the results for the clutch depositions this leads to the conclusion that *R. variabilis* generally recognizes all tadpoles (and avoids them for eggs, since those might be eaten also by non-predatory tadpoles), but regarding tadpole depositions it only differentiates between those tadpoles co-occurring in the same habitat and only poison frog tadpoles are avoided.

After defining due to what characteristics heterospecific tadpoles were recognized by parental *R. variabilis*, the different communication systems analyzed here were defined with the classification system shown in the introduction (figure 1 and 2). The avoided substances of the conspecific tadpoles could be defined as chemical cues, since they are only advantageous to the receiver (the adult frogs), but not to the sender (the tadpoles). The definition of the heterospecific substances, however, was not possible before their chemical analysis and a following comparison with the active compounds of the conspecific tadpoles. For both, the conspecific tadpoles as well as the tadpoles of *Hyloxalus azureiventris*, two active compounds could be described that only triggered avoidance behavior in *R. variabilis* when combined with each other. Although one of the compounds both species had in common, the other



compounds were species-specific. This shows that the avoidance of the heterospecific tadpoles is not triggered by a mere byproduct based on the close evolutionary relationship between the two species. It might rather be defined as a species-specific chemical signal released by *H. azureiventris* tadpoles in order to prevent *R. variabilis* from depositing its predatory tadpoles. Since both species avoid competition this way (the tadpoles of *R. variabilis* are only able to feed on very small *H. azureiventris* tadpoles, while bigger ones can be seen as competitors), the allelochemicals used in this interspecific communication system can be defined as synomones (advantageous for both emitter and receiver).

Another interspecific communication system is the recognition of predator kairomones by prey animals. It was tested if *R. variabilis* was able to recognize and avoid kairomones of phytotelmata breeding damselfly larvae in the same way as shown before for the chemical cues of its predatory conspecific tadpoles. Even if the frogs were able to recognize the invertebrate predators when present in a phytotelm, this was not the case when only chemical substances released by those predators were presented. However, for the recognition of conspecific tadpoles chemical substances were necessary. A merely visible tadpole (i.e. an artificial model) was not avoided by the frogs. This leads to the conclusion that *R. variabilis* is able to use both visual and chemical compounds when choosing an adequate habitat for its offspring, but that different sensory systems are used for the recognition of different predators.

It was then investigated, whether multiple senses might also be used during the mating behaviour of *R. variabilis*. Acoustic communication is known to be the dominant communication system during sexual recognition, but here the intersexual recognition via chemical signals was tested. While a sex-specific recognition could not be shown, there was a general recognition of adult conspecifics. However, there was no attraction as suggested, but rather avoidance of the conspecific chemicals. This might be an artifact due to confinement stress of the releaser animals, emitting disturbance cues that trigger avoidance behaviour in their conspecifics. To find out if chemical communication is conducted during mating behaviour between adult *R. variabilis*, further studies using a different methodology (i.e. larger containers for the signal releasing frogs) are needed.

Returning to parental care, the knowledge obtained thus far about chemical communication between parental *R. variabilis* and conspecific tadpoles was used to gain a deeper insight into

the possible provisioning behaviour in this species. It was tested whether parental care behaviour changed as a function of rain seasonality, as has been shown for the sister species *Ranitomeya amazonica*. While there was no seasonally caused change of clutch deposition decisions, like in *R. amazonica*, tadpole deposition behaviour, however, was altered. While conspecific cues were strictly avoided during the rainy season, they were preferred for tadpole depositions during the dry season. This shows that *R. variabilis*, considered as a species that does not provision its offspring with food, may actually feed its offspring with younger tadpoles. For this feeding strategy, females are not required, but males can provision their offspring by themselves. Accordingly, a new parental care strategy, never shown in another poison frog species before, might be added to the nine strategies listed in the introduction: (10) Eggs are deposited in phytotelmata at the water surface and males transport tadpoles singly to medium/small phytotelmata; sporadic egg or tadpole provisioning.

