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UNIVERSITY OF MIAMI

BEHAVIORAL ECOLOGY OF PARENTAL CARE IN A DENDROBATID FROG
(*OOPHAGA PUMILIO*)

By

Jennifer L. Stynoski

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

May 2012

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BEHAVIORAL ECOLOGY OF PARENTAL CARE IN A DENDROBATID FROG
(*OOPHAGA PUMILIO*)

Jennifer L. Stynoski

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Behavioral Ecology of Parental Care
In a Dendrobatid Frog (*Oophaga pumilio*)

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Parental care is essential to the success of both parents and offspring in many species, and plays an important role in the evolution of animal mating systems and life histories. The mechanisms that regulate the intensity and form of parental care in a species are determined by many factors and are highly variable across taxa. However, our understanding of the behavioral ecology of parental care is built on research that is highly taxonomically biased to birds, with some studies also in mammals, arthropods, and fish. Amphibians display an impressive diversity of form in parental care, yet few studies of parental care have explained the mechanisms behind parental behaviors in this vertebrate class. Accordingly, the research in this dissertation tested four hypotheses about parental care in a species of dendrobatid frog that displays complex parental behavior, the strawberry poison frog (*Oophaga pumilio*). In this species, father frogs guard egg clutches in the leaf litter for approximately one week. When eggs hatch into tadpoles, mother frogs return to transport tadpoles individually to small terrestrial water pools such as those in the axils of a bromeliad plant. For approximately six weeks, mother frogs continue to visit tadpoles. When mother frogs visit, tadpoles display a vibration behavior. Then, mother frogs supply unfertilized eggs to their tadpoles as their principal source of food.

To develop a thorough understanding of the behavioral mechanisms at play between mother and offspring *O. pumilio*, this dissertation tests hypotheses related to offspring discrimination, the use of multimodal sensory cues, honest signaling, and provisioning of potential chemical defenses. Studies mostly made use of behavioral tests and observations in the laboratory and in the field at the La Selva Biological Station in the lowland wet forest of Costa Rica. To facilitate observation of the interactions between mothers and tadpoles in a natural setting as well as easy access to large quantities of tadpoles, an extensive setup of 1000 artificial tadpole-rearing sites (“cups”) was installed on the trees in abandoned plantations adjacent to primary forest. Mother frogs used cups to deposit and feed tadpoles just as they would the natural phytotelmata in their habitat. Furthermore, motion-activated IR video cameras were installed at the site to permit 24-hour observation of mother-offspring interactions.

Findings demonstrated that mother frogs use indirect recognition to locate tadpoles, and rely on honest signals of hunger from tadpoles to allocate nutritive eggs. Tadpole begging signals are likely reliable because of a significant cost of signal production via reduction in growth and as a result of a differential benefit of maternal eggs. Tadpoles use visual and tactile cues to discriminate between mother frogs and other visitors such as other species of frogs and potential predators when deciding whether to signal their level of need or to avoid predation. The eggs that mother frogs provide tadpoles as nourishment may also be provisioned with alkaloids that could provide chemical defenses for offspring. Thus, the behavioral mechanisms described in this research explain how mother *O. pumilio* determine who, when, and how much to feed, as well as how both tadpoles and mothers contribute to reducing the risk of predation to offspring.

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Chapter 1: Introduction

In species with parental care, the combined success of parents, current offspring, and future offspring relies on the effective expression of parental behaviors (Clutton-Brock 1991, Wade & Shuster 2002). Effective parents should only direct parental behaviors at genetically related young, so they must be able to recognize their offspring and discriminate against unrelated young. Parents must also supply their offspring with ample resources that young need to develop and survive to independence. Doing so requires regular assessment of offspring status (hunger, thermal condition, predator risk, etc.) and selection of the appropriate form and intensity of parental behaviors. In general, parental care should be efficient both because it is costly in terms of increased energy expenditure and risk of predation and because it may limit a parent's ability to rear future offspring (Trivers 1974, Godfray 1995).

Interest in studying parental care and its regulation stems from the important role that parental investment plays in animal mating systems (Emlen & Oring 1977, Reynolds 1996), in the evolution of life histories (Roff 1992), and in sexual selection (Williams 1966, Trivers 1972, Kokko et al. 2002). Researchers have suggested that the life history, mating system, and/or pattern of sexual selection of a species determine the intensity and nature of its parental care (Reynolds 1996, Roff 1992, Kokko et al. 2002, Wade & Shuster 2002).

The expression of parental care is regulated by factors at several levels including endogenous factors such as neural and hormonal processes, social factors such as communication with offspring and competition with conspecifics, and ecological factors such as predators and climate. Not surprisingly given all of these influential factors,

parental care is highly diverse among and within taxa (Clutton-Brock 1991, Gross 2005). For that reason, it is important to study parental behavior and its regulation in a comparative context (Gross 2005).

Brooding birds and lactating mammals were the exemplary taxa for initially explaining how endogenous, social, ecological, and other factors influence the nature of parental care (Clutton-Brock 1991). In addition, some species of fish and arthropods have become useful models for studying parental care (Evans 1998, Neff 2003, Gross 2005, Kölliker et al. 2006). However, the regulation of parental care in amphibians has received little research attention and is thus poorly understood, especially in an experimental context (Wells 1981, Crump 1996, Lehtinen & Nussbaum 2003, Wells 2007). This lack of knowledge is surprising given the enormous diversity of forms of parental care in this clade, including parental attendance of eggs or larvae, transport of eggs, larvae, or froglets on the parent's body, and feeding of larvae (reviewed in Wells 2007). The goal of this dissertation is to expand our comparative understanding of parental care by studying behavioral mechanisms behind complex parental care in a dendrobatid frog.

Study Species

Oophaga [=Dendrobates] pumilio, or the strawberry poison frog, is a diurnal, aposematic species that sequesters toxic alkaloids in the skin after eating ants and mites (Saporito et al. 2004, Saporito et al. 2007a). Breeding occurs throughout the year, but shows some seasonality as more juveniles are found in wetter months (May-Dec; Donnelly 1989a, Pröhl 2005). Adult male *O. pumilio* defend territories for up to 4 years (1-2 m²; McVey et al. 1981, Robakiewicz 1992, Pröhl & Berke 2001, Bee 2003), and vocalize from perches to attract females and defend oviposition sites (Bunnell 1973,

Donnelly 1989b, Pröhl & Hödl 1999). Females maintain home ranges (4-20 m²) that overlap the territories of several males (Pröhl & Berke 2001, Bee 2003). Eggs are fertilized in leaf litter on the forest floor, and egg clutches are guarded and hydrated by fathers for 7-12 days (Weygoldt 1980, Brust 1993, Haase & Pröhl 2002). Once eggs develop into tadpoles (stage 25; Gosner 1960), mothers return to their clutch and transport each tadpole singly to its own water-filled axil in a water-holding plant such as a bromeliad (Weygoldt 1980, Brust 1993, Donnelly 1989b). Then, mothers return to each tadpole at 1-8 day intervals for approximately 6 weeks to provision them by laying unfertilized eggs into the water (Brust 1993). Maternal provisioning is obligatory for tadpole survival, as tadpoles only grow when eating eggs (Brust 1993). Mothers maintain only one concurrent clutch (Weygoldt 1980, Brust 1993, Haase & Pröhl 2002, Pröhl 2005). Different mothers use different axils of the same plants for rearing unrelated offspring (Weygoldt 1980, Haase & Pröhl 2002). Mothers move among plants, especially before depositing tadpoles (Brust 1990). When mothers visit tadpole-occupied axils, tadpoles exhibit a vibration behavior that is thought to facilitate egg laying by mothers (Weygoldt 1980).

The research described in this dissertation was conducted at the La Selva Biological Station in Costa Rica. La Selva consists of primary and secondary lowland tropical wet forest, and receives ~4 m of precipitation annually (Sanford et al. 1994). The Huertos Plots at La Selva are abandoned plantations that provide a semi-natural infrastructure of trees in rows adjacent to primary forest. An extensive field setup of artificial tadpole rearing sites (termed “cups”) was constructed in the plots that allows mother frogs to deposit and care for offspring in their natural habitat while being observed. Mother frogs

regularly deposit and rear their tadpoles in these cups as they would in natural bromeliads (Stynoski 2009, Stynoski & Noble 2012).

Objectives

This dissertation aims to elucidate the behavioral ecology of parental care in *Oophaga pumilio* by exploring the mechanisms by which mothers discriminate between, communicate with, allocate resources to, and defend offspring. The research tested 4 hypotheses: (1) mothers discriminate between offspring and unrelated young during parental care, (2) the sensory mode of mother-offspring communication is suited to the habitat of the species, (3) mothers use honest begging signals from offspring to allocate their provisioning resources, and (4) mothers provision offspring with an effective defense against predators. Each hypothesis corresponds to one of the following chapters.

In chapter 2, I discuss the mechanism by which mother frogs avoid feeding unrelated offspring and how they target their own young when supplying eggs. By placing “cups” in pairs and moving tadpoles between cups, I was able to manipulate either the identity or the location of a mother frog’s deposited tadpole. Then, by measuring the subsequent growth of tadpoles, I determined whether mothers continued to feed unrelated tadpoles or their own tadpoles with an adjusted location. I also offered mother frogs a choice between their own tadpole in an adjacent location and an unrelated tadpole in their originally chosen location. Because offspring recognition and discrimination sometimes do not occur until after a certain period of time interacting has passed between the mother and offspring, I also performed an experiment in which tadpole identity was manipulated after mothers had reared a given tadpole for 10 days.

In chapter 3, I investigated the way that tadpoles direct their begging behavior so that they only vibrate at the arrival of adult *O. pumilio* as opposed to other visitors. The vibration behavior that tadpoles perform to facilitate egg laying by mother frogs is conspicuous and extended. If the visitor to their water pool is not, in fact, a mother frog but a potential predator or a heterospecific frog, it could be dangerous or costly to perform the vibration behavior. Thus, using laboratory behavior trials that isolated different sensory cues during mother-offspring interactions, I determined the sensory mode(s) by which tadpoles identify a visitor and choose the appropriate response: vibrating, ignoring the visitor, or freezing in place.

In chapter 4, to determine whether mother frogs use the begging signals of tadpoles to allocate egg resources, I tested three conditions of theoretical models for the maintenance of reliable signals in a begging communication system. First, I determined whether tadpole vibration behavior is limited to when tadpoles are truly hungry by starving tadpoles for various lengths of time and then exposing them to mother frogs and quantifying their vibration behavior. Similarly, I determined whether begging behavior varies across development in the tadpole phase, since presumably tadpoles have different nutritional needs at different times and this should be reflected in an honest signal of need. Then, I conducted field observations of mother visits to tadpoles (using video cameras) to establish whether mother frogs respond to variation in tadpole vibration behavior when supplying eggs. Lastly, I performed an experiment to test the theoretical concept that a sufficient cost of signal production (along with a sufficient differential benefit of the receiver's response) exists that can maintain honesty in a communication system and prevent the signaler from falsely magnifying their needs. To do so, I

compared the growth of tadpoles that were encouraged to beg for a biologically relevant period of time to the growth of tadpoles that were fed the same amount of food but not coerced to beg.

In chapter 5, I investigated the possibility that mother frogs provision tadpoles in the post-hatching phase with the alkaloids that they themselves use as a chemical defense against predators. I collected a full age-series of *O. pumilio* and (with collaborator R.A. Saporito) identified and quantified any alkaloids found in the samples. The appearance of alkaloids in tadpoles would suggest that mother frogs are supplying alkaloids to tadpoles via nutritive eggs because tadpoles do not eat the arthropods from which adult frogs sequester these chemical defenses. To test for further evidence of post-hatching chemical provisioning by mother frogs, we also performed chemical analysis on tadpoles that we collected within a few hours of mother deposition, reared in the laboratory, and fed with eggs of another species of frog that is not known to contain alkaloids.