The sporadic egg provisioning mentioned above was not shown to be seasonally caused. However, when comparing the number of clutch depositions into phytotelmata already housing conspecific tadpoles (or their chemical cues), those phytotelmata containing a tadpole deposited by the frogs themselves received much more depositions than freshly erected artificial phytotelmata containing unfamiliar tadpoles (i.e. their cues). This means that only unknown conspecific tadpoles were strictly avoided by parental *R. variabilis*, while their own offspring were not. This behaviour might be interpreted as an irregular offspring provisioning with fertilized eggs and a putative evolutionary link to biparental feeding behaviour shown in other species of the genus *Ranitomeya*.

Since males in this study showed an unexpectedly strong site fidelity, it was suggested that offspring could be recognized by phytotelmata location. Nonetheless, there was one other possible way of offspring recognition to be tested: chemical kin recognition was shown to be important in other anuran species (see introduction). Here it was tested if adult frogs were able to recognize their own tadpoles chemically and preferred them during provisioning with conspecific larvae. However, using genetic samples of tadpoles (respectively their chemical cues) deposited together (or alone), kin recognition by chemical cues could not be confirmed in *R. variabilis*.

Due to the use of chemical cues only rather than whole tadpoles in the above-mentioned parental choice tests, depositions into phytotelmata already occupied by conspecifics (i.e.

their cues) did not result in cannibalism, so all deposited tadpoles could actually be found. This opens a great possibility to study parental care behaviour in highly cannibalistic species and might be applied for other poison frog species as well (e.g. of the genus *Dendrobates*). The knowledge gained about the importance of chemical communication on an intra- and interspecific base in *R. variabilis* might further stimulate similar investigations in other anurans conducting parental care.

## Summary

Chemical communication is the evolutionary oldest communication system in the animal kingdom that triggers intra- and interspecific interactions. It is initiated by the emitter releasing either a signal or a cue that causes a reaction of the receiving individual. Compared to other animals there are relatively few studies regarding chemical communication in anurans. In this thesis the impact of chemical communication on the behaviour of the poison frog *Ranitomeya variabilis* (Dendrobatidae) and its parental care performance was investigated. This species uses phytotelmata (small water bodies in plants) for both clutch and tadpole depositions. Since tadpoles are cannibalistic, adult frogs do not only avoid conspecifics when depositing their eggs but also transport their tadpoles individually into separated phytotelmata. The recognition of already occupied phytotelmata was shown to be due to chemical substances released by the conspecific tadpoles.

In order to gain a deeper comprehension about the ability of adult *R. variabilis* to generally recognize and avoid tadpoles, in-situ pool choice experiments were conducted, offering chemical substances of tadpole of different species to the frogs (**Chapter I**). It turned out that they were able to recognize all species and avoid their chemical substances for clutch depositions. However, for tadpole depositions only dendrobatid tadpoles occurring in phytotelmata were avoided, while those species living in rivers were not. Additionally, the chemical substances of a treefrog tadpole (Hylidae) were recognized by *R. variabilis*. Yet, they were not avoided but preferred for tadpole depositions; thus these tadpoles might be recognized as a potential prey for the predatory poison frog larvae.

One of the poison frog species which was avoided for both tadpole and clutch depositions, was the phytotelmata breeding *Hyloxalus azureiventris*. The chemical substances released by its tadpoles were analysed together with those of the *R. variabilis* tadpoles (**Chapter II**). After finding a suitable solid-phase extraction sorbent (DSC-18), the active chemical compounds from the water of both tadpole species were extracted and fractionated. In order to determine which fractions triggered the avoidance behaviour of the frogs, in-situ bioassays were conducted. It was found that the biologically active compounds differed between both species. Since the avoidance of the conspecific tadpoles is not advantageous to the releaser tadpoles (losing a potential food resource) the chemicals released by them might be defined as

chemical cues. However, as it turned out that the avoidance of the heterospecific tadpoles was not triggered by a mere byproduct based on the close evolutionary relationship between the two species, the chemical compounds released by *H. azureiventris* tadpoles might be defined as chemical signals (being advantageous to the releasing tadpoles) or, more specifically as synomones, interspecifically acting chemicals that are advantageous for both emitter and receiver (since *R. variabilis* avoids a competition situation for its offspring, too).