This dissertation provides novel empirical evidence using an amphibian species to test hypotheses in behavioral ecology related to offspring discrimination, multimodal sensory integration, and honest signaling that have thus far been studied in limited taxonomic groups. The research explores the ways that mother frogs and tadpoles receive signals and respond to them in their complex habitat in the lowland rainforest. It also provides the first evidence of an animal that supplies its offspring with chemical defenses after birth or hatching. Taken together, these chapters provide a clear view of how *O. pumilio* mothers and offspring interact among themselves and with their environment so that mother frogs can reach the goal of any good mother: keep the kids well fed and safe.

Chapter 2: Discrimination of offspring by indirect recognition¹

Summary

Offspring discrimination - the differential treatment of offspring and unrelated young - functions in numerous animal taxa to ensure that vital and costly parental care behaviors are appropriately directed. Discrimination can be facilitated either by direct (phenotypic) recognition of offspring or by indirect (nonphenotypic) recognition of offspring location. Offspring discrimination and recognition mechanisms have not been identified in an amphibian. In the strawberry poison frog, *Oophaga pumilio*, a dendrobatid frog with obligatory maternal provisioning behavior, I tested whether mothers discriminate between offspring and unrelated young, whether they use direct or indirect recognition cues, and whether prior parental investment plays a contextual role in the differential treatment of young. Mother frogs utilized tadpole-rearing cups attached to tree trunks in a wet tropical forest. After manipulating the identity, location, and/or age of tadpoles in cups, I determined whether maternal provisioning behavior was maintained by measuring tadpole growth and development. Mothers provisioned young regardless of tadpole identity, but were sensitive to location and did not provision tadpoles moved two centimeters to an adjacent cup. When given a choice between related and unrelated tadpoles in originally chosen or adjacent cups, mothers discriminated by location, but not relatedness. Maternal provisioning behavior persisted when a tadpole provisioned for 10 days was replaced with either an age-matched or newly hatched unrelated tadpole, so

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direct offspring recognition does not appear to be dependent on prior parental investment. Together, these results provide strong evidence that mother *O. pumilio* utilize indirect recognition cues to discriminate between offspring and unrelated offspring.

Background

In species that exhibit parental care, the ability to discriminate between offspring and unrelated young has strong and obvious implications for fitness. The success of a parent's offspring is directly related to the quality and amount of care that the offspring receive (Hepper 1986). Also, parental care behaviors are costly to parents and their future offspring, and these costs are fruitless if care is directed at unrelated young (Trivers 1972, Queller 1997, Duckworth et al. 2003). Thus, to ensure that care is beneficial, parents from diverse taxa modulate the intensity and the target of their care according to the genetic relatedness of young. For example, in bluegill sunfish, *Lepomis macrochirus*, fathers adjust the intensity of care on the basis of the prevalence of cuckolding males and on the familiarity of offspring chemosensory cues (Neff 2003). Mother subantarctic fur seals, *Arctocephalus tropicalis*, forage at sea for weeks, and return to provision their offspring after recognizing their pups' voices among hundreds of others (Charrier et al. 2002). Mother crab spiders, *Diaea ergandros*, maintain nests with both related and unrelated young, but only catch prey and produce trophic oocytes for their own offspring (Evans 1998).

For parents to discriminate on the basis of the degree of genetic relatedness of young, a recognition mechanism must be present (Sherman et al. 1997, Tsutsui 2004). Recognition mechanisms can indicate relatedness in one of two ways. In direct

recognition mechanisms, parents employ a phenotypic component of the young (Waldman 1987). The phenotypic cues that are used to recognize offspring can vary widely in modality, from chemosensory (Greenberg 1979, Main & Bull 1996, Evans 1998, Yamazaki et al. 2000, Neff & Sherman 2005) to auditory (Espmark 1971, Jouventin et al. 1999, Charrier et al. 2002, Searby & Jouventin 2003, Knörnschild & Von Helversen 2008) and less often to visual (Lahti & Lahti 2002) modes. In indirect recognition mechanisms, on the other hand, parents make use of a contextual cue such as spatial location or frequency of encounters with young (Waldman 1987). Indirect mechanisms are most commonly used when offspring are not likely to move from where parents have left them, or when offspring are not likely to be confused with unrelated young in an adjacent location (Waldman 1987, Sherman et al. 1997). In these cases, parents use spatial, chemosensory, or visual cues that are derived from the nest itself (Lank et al. 1991, Bonadonna et al. 2003, Neff 2003, Chiu & Kam 2006), or depend on temporal cues, such as in several rodent species that cease infanticidal behaviors while nursing (Elwood 1991). Both direct and indirect mechanisms can signify relatedness (Hamilton 1964), and thus both can mediate the differential treatment of young.

Kin discrimination and recognition mechanisms are known to occur in amphibian species (Blaustein & Waldman 1992), but have not been demonstrated in the context of post-hatching parental care (Gibbons et al. 2003). Amphibian discrimination and recognition have been examined almost exclusively in the contexts of groups of sibling tadpoles (Blaustein & Waldman 1992), cannibalism of larval non-siblings over siblings and first cousins (Pfennig et al. 1993, Pfennig et al. 1994), and cannibalism of unrelated young over offspring (Forester et al. 1983, Gabor 1996, Peterson 2000, Gibbons et al.

2003). Poelman & Dicke (2007) examined but did not find evidence of an ability to discriminate offspring from unrelated young in a poison frog, *Dendrobates ventrimaculatus*. However, another poison frog, *Oophaga* [formerly *Dendrobates* (Grant et al. 2006)] *pumilio* might be more likely than *D. ventrimaculatus* to discriminate between offspring and unrelated young because it exhibits more extreme parental investment that includes provisioning of offspring (Brust 1993). In addition, male *D. ventrimaculatus* defend the territories in which they rear their tadpoles (Poelman & Dicke 2007), whereas female *O. pumilio* rear tadpoles in a social environment with a greater overlap of home ranges and compete with other females for the same tadpole-rearing sites (Pröhl & Hödl 1999, Pröhl & Berke 2001, Haase & Pröhl 2002). For these reasons I decided to investigate whether female *O. pumilio* are able to discriminate between offspring and unrelated young, whether they use direct or indirect recognition cues, and whether prior parental investment plays a role in differential treatment of young.

Moreover, kin recognition is often context-dependent, such that its expression varies with the costs and benefits of discrimination (Sherman et al. 1997). Thus, kin recognition is often expected to vary with environmental conditions (Blaustein & Waldman 1992, Holmes & Mateo 2007). Therefore, I studied offspring recognition in a free-living population of *O. pumilio* at La Selva Biological Station in Costa Rica, where the natural environment is more variable than a laboratory setting.

In *O. pumilio*, eggs are fertilized in leaf litter on the forest floor, and egg clutches are guarded and hydrated by fathers for seven to 12 days (Limerick 1980, Weygoldt 1980, Brust 1993, Haase & Pröhl 2002). Once eggs develop into tadpoles, mothers return to the clutch to transport each tadpole individually to its own water-filled axil in a

bromeliad or other water-holding plant, depositing only one tadpole in each axil (Brust 1993, Maple 2002). Then, mothers return to each tadpole every one to eight days for approximately six weeks to provision them by laying unfertilized eggs into the water (Brust 1993). Even though mothers do not maintain more than one concurrent clutch of offspring (Weygoldt 1980, Brust 1993, Haase & Pröhl 2002, Pröhl 2005), tadpoles of vastly different sizes and stages are found in different axils of the same bromeliads (Weygoldt 1980, personal observation), and different mothers are seen caring for offspring in the same plants (Haase & Pröhl 2002, personal observation). Together these observations suggest that mother *O. pumilio* need to discriminate regularly between offspring and unrelated young. Indeed, mothers spend a significant amount of time searching bromeliads, and always do some searching before depositing nutritive eggs (Brust 1990). Searching behavior may allow mothers to distinguish among axils or among tadpoles.

In the present field study, I tested if and how mother *O. pumilio* discriminate between offspring and unrelated young when provisioning. In a series of three experiments, I determined whether mothers use either tadpole phenotypic cues or spatial location to recognize their young (recognition experiment), the relative importance of indirect and direct recognition cues in offspring discrimination during provisioning (paired discrimination experiment), and whether size and/or age play a role in offspring recognition (post-parental investment recognition experiment). The post-parental investment recognition experiment was necessary because offspring recognition may not occur until after parents have made some investment in their offspring (Lefevre et al. 1998, Mateo 2006).

Methods

This study was conducted with *O. pumilio* at La Selva Biological Station in Costa Rica. The station consists of primary and secondary lowland tropical wet forest, and receives approximately 4 m of rain annually, with slightly more precipitation occurring from May to December than in other months (Sanford et al. 1994). Experiments took place in the abandoned Huertos plantations, a site where *O. pumilio* is very abundant and actively reproduces (Donnelly 1989a), and where rows of large trees provide an organized substrate (Haggar & Ewel 1995). The environmental characteristics of the plantations mimic those of the natural habitat of *O. pumilio* and population densities are similar to those in the adjacent secondary forests at La Selva.

Oophaga pumilio is a diurnal and aposematic litter frog (19-22 mm) ranging from Nicaragua to Panama that is not harmful to humans when handled, but is often rejected by predators (Grant et al. 2006, Saporito et al. 2007b). Both sexes are polygamous, and males defend long-term territories and attract females by calling from perches (Pröhl & Hödl 1999, Limerick 1980). Both sexes show extensive parental care: males guard and hydrate egg clutches within their territories, whereas females transport and then provision tadpoles in water-holding plants (Weygoldt 1980, Brust 1993). Tadpoles are deposited and provisioned in axils of water-holding plants individually; if tadpoles are deposited in multiples, cannibalism occurs (Brust 1990).

In this study, tadpole-rearing cups were used in three experiments. During experiments, tadpoles that were deposited in these cups by a female frog are assumed to be the offspring of that female. Behavioral observations were conducted to provide

evidence that the same female that transported a tadpole is the future source of nutritive eggs for that tadpole. On 10 occasions, mothers observed depositing tadpoles were marked using uniquely colored orthodontic bracket ties (Orthodontic Supply & Equipment Co., Inc.) on the hind legs that fit loosely below the knee and above the calf muscle. In the days following banding, I found mothers each morning and observed them from 0700-1100 hours daily until eggs were deposited in the cup containing the originally deposited tadpole. Female *O. pumilio* only provision their offspring during this time period (Haase & Pröhl 2002). In addition, I considered tadpoles deposited in cups greater than 20 m from each other as being unrelated, because females are reported to have a home range of $X \pm SE = 9.9 \pm 3.0 \text{ m}^2$ in La Selva (Donnelly 1989c) and $27.6 \pm 11.4 \text{ m}^2$ elsewhere in Costa Rica (Haase & Pröhl 2002) and thus are not likely to deposit tadpoles at such a distance from each other. Tadpoles used in experiments were generally found at least 100 m apart. While it would have been ideal to conduct genetic analysis and verify mother-tadpole relationships, such analysis was considered to be outside the scope of the field study.

Prior to the experiments, tadpole-rearing cups (N = 800) made from 15 ml clear polypropylene beakers (VWR, Batavia, IL) were placed in the forest. To prevent flushing of tadpoles from the tops of cups by rain, six small holes were drilled in the sides of each cup approximately 2 cm below the top rim such that cups were generally half full. Cups were affixed to vertically aligned tongue depressors with zip ties to provide a stable base to rest against trees. After removing the natural bromeliads within reaching height, I tied cups in pairs with yarn or monofilament (~2 cm apart, 1-1.5 m high; Figure 2.1A) to trees greater than 15 cm in diameter in order to mimic the natural situation of two adjacent

axils of a bromeliad. Natural bromeliads at this site typically have between 2 and 7 water-filled axils per plant (Maple 2002). Mother frogs were allowed to place their tadpoles in one of these two cups and return to them repeatedly for provisioning without human interference. All cups were checked daily between 1300-1600 hours for the presence of new or old tadpoles and eggs from June 5, 2008 to October, 23 2008, with the exception of August 7, 2008 because a flash flood restricted researcher access to the forest. During daily cup checking, cups with low water levels were refilled with previously collected rainwater, and excess debris was removed. After a mother frog placed a tadpole in one of the two cups of a pair (Figure 2.1B), that tadpole was randomly assigned to one of the seven groups described in the experiments below. Any tadpoles I moved during manipulations were moved with the water (and possible chemical cues) in their cup. Deposited tadpoles were monitored until no longer in the cup. I noted whether tadpole disappearance was a result of metamorphosis, death (carcass visible in cup), predation (tadpole missing but cup water and debris is unaffected), or splash from direct raindrops or canopy drips (tadpole, debris, and water in cup are missing). On the rare occasion (N = 5) that a tadpole was deposited in a cup adjacent to an existing experimental tadpole, it was removed the same day to prevent interference with provisioning of the existing tadpole.

The continuation of maternal provisioning was determined by quantifying tadpole growth and by observing changes in developmental stages (Gosner 1960) every other day for 19 days in the first two experiments and for 10 days in the third experiment. To improve precision of growth measurements in the field, I determined the total body length of tadpoles with digital photograph analysis. Using a 3 ml disposable wide pipette

with the tip removed, I placed tadpoles in a Petri dish aligned in the same plane as a ruler and took a photograph with a digital SLR camera (Canon Rebel XTI) on a tripod. During frequent rainstorms, photos were taken under a large umbrella. Then, using the measure tool in the software program (ImageJ 1.40g, NIH), I measured tadpole lengths in photographs after standardizing the relative pixel distance to 1 cm on the ruler in each image.

In the first experiment, I manipulated tadpole position and identity to determine which recognition cues are used by mothers when making choices about provisioning. Upon deposition, tadpoles were assigned to one of three treatment groups ($N = 12$). In the identity group, tadpoles were replaced with an age- and size-matched tadpole from at least 20 m away. In the location group, tadpoles were moved 2 cm to the other cup in a pair of cups on a given tree. In the control group, the existing tadpole was merely picked up and returned.

In the second experiment, I examined the relative importance of location and identity cues in discrimination between offspring and unrelated offspring. Upon deposition, tadpoles were assigned one of four treatment groups ($N = 13$). For half of the tadpoles, the offspring placed into a cup by a mother was moved to the adjacent cup in a pair of cups on a tree, and an unrelated tadpole of the same age was put in its place. For the other half of the tadpoles, the offspring was merely picked up and returned, and an unrelated tadpole was placed in the adjacent cup.

In some taxa, mechanisms of offspring recognition are not used until some minimum level of parental investment occurs (Lefevre et al. 1998, Mateo 2006); in the third experiment, I tested whether mother frogs can recognize their offspring after

providing 10 days of care. Ten days after the initial tadpole deposition, tadpoles ($N = 12$) were replaced with either a one-day-old unrelated tadpole or a 10-day-old unrelated tadpole.

Parametric statistics were used because F-tests for equality of variances of groups showed no significant differences (all $P > 0.05$). For the first two experiments, I compared tadpole growth with univariate, repeated measures ANOVAs. Tukey HSD tests were used post-hoc to determine differences between individual groups. In the third experiment, paired t-tests were used to compare the growth of 1 day-old and 10 day-old tadpoles that replaced related offspring to the growth of unmanipulated tadpoles of the same age (either from one to nine days old or from 10 to 19 days old, respectively). One-way ANOVAs were used to compare changes in developmental stages, the number of days with eggs present in a cup, and the days of survival for the first two experiments, and changes in developmental stages after manipulations in the third experiment. Also, a multiple regression was used to determine whether a correlation exists between daily rainfall, average temperature, maximum temperature, minimum temperature, or relative humidity and the number of tadpoles deposited in cups on a given day. All data were analyzed in StatView 4.0 (SAS Institute Inc., Cary, NC, U.S.A.).

Results

Tadpoles in the recognition experiment that had been relocated 2 cm adjacent to where mothers had placed them into tadpole-rearing cups (see Figure 2.1A) differed after 19 days of cup occupancy from control tadpoles in growth, developmental stage progression (Gosner 1960), and the number of days with nutritive eggs present (Table

2.1; Figure 2.2). Unrelated tadpoles that were exchanged with a mother-deposited tadpole but were not relocated did not differ from control tadpoles in these three characteristics (Table 2.1; Figure 2.2).

In the paired discrimination experiment, when tadpoles differed in whether they occupied the mother's originally chosen location or the adjacent location, they also differed in growth, developmental stage progression, and the number of days with nutritive eggs present (Table 2.2; Figure 2.3). However, when related and unrelated tadpoles occupied the same relative location (either the mother's originally chosen location or an adjacent location), they did not differ in these characteristics (Table 2.2; Figure 2.3). In no case were differences dependent on whether tadpoles were related to mothers or not (Table 2.2; Figure 2.3).

Tadpole growth and developmental stage progression in the post-parental investment recognition experiment did not differ from that of unmanipulated tadpoles when tadpoles were replaced after 10 days of cup occupancy by either non-age-matched tadpoles (one day-old; growth, Paired t test: $t_{12} = 0.941$, $P = 0.342$; stages, Paired t test: $t_{12} = 0.861$, $P = 0.363$; Figure 2.4) or age-matched tadpoles (10 day-old; growth, Paired t test: $t_{12} = 0.180$, $P = 0.675$; stages, Paired t test: $t_{12} = 0.240$, $P = 0.629$; Figure 2.4).

Throughout the study, 196 tadpoles were deposited into tadpole-rearing cups, and of those, 51 were predated (26% as compared to 68% predation in natural bromeliads; Maple 2002), 62 were splashed out by raindrops, 62 died, and 21 metamorphosed. Metamorphosis took an average of 45 days (range: 41-56). The duration of survival for tadpoles in recognition and discrimination experiments did not differ between groups (recognition, ANOVA: $F_{2,33} = 0.292$, $P = 0.748$; discrimination, ANOVA: $F_{3,48} = 0.987$,

$P = 0.406$); this was likely the result of the relatively large within group variance caused by external factors such as predation and splash. Tadpoles measured on the day of deposition in a cup had a total length $X+SE = 9.66+0.78$ mm, $N = 196$, range 6.3-11.9. Control tadpoles progressed through an average of $X+SE = 2+2.6$, $N = 12$ developmental stages in 19 days, and in that time, eggs were found in their cups an average of $X+SE = 8+4.2$, $N = 12$ days. The number of days that eggs were found in a cup is not the same as the number of eggs that a tadpole was fed because it was difficult to determine whether eggs were freshly deposited or remained from the previous day. Moreover, eggs were sometimes eaten within a short interval after provisioning. During observations of marked females depositing and later provisioning tadpoles in cups ($N = 10$), females deposited eggs in the same cup in which they had previously deposited a tadpole within $X+SE = 2.5+1.3$ days. In no case was an unmarked female seen in the cup with a marked female's tadpole, and nutritive eggs were never seen in tadpole cups before observation of a marked female's first nutritive egg deposition. In four and a half months of cup checking, nutritive eggs were never found in cups that did not contain a tadpole. A multiple regression showed no combined correlation or individual correlation between average temperature, maximum temperature, minimum temperature, or relative humidity and the number of tadpoles deposited in cups on a given day (Multiple regression: $F_{5,94} = 1.889$, $P = 0.104$, $R^2 = 0.091$). However, a correlation did exist between the amount of daily rainfall and the number of tadpoles deposited (Linear regression: $F_{1,98} = 5.250$, $P = 0.024$, $R^2 = 0.225$).

Discussion

During provisioning, mother *O. pumilio* discriminated between offspring and unrelated young using indirect spatial recognition, but not direct recognition.

Provisioning of tadpoles continued both when tadpoles were picked up and returned and when they were exchanged with an unrelated tadpole, but not when they were moved to an adjacent tadpole-rearing cup. When given a choice between provisioning unrelated and related tadpoles in either original or adjacent cups, mothers chose to provision the tadpole in the original location regardless of tadpole identity. Furthermore, if mothers' tadpoles were replaced on day 10 with either one-day-old unrelated tadpoles or 10-day old unrelated tadpoles, maternal provisioning was not interrupted, suggesting that mothers do not recognize offspring even after a period of parental investment has taken place. These conclusions assume that a female provisioning a tadpole was indeed its genetic mother.

These findings suggest that mother *O. pumilio* do not discriminate between offspring and unrelated tadpoles using direct recognition of phenotypic cues of offspring. However, previous studies have shown that the ability to recognize offspring is often context dependent (Beecher 1991, Gibbons et al. 2003), and the results of the present study of *O. pumilio* do not prove that mothers of this species cannot recognize offspring. Nonetheless, because the study was conducted in the natural habitat of *O. pumilio*, and because offspring age, size, location, and chemosensory cues were accounted for and did not appear to influence maternal behaviors, it is clear that indirect recognition determined by a location cue is the primary mechanism used in offspring discrimination during

provisioning in this amphibian. The spatial accuracy with which mother *O. pumilio* appear to navigate their tadpole-rearing environment is noteworthy. The capacity of *O. pumilio* to navigate spatially is also supported by the demonstration that individuals are able to return to their territory or home range following experimental displacement (McVey et al. 1981).

Indirect rather than direct recognition is the likely mechanism for offspring discrimination in female *O. pumilio* because tadpoles are not capable of moving from one bromeliad axil to another (Waldman 1987, Sherman et al. 1997). Thus, indirect spatial cues are presumably reliable enough to ensure that mothers are provisioning their own offspring. However, both phenotypic and nonphenotypic cues appear to play a role in maternal care in this system; mothers may also use tadpole behavior as an auxiliary measure to verify that they are provisioning in the appropriate location. In *O. pumilio*, tadpoles perform a vibration behavior when an adult frog approaches their axil (Weygoldt 1980, personal observation), and this behavior appears to stimulate egg deposition into the axil. In both the present study and in a laboratory study (Weygoldt 1980), eggs were never found in cups or axils that did not contain tadpoles or that contained dead tadpoles. Yet, the indirect recognition mechanism shown by *O. pumilio* mothers leaves open the possibility for intraspecific reproductive parasitism, in which a female might consume the existing tadpole in an axil and deposit her own in its place to be fed by the other tadpole's mother. Adult *O. pumilio* consume unrelated eggs and perhaps tadpoles (Weygoldt 1980, Haase & Pröhl 2002), and in this study, mothers still provided eggs when their 10-day-old tadpoles were replaced with one-day-old tadpoles. Reproductive parasitism is speculated to occur in *O. pumilio* females (Haase & Pröhl 2002) and occurs in the related

D. ventrimaculatus (Summers 1999). Nothing is yet known about the relatedness of adult and young individuals in *O. pumilio* populations, but the hypothesis on reproductive parasitism would benefit from information on genetic relatedness.

Table 2.1 Results of ANOVAs in a recognition experiment

Factor(s)		Tadpole Growth	Developmental Stage	Number of Days with Eggs
Whole Model		$F_{2,33} = 6.176$; $P = 0.005$	$F_{2,33} = 8.458$; $P = 0.001$	$F_{2,33} = 13.811$; $P < 0.0001$
Location	Control	$P = 0.0002$	$P = 0.001$	$P = 0.0003$
Location	Identity	$P < 0.0001$	$P = 0.0009$	$P < 0.0001$
Identity	Control	$P = 0.668$	$P = 0.823$	$P = 0.343$

Tadpoles ($N = 12$) were either picked up and returned (control), replaced with an unrelated tadpole (identity), or moved to a location 2 cm adjacent to where they were originally placed by a mother frog (location). The bottom three rows indicate results of post-hoc tests between these groups.