Another interspecific communication system investigated in this thesis was the avoidance of predator kairomones (**Chapter III**). Using chemical substances from damselfly larvae, it could be shown that *R. variabilis* was unable to recognize and avoid kairomones of these tadpole predators. However, when physically present, damselfly larvae were avoided by the frogs. For the recognition of conspecific tadpoles in contrast, chemical substances were necessary, since purely visible artificial tadpole models were not avoided.

If *R. variabilis* is also capable to chemically communicate with adult conspecifics was investigated by presenting chemical cues/signals of same-sex or opposite-sex conspecifics to the frogs (**Chapter IV**). It was suggested that males would be attracted to chemical substances of females and repelled by those of conspecific males. But instead all individuals showed avoidance behaviour towards the conspecific chemicals. This was suggested to be an artefact due to confinement stress of the releaser animals, emitting disturbance cues that triggered avoidance behaviour in their conspecifics.

The knowledge gained about chemical communication in parental care thus far, was used to further investigate a possible provisioning behaviour in *R. variabilis*. In-situ pool-choice experiments with chemical cues of conspecific tadpoles were carried out throughout the change from rainy to dry season (**Chapter V**). With a changepoint analysis, the exact seasonal change was defined and differences between frogs' choices were analysed. It turned out that *R. variabilis* does not avoid but prefer conspecific cues during the dry season for tadpole depositions, what might be interpreted as a way to provide their tadpoles with food (i.e. younger tadpoles) in order to accelerate their development when facing desiccation risk.

That tadpoles were also occasionally fed with fertilized eggs could be shown in a comparative study, where phytotelmata that contained a tadpole deposited by the frogs themselves received more clutch depositions than freshly erected artificial phytotelmata containing unfamiliar tadpoles (i.e. their chemical cues; **Chapter VI**). Conducting home range

calculations with ArcGIS, it turned out that *R. variabilis* males showed unexpectedly strong site fidelity, leading to the suggestion that they recognize their offspring by phytotelmata location.

However, in order to test if *R. variabilis* is furthermore able to perform chemical offspring recognition, frogs were confronted in in-situ pool-choice experiments with chemical cues of single tadpoles that were found in their home ranges (**Chapter VII**). Genetic kinship analyses were conducted between those tadpoles emitting the chemical cues and those deposited together with or next to them. The results, however, indicated that frogs did not choose to deposit their offspring with or without another tadpole due to relatedness, i.e. kin recognition by chemical cues could not be confirmed in *R. variabilis*.

# Zusammenfassung

Chemische Kommunikation ist das evolutionär älteste Kommunikationssystem im Tierreich, das intra- und interspezifische Wechselwirkungen auslöst. Es wird vom Senderindividuum eingeleitet, das einen chemischen Stoff bzw. ein chemisches Signal abgibt, welches eine Reaktion beim Empfänger auslöst. Im Vergleich zu anderen Tiergruppen gibt es bezogen auf Anuren nur verhältnismäßig wenige Studien über die Bedeutung von chemischer Kommunikation. In dieser Arbeit werden die Auswirkungen von chemischer Kommunikation auf das Verhalten des Pfeilgiftfrosches *Ranitomeya variabilis* (Dendrobatidae) insbesondere im Hinblick auf die Brutpflege untersucht. Diese Art nutzt sowohl für die Eiablage als auch für das Absetzen von Kaulquappen Phytotelmen (kleine Wasseransammlungen in Pflanzen). Da die Kaulquappen kannibalistisch sind, meiden die adulten Frösche konspezifische Larven nicht nur bei der Eiablage, sondern sie transportieren auch ihre Kaulquappen einzeln in separate Phytotelmen. Die Erkennung, ob ein Phytotelm bereits besetzt ist, erfolgt mithilfe von von den Kaulquappen abgegebenen chemischen Substanzen.