Table 2.2 Results of ANOVAs for effects of tadpole relatedness and location in a paired discrimination experiment

Factor(s)			Tadpole Growth	Developmental Stage	Number of Days with Eggs
Whole Model			$F_{3,48} = 12.816$; $P < 0.0001$	$F_{3,48} = 24.574$; $P < 0.0001$	$F_{3,48} = 6.362$; $P = 0.001$
<i>Location:</i>	Original	Adjacent			
<i>Tadpole:</i>	Related	Unrelated	$P < 0.0001$	$P < 0.0001$	$P = 0.011$
	Related	Related	$P < 0.0001$	$P < 0.0001$	$P = 0.006$
	Unrelated	Related	$P < 0.0001$	$P < 0.0001$	$P = 0.001$
	Unrelated	Unrelated	$P < 0.0001$	$P < 0.0001$	$P = 0.002$
	Related, Unrelated		$P = 0.779$	$P = 0.484$	$P = 0.511$
		Related, Unrelated	$P = 0.294$	$P = 0.617$	$P = 0.834$

Tadpoles ($N = 13$) were either unrelated or related to mothers, and lived individually in either the original mother-chosen location or 2 cm adjacent to the original location. The bottom six rows indicate results of post-hoc tests between these four groups.



Figure 2.1 Field setup of tadpole-rearing cups, which mimics two adjacent natural bromeliad axils, and thus requires that mothers make the same choices they would in a natural bromeliad. Photo A: Pair of cups tied to a tree with a bathing male *Oophaga pumilio*. Photo B: Mother *O. pumilio* with a recently deposited tadpole below her forelimb.

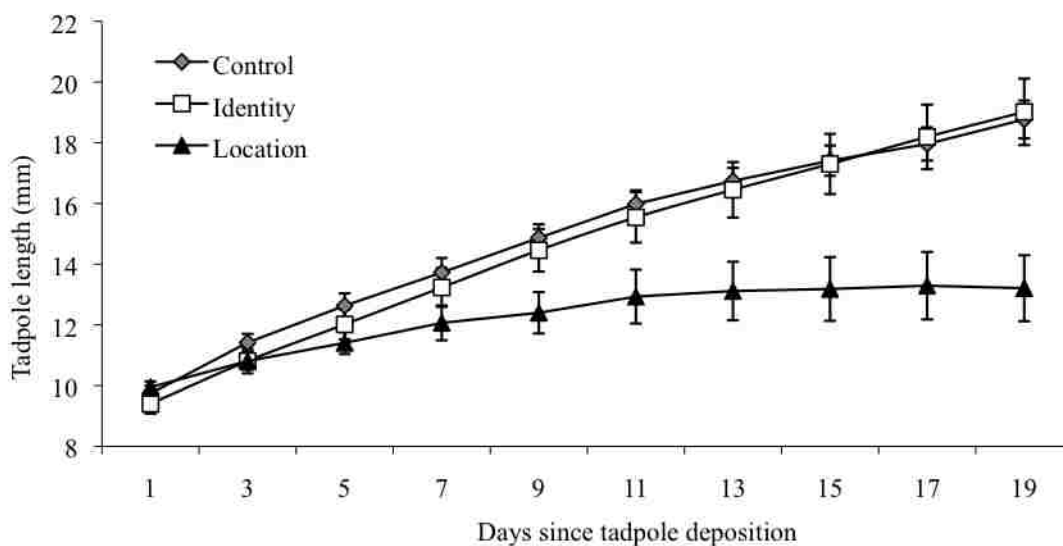


Figure 2.2 Change in total body length ($X \pm SE$) of *Oophaga pumilio* tadpoles in a recognition experiment. Tadpoles in the identity group were exchanged with unrelated tadpoles. Tadpoles in the location group were moved 2 cm to an adjacent tadpole-rearing cup. Control tadpoles were picked up and returned.

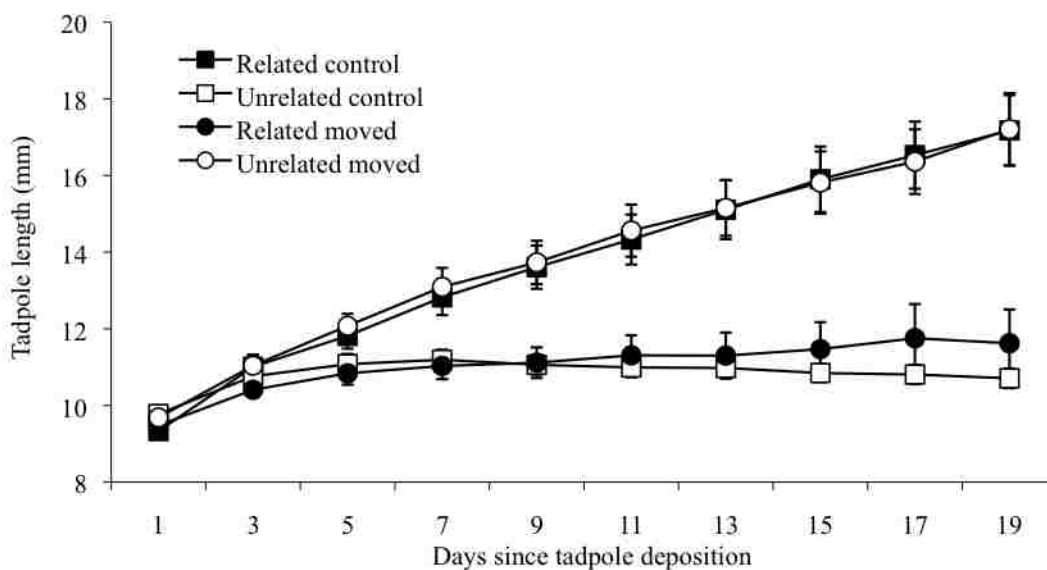


Figure 2.3 Change in total body length ($X \pm SE$) of *Oophaga pumilio* tadpoles in paired choice discrimination tests. Mothers placed tadpoles into one of two tadpole-rearing cups 2 cm apart. Related tadpoles in their original cups were either moved to the adjacent cup and replaced with an unrelated tadpole or picked up and returned and an unrelated tadpole was placed in the adjacent cup.

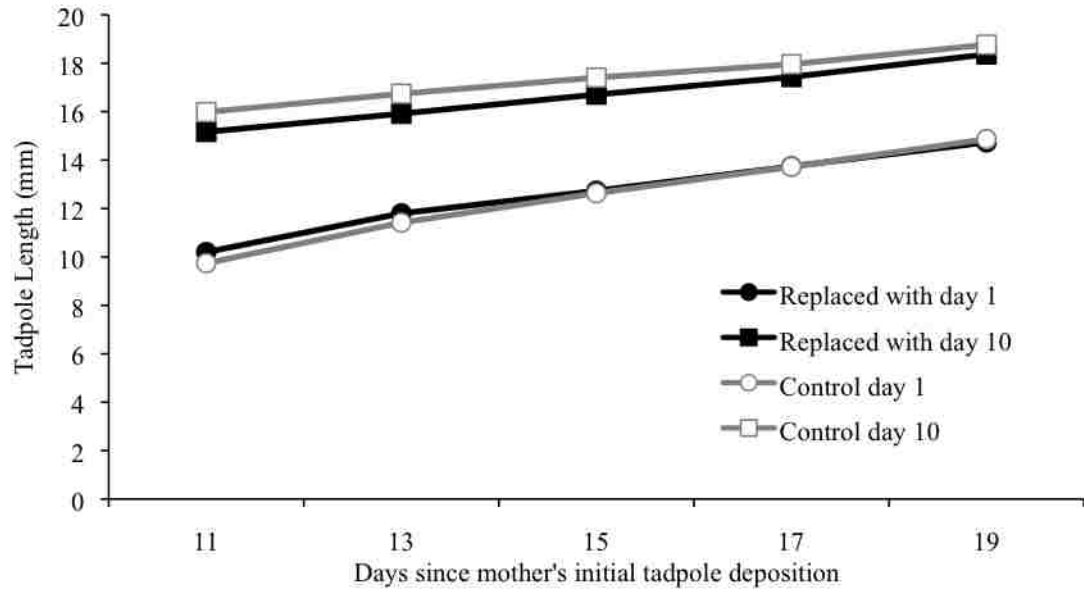


Figure 2.4 Change in total body length ($X \pm SE$) of *Oophaga pumilio* tadpoles in a post-parental investment recognition experiment. Mother-deposited tadpoles were replaced after 10 days of care with either 10 day-old tadpoles or one day-old tadpoles. Growth of unmanipulated tadpoles of the same age is also shown in grey.

Chapter 3: Multimodal sensory integration directs tadpole behavior²

Summary

Effective coordination of behaviors such as foraging and avoiding predators requires an assessment of cues provided by other organisms. Integrating cues from multiple sensory modalities may enhance the assessment. We studied cue integration by tadpoles of *Oophaga pumilio*, which occur in small water pools in plants (phytotelmata). In this species, mothers periodically visit their tadpoles and feed them with unfertilized eggs. When mothers visit, tadpoles beg conspicuously by vibrating until fed. However, animals other than mother frogs including potential predators may visit water pools. Thus, when a visitor appears, tadpoles must use visitor cues to decide whether to beg or to remain inactive to avoid predation. To elucidate the cues that prompt these behaviors, we videotaped behavior of *O. pumilio* tadpoles in response to isolated and multimodal cues. Tadpoles swam more when exposed to visual or visual and chemical cues of adult *O. pumilio*, but only exhibited begging when exposed to visual, chemical, and tactile cues together. Visual, chemical, and tactile cues from either male or female adult *O. pumilio* stimulated swimming and begging, but the same cues from similarly sized heterospecific frogs did not. Lastly, tadpoles exposed to a potential predator did not beg and swam less than tadpoles with no stimulus. Together, these findings suggest that *O. pumilio* tadpoles use multimodal cues to modulate swimming behavior accordingly in the presence of egg provisioners, predators, and other visitors, and that tadpole begging is induced by

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multimodal cues of conspecific frogs such that tactile and perhaps chemical cues supplement visual cues.

Background

How an animal uses the information provided by cues from other organisms to select among behaviors that are critical to its survival is an important question in behavioral ecology (Stauffer & Semlitsch 1993, Mathis & Vincent 2000, Warkentin 2005). For example, an animal must evaluate cues before deciding between foraging and anti-predator behaviors. Nestling white-browed scrubwrens (*Sericornis frontalis*) assess acoustic cues before displaying these two behaviors: they beg more vigorously during playbacks of parental food calls, but become silent during playbacks of predator footsteps (Magrath et al. 2007). To maximize detection and clarity of cues, an animal should use the sensory modality or modalities (chemical, acoustic, visual, and/or tactile) that most successfully transfer information within their specific habitat conditions (Scheffer et al. 1996, Kolliker et al. 2006).

Multimodal cues combine information from two or more sensory modalities, and can often provide more accurate information than cues in a single modality because they improve detection and localization, reduce ambiguity, and amplify the information content (Narins et al. 2005, Partan & Marler 2005). For example, during an otherwise identical visual display, female red-winged blackbirds (*Agelaius phoeniceus*) use male acoustic vocalizations to distinguish between the contexts of aggression and courtship (Beletsky 1983). Multimodal systems are most likely to evolve when information is transferred a short distance across habitat substrates that facilitate multimodal

transmission (Partan & Marler 2005). For example, male wolf spiders (*Schizocosa* sp.) court females using visual display and vibration cues, but switch to using only visual cues when the substrate does not transmit vibrations to females (Scheffer et al. 1996).

Multimodal integration is a topic of much recent theoretical and empirical study because of advances in experimental audio-visual technology (Stauffer & Semlitsch 1993, Uetz & Roberts 2002, Narins et al. 2005, Partan & Marler 2005, Smith & Evans 2008, Bro-Jorgensen 2010, de Luna et al. 2010).

Multimodal systems are categorized as either redundant or non-redundant (Partan & Marler 2005). In redundant systems, cues produce the same response whether presented alone or together, such as in honeybees (*Apis mellifera*), in which both the waggle (visual/tactile) and wingbeat (acoustic) components of the waggle dance independently serve to recruit other bees (Michelsen et al. 1992). In non-redundant systems, cues produce different responses when presented alone versus when presented together. Non-redundant systems can either be simultaneous, such as in the dart-poison frog *Epipedobates femoralis*, in which males display full aggression only when acoustic and visual cues are spatially and temporally synchronous (Narins et al. 2005), or sequential, in which the first modality attracts attention and the second provides more subtle information (Wilczynski et al. 1989, Stauffer & Semlitsch 1993, Parris et al. 2006).

Research suggests that larval amphibians, like many other aquatic taxa, primarily acquire information from chemical cues and only rarely or secondarily from other types of cues such as visual, tactile, and vibrational (Waldman 1985, Petranka et al. 1987, Stauffer & Semlitsch 1993, Kiesecker et al. 1996, Mathis & Vincent 2000, Hickman et al. 2004, Waldman & Bishop 2004, Rot-Nikcevic et al. 2005, Warkentin 2005, Parris et al.

2006). The relative importance of chemical over visual cues for aquatic organisms such as tadpoles may be a consequence of environmental conditions such as poor visibility in sediment-filled pond waters (Stauffer & Semlitsch 1993, Mathis & Vincent 2000, Parris et al. 2006). Tadpole vision is known to be functional to some degree (Rot-Nikcevic et al. 2005, Parris et al. 2006, Gouchie et al. 2008), but it may be myopic with poor acuity (Manteuffel et al. 1977, Mathis et al. 1988). Terrestrial adult frogs sometimes use visual, vibrational, chemical, or tactile cues as well, but are better known for conspicuous acoustic communication (Summers et al. 1999, Heying 2001, Ryan 2001, Waldman & Bishop 2004, Narins et al. 2005, Caldwell et al. 2010).

For tadpoles, chemical cues can stimulate various changes in morphology (McCollum & Leimberger 1997, Relyea 2001), life history (Skelly & Werner 1990), and behavioral decisions (Petranka et al. 1987, Lima & Dill 1990, Horat & Semlitsch 1994, Skelly 1995, Eklov 2000, Mathis & Vincent 2000, Bridges 2002). When tadpoles detect chemical cues of predators, they often rapidly respond by minimizing activity or by not moving (freezing), which are behavioral responses that can greatly reduce the chance of being detected and eaten by a predator (Wassersug & Sperry 1977, Lawler 1989, Azevedo-Ramos et al. 1992, Stauffer & Semlitsch 1993, Skelly 1994). However, anti-predatory freezing can reduce the time spent performing other important behaviors such as feeding (Skelly & Werner 1990, Bridges 2002), air breathing (Baird 1983, Feder 1983), and competing with conspecifics (Werner & Anholt 1996). Reducing the time spent performing these other behaviors can have long-term detrimental effects on the growth and metamorphosis of tadpoles (Smith 1987, Skelly & Warner 1990, Skelly 1994, Bridges 2002). Because of the possible negative fitness consequences of exhibiting anti-

predator behaviors when no predator is present, it is highly adaptive for tadpoles to correctly identify predator cues.

Many species of tadpoles, especially in the tropics where most anuran species are found, do not live in large water bodies such as ponds or swamps but instead complete their development in small pools of water such as those in treeholes or in the axils of a bromeliad plant (i.e. phytotelmata; Crump 1996, Lehtinen & Nussbaum 2003, Wells 2007). In a few species with phytotelm-dwelling tadpoles, parent frogs regularly provision their offspring with unfertilized eggs (*Osteopilus brunneus*, Lannoo et al. 1987; *Dendrobates granuliferus*, van Wijngaarden & Bolaños 1992; *Osteocephalus oophagus*, Jungfer & Weygoldt 1999; *Mantella laevigata*, Heying 2001; *Phrynohyas resinifictrix*, Schiesari et al. 2003; *Leptodactylus fallax*, Gibson & Buley 2004). In some of these provisioning species, bidirectional mother-offspring communication during feeding bouts has been described (*Oophaga pumilio*, Weygoldt 1980; *Anotheca spinosa*, Jungfer 1996; *Dendrobates vanzolinii*, Caldwell & deOliveira 1999; *Colostethus beebei*, Bourne et al. 2001; *Chirixalus eiffingeri*, Kam & Yang 2002). In these species, once the tadpoles have received a cue that a mother frog has arrived, they display a conspicuous behavior that can be likened to the begging behavior of the young of some mammalian and avian species. For example, in *Chiraxalus eiffingeri*, a nocturnal rhacophorid frog in which mothers rear their group-dwelling tadpoles in bamboo stumps, mother frogs communicate with offspring via chemical and tactile cues and tadpoles respond by swimming vigorously towards the mother's body (Kam & Yang 2002). In *Oophaga pumilio*, the strawberry poison frog, females return intermittently to tadpoles living in the axils of bromeliads and lay unfertilized eggs following a pronounced bout of tadpole vibration

behavior. Rather than swimming normally with undulating tail movements, vibrating *O. pumilio* tadpoles stiffen their tails and move their entire body vigorously in one place, typically against the adult's body (Weygoldt 1980). However, the cues that *O. pumilio* tadpoles use to detect the presence of their mothers prior to exhibiting vibration behavior are not known.

Because maternal feeding behavior in *O. pumilio* only occurs during daylight hours (Graves 1999, Haase & Pröhl 2002), visual cues may play a larger role in perception by tadpoles than in nocturnal species that exhibit mother-tadpole communication (Kam & Yang 2002). The goal of the present study was to determine the sensory mode or modes that *O. pumilio* tadpoles use to perceive mother frogs before expressing begging behavior. We also investigated whether *O. pumilio* tadpoles can distinguish between male and female adult conspecifics and between adult conspecifics and other species of sympatric adult frogs. Lastly, we examined tadpole response to the presence of potential predators. We hypothesized that *O. pumilio* tadpoles use multimodal cues to decide whether to beg in the presence of a potential egg-feeding frog or to freeze in the presence of a potential predator.

Methods

Study Site and Species

This study was conducted at the La Selva Biological Station in Costa Rica between October 2009 and February 2011. La Selva is comprised of primary and secondary lowland tropical wet forests and receives approximately 4 m of rain annually, with more precipitation falling from May to December (Sanford et al. 1994).

Oophaga [=Dendrobates] pumilio, or the strawberry poison frog, is a leaf-litter dwelling dendrobatid frog that is found in Atlantic lowland forests ranging from Nicaragua to Panama. It is a diurnal, aposematic species that sequesters toxic alkaloids in the skin after eating ants and mites (Saporito et al. 2007a). Adult *O. pumilio* at La Selva are red with blue hind limbs, and this conspicuous coloration serves as an aposematic signal to predators (Saporito et al. 2007b). After courtship, this species displays complex parental behavior (Weygoldt 1980, Brust 1993). Eggs are fertilized in leaf litter on the forest floor, and egg clutches are guarded and hydrated by fathers for 7-12 days (Weygoldt 1980, Brust 1993, Haase & Pröhl 2002). Once tadpoles hatch (stage 25, Gosner 1960), mothers return to their clutch and transport each tadpole singly to its own water-filled axil in a water-holding plant such as a bromeliad (Brust 1993, Weygoldt 1980). Then, mothers return to each tadpole at 1-8 day intervals for approximately 6 weeks to provision them by laying unfertilized eggs into the water (Brust 1993). Maternal provisioning is obligatory for tadpole survival, as tadpoles only eat eggs (Brust 1993). Mother frogs do not recognize their offspring, but rely on specific location cues to ensure that provisioning is provided for their own offspring (Stynoski 2009). In *O. pumilio*, adults are diurnal and the majority of maternal provisioning takes place between 0700 and 1100 (Graves 1999, Haase & Pröhl 2002). When mothers visit tadpole-occupied axils, tadpoles exhibit a conspicuous vibration behavior against the mother's body, which likely facilitates egg laying by mother frogs (Weygoldt 1980, Stynoski 2009). Eggs are not deposited in sites that do not contain a live tadpole (Stynoski 2009). Tadpoles will beg from unrelated mothers visiting their bromeliad axil when housed in aquaria, but

unrelated mothers do not provide eggs (Weygoldt 1980). Predation is a major source of tadpole mortality in bromeliads (67%; Maple 2002).

Other frog species used in this study to elicit responses by tadpoles of *O. pumilio* included *Rhaebo* (= *Bufo*) *haematiticus*, *Craugaster* (= *Eleutherodactylus*) *bransfordii* and *C. fitzingerii*. These species are common brown litter frogs that are sometimes found in bromeliads at La Selva. Only frogs of similar size to *O. pumilio* and located on bromeliads were collected (N = 4 of each species).

Cupiennius getazi and *C. coccineus* in the spider family Ctenidae are nocturnal wandering spiders commonly found in bromeliads of Central American forests (Barth & Seyfarth 1979, Barth & Cordes 1998). They spend the majority of time facing prosoma-down in a high humidity retreat such as the area above the water in a bromeliad axil, but turn to face outward from their retreat at dusk and hunt insects in this sit-and-wait position for a few hours thereafter (Barth & Seyfarth 1979). Wandering spiders use some visual cues but rely most heavily on mechanosensory cues to detect their prey when it moves (Schmid 1998). The eyes of *Cupiennius sp.* function well in dim moonlight (Barth et al. 1993). Maple (2002) suggested that wandering spiders are the primary predator of *O. pumilio* tadpoles, and these spiders are frequently seen at the field site. Furthermore, Maple (2002) demonstrated that *O. pumilio* tadpoles are most frequently found in axils of bromeliads that are of the size and species in which spiders are seen least frequently, suggesting that spiders eat the tadpoles or that mothers place tadpoles in these axils in the interest of avoiding predation by spiders. Spiders in general are known predators of other species of tadpoles (Caldwell & Araújo 1998, Vonesh & Warkentin 2006, Jara 2008).

Experimental Design

We collected animals from the Huertos Plots at La Selva, which are abandoned plantations that provide a semi-natural infrastructure of trees in rows adjacent to primary forest. Locating tadpoles in natural bromeliads is disruptive to the microcommunity of the plant, is inefficient, and sometimes results in injured tadpoles. Thus, during previous studies, an extensive field setup of artificial phytotelmata was established that allows mother frogs to deposit and care for offspring in their natural habitat while being easily observed (Stynoski 2009). Artificial bromeliad axils made from 15 ml polypropylene beakers (“cups”; N = 800) were tied to trees in pairs. Holes drilled in the sides of cups maintain rainwater volume at approximately 8 ml and cups are wiped out every 2-3 months to prevent buildup of algae and debris. Mother frogs regularly deposit and rear their tadpoles in these cups as they would in natural bromeliads (Stynoski 2009). Tadpoles in cups develop to metamorphosis unless they die or are eaten by predators.

We caught frogs and spiders by hand, with an aquarium net, or with a plastic bag from bromeliads within the Huertos Plots and temporarily placed them in plastic bags with leaf litter for transport to the laboratory. We collected tadpoles with a 3 ml wide-mouthed pipette and placed them individually in glass vials with rainwater. Once in the laboratory, tadpoles acclimated to laboratory conditions for at least 2 hours in rainwater in a testing apparatus that consisted of a lidded clear plastic cup (1 oz) that was seated within a larger lidded clear plastic container also filled with water to the same level. Then, we placed these cups with the tadpole in the position where the trial was to take place, let the tadpole acclimate for an additional 5 minutes, and then performed experimental manipulations as outlined below. Immediately following manipulations,

tadpoles were videotaped for 10 minutes with a video camera (JVC) or an infrared video camera (ActiveVision) in the case of trials that took place in a dark box. We decided upon a trial duration of 10 minutes for all behavioral trials following observations of a similar average duration of mother visits to cups in the field (JLS, unpublished data). Animals were used in only one trial across all experiments. We returned animals to their site of capture after behavior trials. We scored tadpole behavior using the program JWatcher 1.0 to determine the percentage of time that tadpoles were resting (not moving), swimming (moving with flexible tail movements), or vibrating (stiffening the tail and moving rapidly in one place). Tadpole vibration behavior can vary greatly in tadpoles of relatively early and late stages (JLS, unpublished data), so we only used tadpoles from stages 30 to 40 (Gosner 1960).