Um herauszufinden, ob *R. variabilis* in der Lage ist, Kaulquappen generell zu erkennen und zu meiden, wurden in situ Experimente durchgeführt, bei denen den Fröschen chemische Substanzen von Kaulquappen verschiedener Froscharten angeboten wurden (**Kapitel 1**). Es stellte sich heraus, dass die Frösche dazu fähig waren die Substanzen von allen getesteten Arten zu erkennen und bei der Eiablage zu meiden. Beim Absetzen der Kaulquappen hingegen wurden nur andere Pfeilgiftfroschkaulquappen gemieden, die ebenfalls in Phytotelmen vorkommen, während Kaulquappen, die in Flüssen leben keine Meidung erfahren haben. Außerdem wurden die chemischen Substanzen der Kaulquappen einer Laubfroschart (Hylidae) von *R. variabilis* zwar erkannt, jedoch nicht gemieden, sondern bevorzugt. Dies könnte bedeuten, dass diese Kaulquappen als potentielle Beute für die predatorischen Pfeilgiftfroschlarven erkannt wurden.

Eine der Pfeilgiftfroscharten, die sowohl für die Eiablage als auch für das Absetzen der Kaulquappen gemieden wurde, war die in Phytelmen brütende Art *Hyloxalus azureiventris*. Die chemischen Substanzen, die von den Kaulquappen dieser Art abgegeben werden, wurden gemeinsam mit denen der Kaulquappen von *R. variabilis* genauer analysiert (**Kapitel II**). Nachdem ein passendes Extraktionssorbens gefunden wurde (DSC-18), wurden die aktiven

chemischen Substanzen aus dem Kaulquappenwasser extrahiert und fraktioniert. Um herauszufinden, welche der Fraktionen das Meidungsverhalten bei den Fröschen auslöst, wurden in situ Biotests durchgeführt. Dabei stellte sich heraus, dass die biologisch aktiven Substanzen sich bei den beiden Arten unterscheiden. Da die Meidung der konspezifischen Kaulquappen für selbige nicht von Vorteil ist (da sie so um eine potentielle Futterquelle gebracht werden), können die von ihnen abgegebenen Substanzen nur als chemische Stoffe, nicht als Signale definiert werden. Bei den heterospezifischen Kaulquappen kann man hingegen von chemischen Signalen sprechen, da es sich den Ergebnissen der chemischen Analyse zufolge nicht um ein taxonomisch bedingtes Beiprodukt handelt, dass alle Pfeilgiftfrösche gemein haben, sondern um einen artspezifischen Stoff, der den Kaulquappen zum Vorteil gereicht. Da *R. variabilis* jedoch ebenfalls einen Vorteil von der Meidung der heterospezifischen Kaulquappen hat (da es sich hierbei um Konkurrenten für ihren Nachwuchs handelt) kann man hier von Synomonen sprechen, interspezifisch agierende Botenstoffe, die sowohl für den Sender, als auch für den Empfänger von Vorteil sind.

Eine weitere hier untersuchte Form von interspezifischer Kommunikation ist die Meidung von Predatorenkairomonen (**Kapitel III**). Es konnte gezeigt werden, dass *R. variabilis* unfähig ist Libellenlarven anhand von chemischen Substanzen zu erkennen, obwohl diese Kaulquappenpredatoren erkannt und gemieden werden, wenn sie physisch präsent sind. Für die Erkennung von konspezifischen Kaulquappen hingegen waren chemische Stoffe essentiell, da ausschließlich visuelle Kaulquappenmodelle nicht gemieden wurden.

Ob *R. variabilis* außerdem dazu fähig ist auf chemische Art und Weise mit adulten Artgenossen zu kommunizieren, wurde getestet, indem den Tieren chemische Substanzen von gleich- oder andersgeschlechtlichen Fröschen angeboten wurden (**Kapitel VI**). Es wurde angenommen, dass Männchen sich von den chemischen Substanzen der Weibchen angezogen fühlen, während solche von anderen Männchen sie abstoßen. Stattdessen stellte sich jedoch heraus, dass alle getesteten Individuen die chemischen Substanzen ihrer Artgenossen mieden. Eine mögliche Erklärung hierfür ist, dass die Behältnisse der Sendertiere zu klein waren und es so zu einer Abgabe von stressbedingten Substanzen gekommen ist, welche zur Meidung durch die Empfänger geführt haben.

Das erlangte Wissen über die Nutzung von chemischer Kommunikation im Brutpflegeverhalten von *R. variabilis* konnte für eine umfassendere Untersuchung der



Nachwuchsversorgung in dieser Art genutzt werden. In situ Experimente mit chemischen Stoffen von konspezifischen Kaulquappen wurden während des Wechsels von Regen- zu Trockenzeit durchgeführt (**Kapitel V**). Mithilfe einer „change point“ Analyse wurde der genaue Zeitpunkt des jahreszeitlichen Wechsels berechnet und die Unterschiede im Brutpflegeverhalten der Frösche untersucht. Es stellte sich heraus, dass *R. variabilis* in der Trockenzeit die chemischen Stoffe beim Absetzen der eigenen Kaulquappen nicht mied, sondern vielmehr bevorzugte. Dieses Verhalten kann als eine Art Nachwuchsverpflegung (Fütterung mit jungen Kaulquappen) interpretiert werden, die der Entwicklungsbeschleunigung bei steigender Austrocknungsgefahr dient.

Das Kaulquappen außerdem sporadisch mit befruchteten Eiern versorgt wurden, konnte in einer vergleichenden Studie gezeigt werden, in der Phytotelmen mit von den Fröschen selber abgesetzten Kaulquappen wesentlich häufiger zur Eiablage genutzt wurden als solche, die unbekannte Kaulquappen (bzw. deren chemischen Stoffe) enthielten (**Kapitel VI**). Bei der Berechnung der Aktivräume der Frösche in ArcGIS zeigte sich, dass die Männchen von *R. variabilis* eine unerwartet starke Ortstreue aufwiesen, was zu der Vermutung führt, dass die Tiere ihren Nachwuchs anhand der Lage der Phytotelmen erkennen.

Trotzdem wurde in einer weiteren Studie getestet, ob *R. variabilis* auch in der Lage ist, die eigenen Kaulquappen anhand von chemischen Substanzen zu identifizieren. Hierfür wurden den Fröschen in in situ Experimenten die chemischen Stoffe einzelner Kaulquappen aus dem eigenen Aktivraum angeboten (**Kapitel VII**). Genetische Verwandtschaftsanalysen zwischen diesen Kaulquappen, sowie denen, die von den Fröschen abgesetzt wurden, führten jedoch zu dem Ergebnis, dass *R. variabilis* den eigenen Nachwuchs nicht anhand von chemischen Stoffen erkennt und bevorzugt füttert.

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- 07/2009 – 02/2010**      Wissenschaftliche Hilfskraft (mit Abschluss) an der Umweltprobenbank des Fachbereichs VI der Universität Trier:  
Pilotstudie „Klimabedingte Allelveränderung in ausgewählten UPB-Probenarten“
- 01/2009 – 12/2011**      Redakteurin der Reptilien- und Amphibienzeitschrift "Elaphe" des DGHTs (Deutsche Gesellschaft für Herpetologie und Terrarienkunde e.V.)
- 01/2012 – 06/2012**      Wissenschaftliche Hilfskraft (mit Abschluss) an der Biogeographie des Fachbereichs VI der Universität Trier
- 07/2010 – 06/2013**      Promotionsstipendium durch die Studienstiftung des Deutschen Volkes
- 11/2009.12/2013**      Dissertation in der Biogeographie im Fachbereich VI,  
Betreuer: PD Dr. Stefan Lötters  
  
In Kooperation mit dem UFZ (Zentrum für Umweltforschung) Leipzig, Wirkungsorientierte Analytik, Dr. Werner Brack  
  
Titel: “Chemical communication in the reproductive behaviour of Neotropical poison frogs (Dendrobatidae)”

## Erklärung

Hiermit versichere ich, dass mir die derzeitige Promotionsordnung bekannt ist und dass ich die vorliegende Dissertation selbständig verfasst habe. Ich habe für die Arbeit keine anderen als die angegebenen Quellen und Hilfsmittel genutzt und die Ergebnisse anderer Beteiligter sowie inhaltlich und wörtlich aus anderen Werken entnommene Stellen und Zitate wurden als solche kenntlich gemacht. Die Arbeit hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen oder wurde von dieser als Teil einer Prüfungsleistung angenommen.

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Ort, Datum

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Unterschrift