Experiment 1: Sensory perception of a conspecific adult frog

Seven treatment groups (chemical, visual, visual + chemical, tactile + chemical, visual + tactile + chemical, dark control, and control; N=12) were arranged as follows. In the chemical cue group, we put 20 drops of water possibly containing adult chemical cues into the tadpole's cup. We prepared this water by pooling the water from 6 vials within each of which an adult frog had soaked for 4 hours in 15 ml of rainwater prior to the trial (as in Kam & Yang 2002 where water similarly conditioned with adult chemical cues elicited a strong tadpole response). In the visual cue group, we placed an adult *O. pumilio* in the larger container holding the tadpole's cup such that tadpoles could see the adult frog but not touch it or receive chemical cues (but could potentially receive vibrational cues). In the visual + chemical group, tadpoles were simultaneously exposed to 20 drops

of adult conditioned water and an adult in the larger container surrounding the tadpole's cup. In the tactile + chemical cue group, we placed an adult *O. pumilio* in the tadpole's cup, but the trial took place in a dark box. In the visual + tactile + chemical group, an adult *O. pumilio* was placed in the tadpole's cup in ambient lighting. No cues were presented to dark control and control groups, but we videotaped the tadpoles in cups in a dark box or in ambient lighting, respectively. During trials in a dark box, we allowed tadpoles to acclimate to the laboratory for 2 hours in ambient lighting and then to the trial position for 5 minutes inside of the box before videotaping them with an infrared camera. Because tactile cues of adult frogs cannot be presented without also introducing potential chemical cues, we could not examine the effects of visual + tactile or tactile cues alone using live adult frogs. In preliminary tests with models of frogs, tadpoles did not exhibit a begging response, presumably because models did not move and/or because they were inadequate replicas of adult *O. pumilio*; thus, we did not use models in the current study.

Experiment 2: Distinguishing between provisioning and non-provisioning adult frogs

To test for an ability of tadpoles to distinguish between adult frogs of the same or different species, as in the visual + tactile + chemical trials described above, we placed either conspecific or heterospecific frogs in the small cup with the tadpole (N=12). Similarly, we tested for the ability of tadpoles to distinguish between sexes of adult *O. pumilio*, which, unlike heterospecific frogs, are visually similar in terms of shape and movement, by placing either a male or a female adult in the cup with the tadpole (N=12).

Experiment 3: Behavior modulation with the perception of a potential predator

We compared the behavior of tadpoles with a spider placed in their cup at the beginning of the trial to that of tadpoles with no stimulus (N=10). Spiders were held in the lab without food for 7 days prior to the trial to ensure that all spiders were hungry. We noted whether the spider struck at the water's surface with its mouthparts and whether the spider entered the water. In addition, we allowed the video camera to run for a total of 20 minutes after the 5-minute pre-trial acclimation period in order to view tadpole behavior both in a first and second 10-minute time bout.

Statistical Analysis

All analyses were completed in R 2.10.1 (2009). We subjected the data to an arcsine transformation as is appropriate for proportion data (Crawley 2005), performed tests for homogeneity of variances, and then ran linear models of the transformed data for each experiment separately. We used the proportion of time that tadpoles were either swimming or vibrating as response variables when testing the effects of sensory mode, sex of adult frog, and species of adult frog (whether conspecific or heterospecific). To examine the effects of predator presence, we only compared the proportion of time tadpoles were swimming because tadpoles did not vibrate in any of these trials.

Results

The sensory modality of cues from adult *O. pumilio* had a significant overall effect on the percentage of time that tadpoles spent swimming and vibrating (swim: $F_{6,85}=19.54$, $p<0.0001$; vibrate: $F_{6,85}=14.8$, $p<0.0001$; Figure 3.1). The percentage of time

tadpoles spent swimming or vibrating did not differ among light control, dark control, chemical, or tactile + chemical treatments, but tadpoles spent significantly more time swimming in the visual treatment (model coefficient: $t=6.67$, $p<0.0001$), visual + chemical treatment ($t=6.00$, $p<0.0001$), and visual + tactile + chemical treatment ($t=5.19$, $p<0.0001$) than in all other treatments, and significantly more time vibrating in the visual + tactile + chemical treatment ($t=7.39$, $p<0.0001$) than in all other treatments. In the visual + tactile + chemical treatment, tadpoles first tended to swim actively after seeing the movements of the adult frog, and then after they happened to touch the adult frog while swimming, they commenced vibration behavior. Tadpole vibration generally occurred while in contact with the body of the adult frog, but did not include nipping as suggested in previous literature (Weygoldt 1980). Adult frogs did not deposit eggs in any trials and consistently attempted to leave the cup (but were prevented from doing so by the plastic lid); they either appeared uninterested or were disturbed by tadpoles (moved away when touched).

Tadpole swimming and vibration behavior did not differ when in the presence of either a male or female adult *O. pumilio* (swim: $F_{22,1}=0.80$, $p=0.38$; vibrate: $F_{22,1}=1.59$, $p=0.22$). However, tadpoles did spend significantly more time swimming and vibrating in the presence of conspecific adult frogs than in the presence of heterospecific adult frogs (swim: $F_{24,1}=30.9$, $p<0.0001$; vibrate: $F_{24,1}=12.87$, $p=0.001$; Figure 3.2). All adult frogs in these trials behaved as those described in the paragraph above.

Tadpoles spent significantly less time swimming in the presence of a spider ($F_{40,1}=14.27$, $p=0.0005$) and more time swimming in the second bout of 10 minutes than in the first bout ($F_{40,1}=5.54$, $p=0.02$; Figure 3.3). No significant effect of the interaction of

stimulus and bout was detected ($F_{40,1}=0.13$, $p=0.071$). In 2 of 10 trials, spiders appeared to attack with their mouthparts at the water's surface without touching the tadpole, but retreated to the vial lid soon after. Tadpoles remained motionless during spider movements. Spiders did not enter the water.

Discussion

This study shows that *Oophaga pumilio* tadpoles use multimodal cues to assess the arrival of an adult frog that could lay nutritive eggs as opposed to the arrival of a non-provisioning animal or potential predator. We found that tadpoles swam more when presented with visual cues from adult *O. pumilio*, but did not swim more in the presence of other species of frogs. We also found that tadpoles swam less when a potential predator was above the water. After an adult *O. pumilio* entered the water, tactile and possibly chemical cues supplemented visual cues to induce tadpole begging. Reliance on such sequential non-redundant multimodal cues may act to ensure recognition of adult *O. pumilio* before a tadpole performs a conspicuous and potentially costly begging behavior at a heterospecific frog or predator. Similarly, tadpoles and embryos from species in the genus *Lithobates* (= *Rana*) were found to use mostly chemical cues to detect predators, although they showed more intense anti-predator responses when chemical cues were further amplified by tactile cues (Stauffer & Semlitsch 1993, Smith & Fortune 2009).

The results of the present study also suggest that visual and tactile cues are of more importance to *O. pumilio* tadpoles than chemical cues in differentiating among animals; this is noteworthy because tadpoles of other species have been found to use primarily chemical cues (Mathis & Vincent 2000). *Oophaga pumilio* tadpoles begged

when presented at the same time with visual, tactile, and chemical cues but did not beg when presented with both tactile and chemical cues, with both visual and chemical cues, or with chemical cues alone. Furthermore, tadpoles presented with adult *O. pumilio* cues swam more than control tadpoles when presented with both visual and chemical cues or with visual cues alone, but not when presented with chemical cues alone. It is possible that *O. pumilio* tadpoles use visual cues to assess visitors to their water pools before deciding whether to increase or decrease swimming behavior because there is potential danger in waiting for possible tactile or chemical cues from the visitor after it enters the water because the visitor might be a predator. Additionally, visual cues may be of key importance in *O. pumilio* because the adults are diurnal and their tadpoles develop in phytotelmata, which contain small volumes of clear water and often lack refuges. To our knowledge, only one other study has specifically investigated the communication between egg-fed tadpoles and their mothers and this was the study of *Chirixalus eiffingeri* by Kam & Yang (2002). These investigators found that *C. eiffingeri* tadpoles did not respond to visual cues of an adult frog in a surrounding container or to a model of an adult frog, but instead used only chemical cues to detect the arrival of mother frogs (Kam & Yang 2002). It could be that *C. eiffingeri* tadpoles do not respond to visual cues because parent frogs in this species only provide nutritive eggs at night.

It is possible that provisioning mother *O. pumilio* only release a chemical, acoustic, or vibrational cue under particular spatial conditions with their own tadpoles, and tadpole responses to such cues would not be detected with our experimental design. But, it seems unlikely that additional cues are necessary for mother-to-tadpole communication in *O. pumilio*; tadpoles still begged vigorously from adult conspecific

frogs even though these adults appeared to be highly motivated to escape from the testing cups and tadpoles begged equally from adults of both sexes even though only mother frogs provide eggs.

Tadpoles of *Oophaga pumilio* begged from both male and female conspecifics, but did not beg from similarly sized individuals of other species of bromeliad dwelling frogs. Presumably, *O. pumilio* tadpoles have the ability to distinguish between potential predators and egg provisioners, or at least between non-provisioning and potentially provisioning organisms, using cues such as body shape or movement patterns. It is important to note that *O. pumilio* tadpoles can be reared in the laboratory by unrelated conspecific frogs from very differently colored polymorphic populations (C. Richards-Zawacki, personal communication). Thus, it is unlikely that color plays a role in recognition of conspecific adults by *O. pumilio* tadpoles.

Oophaga pumilio tadpoles presented with a spider stimulus swam approximately 5 times less than tadpoles presented with no stimulus. Freezing is a common response of tadpoles to the detection of a potential predator (Horat & Semlitsch 1994, Skelly 1995, Bridges 2002), even though it may reduce the time spent performing other important behaviors. For example, *Anaxyrus (=Bufo) americanus* tadpoles moved 41% less in the presence of predators, and this decline in movement was associated with a 28% decrease in growth rate that results from reduced feeding (Skelly & Werner 1990). On the other hand, *Pseudacris triseriata* tadpoles were found to swim less in the presence of predators, but did not spend less time feeding (Bridges 2002). *Oophaga pumilio* tadpoles spend much less time swallowing maternal eggs each day than the 11% of the time that *P. triseriata* spend eating plant material (Bridges 2002; JLS, unpublished data), so it is

unlikely that the time that *O. pumilio* tadpoles spend feeding is reduced as a result of freezing when a potential predator arrives. Reduction of swimming by *O. pumilio* tadpoles might only occur in the presence of predators because it might interfere with cutaneous respiration or surface breathing, and because it might lower the likelihood of detecting mother frogs that arrive to provision. Freezing in response to the presence of a predator might be more likely to occur in species such as *O. pumilio* in which tadpoles are located in relatively small pools of water in phytotelmata than in species in which tadpoles dwell in relatively large aquatic habitats. Future studies might address the degree to which movement affects tadpole survival if a predator is already sitting above the phytotelm water surface.

The freezing behavior that tadpoles exhibit in the presence of a predator may attenuate with time. Although tadpoles exposed to spiders swam significantly less than control tadpoles in the first 10 minutes, they did not swim less than control tadpoles in the second 10 minutes. Tadpoles may only see spiders as a threat worth responding to when the spider first appears above the water pool.

The present study shows that *O. pumilio* tadpoles use multimodal cues to distinguish between organisms that appear above their water pool and to modulate their behavior according to visitor identity. First, they use visual cues to determine whether to freeze because a visitor is a potential predator or to swim more because a visitor is a potential egg provider. Once a non-predatory visitor enters the water pool, *O. pumilio* tadpoles use tactile and perhaps chemical cues to determine whether or not the visitor is a conspecific adult and whether or not to exhibit begging behavior. Thus, tadpoles of *O.*

pumilio supplement visual cues with tactile and perhaps chemical cues to fine-tune their behavior in the interest of eating but not being eaten.

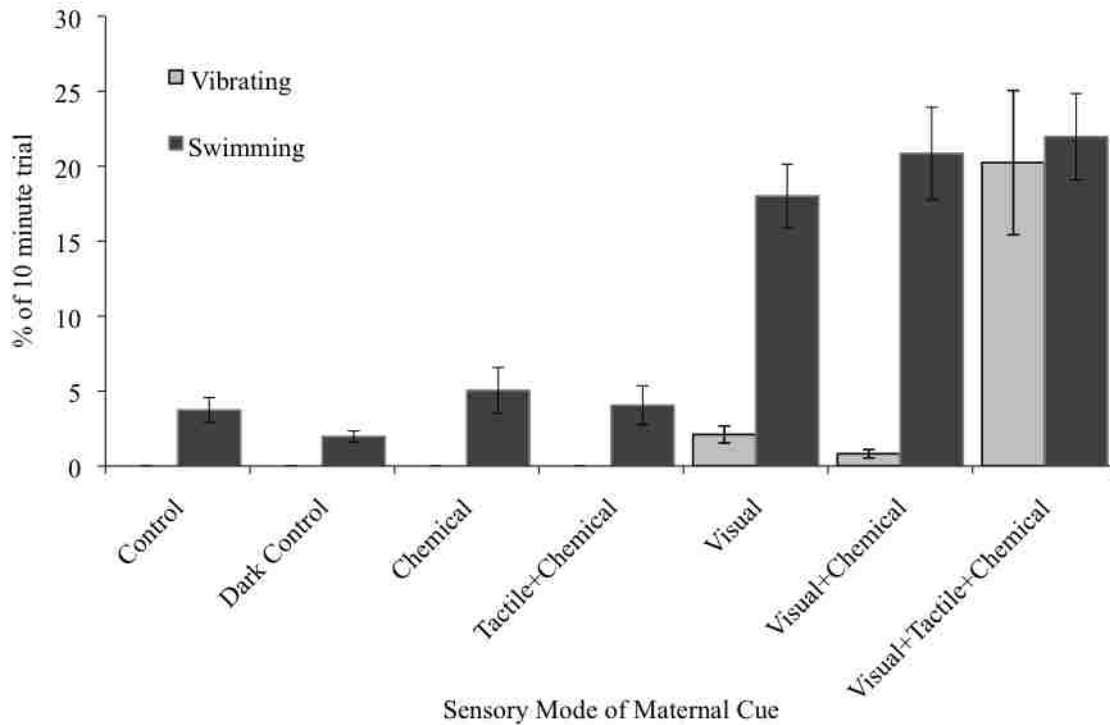


Figure 3.1 Behavior of *O. pumilio* tadpoles (N=12) during 10 minutes of exposure to combinations of adult *O. pumilio* cues. Tadpoles swam significantly more when presented with visual cues alone, visual and chemical cues together, or with visual, tactile, and chemical cues together than in all other cue combinations. Tadpoles vibrated significantly more when presented with visual, tactile, and chemical cues together than in all other cue combinations. Error bars show SEM.

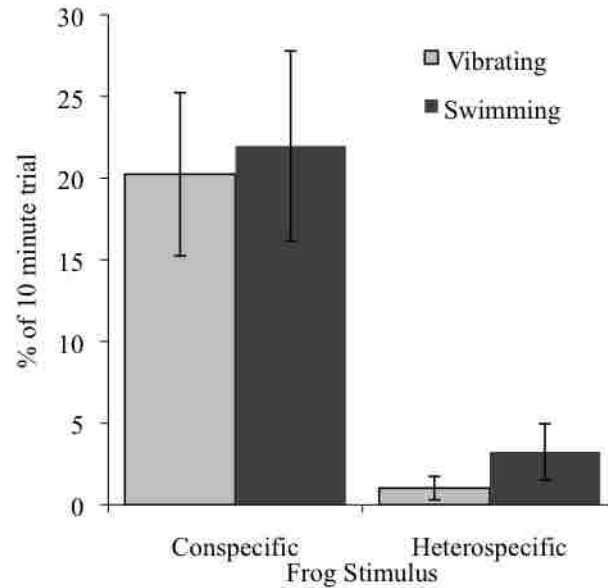


Figure 3.2 Behavior of *O. pumilio* tadpoles (N=12) during 10 minutes with either a conspecific adult frog or a similarly sized heterospecific frog. Tadpoles swam and vibrated significantly more in trials with adult *O. pumilio* than in those with heterospecific frogs. Error bars show SEM.

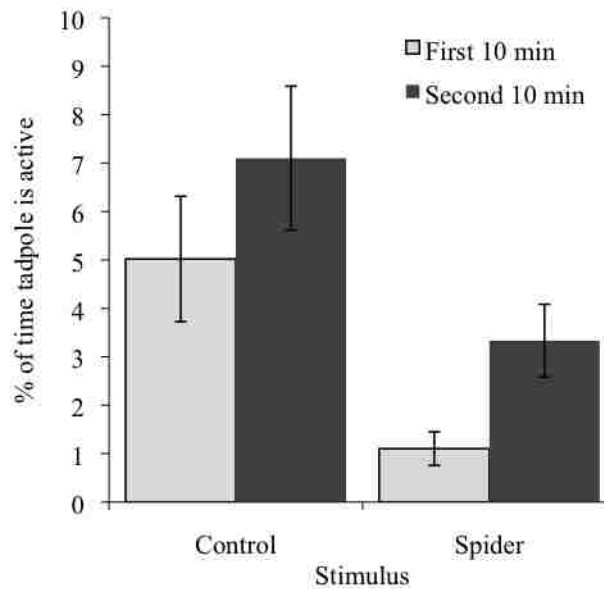


Figure 3.3 Behavior of *O. pumilio* tadpoles (N=10) with either no stimulus or with a potentially predatory spider. Over the first 10 minutes of trials, tadpoles swam approximately 5 times less in the presence of a spider than with no stimulus. Error bars show SEM.

Chapter 4: Begging is honest and costly in tadpoles³

Summary

Begging signals convey the nutritive needs of offspring to parents so that parents can appropriately supply resources. Theoretical models suggest that parents can rely on offspring signals because costs of signal production, such as reduced growth or risk of predation, discourage exaggeration of need. Empirical studies have focused on group-reared avian, arthropod, and mammalian systems that are affected by sibling influences on offspring behavior. Here, we investigated three predictions of honest signaling models in the strawberry poison frog (*Oophaga pumilio*). Mother *O. pumilio* rear their tadpoles by placing them in individual arboreal water pools and provisioning them with nutritive eggs during a period of six weeks. Tadpoles display vibrational begging behavior before mother frogs deposit eggs. In laboratory behavior tests, relative duration of begging behavior was dependent on both short-term need and developmental stage. In a laboratory growth experiment, tadpoles grew less when coerced to beg for a biologically relevant period before being fed. Lastly, video cameras in the field revealed that mother frogs only deposit eggs during visits in which tadpoles beg. Together these experiments show that begging signals in this amphibian are honest and metabolically costly, and mother *O. pumilio* utilize tadpole signals of need to appropriately allocate resources.

³ This chapter was submitted for publication with authors: Stynoski, J.L., Stynoski, P.B., and Strider, M.

Background

Parents and offspring are at odds over the amount of care that parents should invest because of their difference in relatedness (Trivers 1974). Begging signals function as a mechanism for offspring to influence the outcome of this conflict by indicating their level of need to parents (Godfray 1991, 1995, Kilner & Johnstone 1997, Bell 2008). Offspring could potentially exploit parents by exaggerating their begging, but parents would eventually ignore dishonest signals and the communication system would unravel (Kilner & Johnstone 1997). For this reason, begging signals have become a popular framework for theoretical and empirical studies that explore the mechanisms that maintain honesty in communication and evolutionary stability of signals (reviewed in Searcy & Nowicki 2005). Theoretical models suggest three conditions that would allow begging signals to persist in a population: (1) begging displays should truly reflect offspring need, (2) parent provisioning should reflect begging intensity, and (3) begging displays should be sufficiently costly to discourage false use of signals (Godfray 1991, 1995, Kilner & Johnstone 1997). In addition, it is necessary that deprived offspring receive a higher benefit from being provisioned by parents than well-fed offspring so that the system does not stabilize in such a way that the strongest begging signals come from well-fed offspring (Searcy & Nowicki 2005). With such differential benefits, the reliability of signals can be maintained in a system with only a minimal cost associated with begging (Searcy & Nowicki 2005).

Many empirical studies have examined whether begging signals reflect offspring status by manipulating the nutritional needs of offspring (Royle et al. 2002; Mas & Kölliker 2011). From this work, we know that the nature of an individual's begging

display can be dependent on a suite of factors including short-term hunger level (Weary & Fraser 1995, Iacovides & Evans 1998, den Boer & Duchateau 2006), long-term nutritional status (Weary & Fraser 1995, Price et al. 1996, Kölliker et al. 2006, Wright et al. 2010), past experience with caretakers (Kedar et al. 2000, Bell 2008), and stage of development (Iacovides & Evans 1998, Kilner 2001, Royle et al. 2002). There is also evidence that parents in many taxa use reliable signals of offspring need to appropriately adjust their provisioning behavior and account for offspring level of need while allocating resources (Ottoosson et al. 1997, Quillfeldt 2002, Laurien-Kehnen & Trillmich 2003, Kölliker et al. 2006, Tarwater et al. 2009).

Costs that result from producing begging signals, such as increased predator risk or energy use, are thought to maintain signal reliability because they discourage offspring from misrepresenting their needs to acquire extra resources (Godfray 1991, 1995, Kilner & Johnstone 1997). Offspring may reduce begging activity in the presence of predators, because begging as well as subsequent increases in parental activity can attract predators and thus increase the risk of predation (Haskell 1994, Leech & Leonard 1997, Martin et al. 2000, McDonald et al. 2009). Similarly, the honesty of begging signals may be maintained through costs associated with increased use of metabolic energy and reduced growth of offspring (Kedar et al. 2000, Kilner 2001, Rodríguez-Gironés 2001), which may be mediated via concurrent hormonal regulation of behavior and energy use or growth (reviewed in Smiseth et al. 2011). However, controversy exists over whether or not the magnitude of any cost is sufficiently detrimental to fitness relative to the substantial benefit of receiving food such that it would result in selection against non-reliable begging signals (McCarty 1996, Bachman & Chappell 1998, Rodríguez-Gironés

2001, Smiseth & Parker 2008, Wright et al. 2010). Alternative theoretical models argue that factors other than costs of begging are sufficient to maintain reliability such as the benefits of receiving resources, long-term costs to dishonesty, sibling competition, or a sufficient overlap of interest between parents and offspring (reviewed in Searcy & Nowicki 2005). Despite a wealth of studies, the mechanisms responsible for maintaining reliability of begging signals have not yet been elucidated, and in fact there may be species- and context-specific mechanisms at play.

Research on begging signals and their costs is highly biased taxonomically, with an overwhelming majority of studies on birds (Searcy & Nowicki 2005, Smiseth & Parker 2008), and many fewer investigations on insects (Treehopper *Umberia crassicornis*, Cocroft 1999; Ponerine ant *Gnamptogenys striatula* Kaptein et al. 2005; Bumblebee *Bombus terrestris* den Boer & Duchateau 2006; Burrower bug *Sehirus cinctus* Kölliker et al. 2006; Burying beetle *Nicrophorus vespilloides* Smiseth & Parker 2008; European earwig *Forficula auricularia* Mas & Kölliker 2011) and on mammals (Meerkat *Suricata suricatta* Manser & Avey 2000; Grey seal *Halichoerus grypus* Smiseth & Lorentsen 2001; Guinea pig *Cavia porcellus* Laurien-Kehnen & Trillmich 2003; Domestic pig *Sus scrofa* Weary & Fisher 1995; Banded mongoose *Mungos mungo* Bell 2008). Furthermore, studies of begging signals and their costs in mammals, birds, and insects have generally examined species that raise their young in groups. In such groups, the combined signals of siblings can function either competitively or cooperatively to alter both sibling signals and the provisioning behaviors of parents (Dreiss et al. 2010, Mas & Kölliker 2011, reviewed in Searcy & Nowicki 2005). Models and empirical studies have developed ways to explain or account for sibling interaction

effects (Royle et al. 2002, Dreiss et al. 2010), but few empirical studies have included species in which young are reared individually rather than in a group (Smiseth & Lorentsen 2001, Quillfeldt 2002). Thus, we have a limited understanding of begging behavior that is not influenced by sibling interactions and hatching asynchrony.

In this study, we sought to expand our understanding of the reliability and cost of begging behavior by studying begging signals in an amphibian in which the behavior of individual offspring is not directly influenced by siblings. Amphibians display an impressive array of parental care modes, ranging from nest making and guarding to provisioning with unfertilized eggs (reviewed in Crump 1996, Wells 2007). In a few frog species that provision their offspring, tadpoles live in small arboreal water pools and display a conspicuous begging behavior before they are provided with unfertilized eggs by visiting parental frogs [*Oophaga pumilio*, Weygoldt 1980; *Anotheca spinosa*, Jungfer 1996; *Osteopilus brunneus*, Thompson 1996; *Colostethus beebei*, Bourne et al. 2001; *Chirixalus eiffingeri*, Liang et al. 2002]. Mothers of the Neotropical strawberry poison frog, *Oophaga pumilio*, transport tadpoles individually from egg clutches in the leaf litter to water-filled axils of bromeliad plants on the sides of trees. Mothers place only a single tadpole per water-filled axil and make return visits to feed each tadpole for a period of six weeks (Weygoldt 1980, Brust 1993, Stynoski 2009). When mothers arrive, tadpoles typically display a conspicuous vibration behavior that is thought to stimulate egg deposition because eggs are only found in sites with live tadpoles (Weygoldt 1980, Stynoski 2009). Similar tadpole vibration behavior stimulates egg laying in mother frogs of other species that provision tadpoles with eggs (Jungfer 1996, Bourne et al. 2001, Kam & Yang 2002). In *O. pumilio*, each tadpole from an egg clutch is reared individually in a

different location from its siblings and thus does not beg concurrently with siblings. The number of nutritive eggs provided and the time interval between feeding visits varies (Weygoldt 1980, Brust 1993, Stynoski 2009).

Here we used the strawberry poison frog (*O. pumilio*) to test three major predictions that theoretical models suggest must be met in order for begging signals to remain honest in a communication system. Specifically, we tested the predictions that tadpole begging behavior: 1) varies according to the level of short-term need and developmental stage of tadpoles, 2) is costly in terms of tadpole growth, and 3) plays a role in egg allocation decisions by mother frogs.

Methods

Study site and species

Oophaga pumilio is an aposematic dendrobatid frog found in lowland forests along the Atlantic versant of lower Central America that is chemically defended by the alkaloids it obtains from a diet of ants and mites and sequesters in skin glands (Saporito et al. 2004, Saporito et al. 2007a). This diurnal and territorial species displays the most advanced form of parental care within the dendrobatid frogs, a group well known for its diverse range of parental modes (Grant et al. 2006). In *O. pumilio*, males guard the eggs they have fertilized for a period of 7-10 days, and then mother frogs return to the clutch to transport each tadpole on their back to its own small water pool known as a phytotelm (Weygoldt 1980, Brust 1993). Mother frogs return at 1-8 day intervals for a period of about six weeks to each phytotelm containing one of their tadpoles. After apparently communicating with the tadpole, they provide unfertilized eggs that are eaten

immediately or in the days following deposition (Stynoski 2009). Communication involves an increase in tadpole activity followed by a conspicuous vibration behavior performed against the mother frog's body, and this vibratory action seems to trigger egg release by the mother (Weygoldt 1980, Bourne et al. 2001, Kam & Yang 2002, Stynoski 2009). Tadpoles are thought to only eat eggs and are cannibalistic (Brust 1993, Stynoski 2009).

The present study was conducted in 2009-2011 in the abandoned tree plantations known as the Huertos Plots at the La Selva Biological Station in Sarapiquí, Costa Rica. Tadpoles in the study were collected from a long-term field setup of artificial rearing sites used in a series of studies. The field setup consists of 1000 15 ml polypropylene beakers ("cups") tied to trees in pairs at a 1 m height. Mother frogs rear tadpoles in these cups just as they do in natural bromeliads such that tadpoles remain in cups until they die, are eaten by predators, or metamorphose (Stynoski 2009).

Field Observations with Motion Detecting Video Cameras

To observe the response of mother frogs to tadpole vibration behavior in the field, we placed eight motion-detecting weatherproof infrared-capable video cameras (ActiveVision Inc., Ronkonkoma, NY) above cups containing tadpoles to observe mother-tadpole interactions. Power lines installed specifically for this project supplied electricity. We adjusted the digital video recorders (Advance Security, Belleville, IL) connected to the video cameras so that they recorded only during events with substantial motion within the cup; they detected frogs and other organisms entering the cup but not rain, debris, or items moving outside of the cup in the viewing screen. For each recorded

event, we noted the identity of the visitor, the time of day, the tadpole's behavior, and the visitor's response.

Laboratory Behavior Trials

We conducted laboratory behavior trials to investigate the reliability of begging signals as an indicator of tadpole need. Tadpoles in all trials were brought to the laboratory in rainwater in glass vials. In trials aimed at determining the effect of short-term need on tadpole begging behavior, we gave each tadpole one *O. pumilio* egg. All of the provided eggs had been collected at the same time as the tadpole from another cup in the field. We then randomly assigned tadpoles in terms of whether they were held in the laboratory without any additional food during a starvation period of either 2 hours, 1 day, 5 days, or 10 days prior to behavior trials (N = 12 per treatment). In trials aimed at determining the effect of developmental stage on tadpole begging behavior, we randomly selected 60 tadpoles from field cups at developmental stages representing the full range of tadpole development (stages 25-44, Gosner 1960). We brought these tadpoles to the laboratory but did not give them eggs or hold them for a starvation period in the laboratory prior to trials as in the experiment explained above. Before video trials in both the short-term need and development stage experiments, we poured tadpoles and their rainwater into a 1-ounce clear plastic cup and allowed them to acclimate to laboratory conditions for at least two hours. Then, tadpoles were placed in a staging area in front of a video camera (JVC) and allowed to acclimate for five minutes. We then added an adult frog (collected from the field) to the cup, placed a lid on the cup, and waited 30 seconds before starting the video camera to record behavior for 10 minutes. We used adult frogs

from both sexes because it was sometimes not possible to find a sufficient number of female frogs on a given day prior to trials and because previous studies show that there is no difference in tadpole begging in response to male or female conspecific adults (Stynoski & Noble 2012). We selected a trial duration of 10 min on the basis of the observed average duration of maternal visits to tadpoles in field videos in the current study. All tadpoles were returned within a few hours of trials to their original cup in the field, and mother frogs continued to rear them naturally. Each animal participated in only one trial. We used the software program Jwatcher 1.0 to score behavior and quantify the proportion of time that each tadpole spent resting (not moving), swimming (moving but not vibrating), or vibrating (vigorously moving the whole body side to side without changing average position in the water). We considered tadpoles to be begging only when they were vibrating.

Growth Study

We collected 30 tadpoles from field cups at stage 28, photographed them in the same plane as a ruler, and placed them in individual plastic cups with rainwater in ambient conditions. We fed tadpoles with a single yolk of an egg of the red-eyed treefrog (*Agalychnis callidryas*) collected from swamps at La Selva. These eggs are easier to obtain in large quantities than *O. pumilio* eggs and may be a better option for promoting normal tadpole growth than the chicken egg yolk used to feed oophagous tadpoles in other studies (Weygoldt 1980, Liang et al. 2002). During a 10-day pilot study, growth of tadpoles (N = 10 per treatment) fed with *A. callidryas* egg yolks (mean \pm SE, 2.0 ± 0.4 mm) was more similar to growth of mother-fed field tadpoles (4.9 ± 0.3 mm) than to

growth of tadpoles fed chicken egg yolk (1.1 ± 0.3 mm). To determine whether begging impacts tadpole growth, we fed tadpoles every 48 hours for 2 weeks. Prior to each feeding, we presented tadpoles (N=12 per treatment) with an adult male *O. pumilio* either for 10 seconds as a control (i.e., should elicit little or no begging) or for 10 minutes to stimulate biologically relevant quantities of begging behavior. Yolk remaining from previous feedings was removed prior to each feeding. After 2 weeks, we took additional photos of each tadpole. We used the line tool in the software program ImageJ (NIH) to determine tadpole growth from tadpole photos (as in Stynoski 2009). Lastly, we took length photos of 100 tadpoles at various developmental stages in the field to better interpret the results of the experiment that tests the effects of developmental stage on begging behavior and to establish a normal growth curve for tadpoles of this species.

Statistical Analysis

To determine the effect of short-term nutritional need on begging behavior, as appropriate for categorical proportional behavior data, we conducted linear models on arcsine-transformed proportions of time that tadpoles spent either swimming or begging (vibrating). To determine the effect of developmental stage on begging behavior, we compared the AIC values of linear and quadratic Generalized Linear Models (GLM) and performed Chi-squared test comparisons of the two model types. To determine whether begging behavior reduced tadpole growth, after determining that variances of the data were not homogeneous, we used a non-parametric Mann-Whitney U test. We conducted all statistics in R (2.10.1).

Results

Field video observations

We collected 64 videos of mother frog visits to cups containing tadpoles, and many other videos of visits to cups by ants, harvestmen, leeches, anoline lizards, and snakes. Mother visits to tadpoles averaged 9 min and 50 sec \pm 1 min and 2 sec (range: 2 min and 2 sec - 50 min and 31 sec), and occurred an average of every 2.2 ± 0.3 days (range: 1 - 5 days). It was sometimes difficult to count deposited eggs in videos because they are transparent, but mothers in most cases appeared to lay 1-2 eggs per visit and rarely up to 6 eggs. Mother visits took place between 0605 h and 1543 h, with approximately 90% of visits occurring between 0600 h and 1100 h. In the 54 videos in which tadpoles vibrated, mothers provided eggs in 48 cases but did not in 6 cases. In all 10 videos in which tadpoles did not vibrate during a mother frog's visit, mothers did not provide eggs and had already deposited eggs the same or previous day. When predators ate focal tadpoles on two occasions, mother frogs continued to visit the empty cups; one cup was visited 3 times in 4 days and the other 4 times in 6 days, with an average time per visit of 16 min and 18 sec \pm 4 min and 2 sec (range: 3 min and 1 sec - 23 min and 51 sec). The fact that these mothers remained in the empty cups for a longer than normal time suggests that mothers delayed their exit because they were waiting for a tadpole to vibrate.

Laboratory Behavior Trials

When exposed to an adult frog, tadpoles that had been starved for 5 or 10 days swam significantly more than tadpoles that had been starved for 0 or 1 days (overall linear model: $R^2 = 0.21$, $F_{3,44} = 3.91$, $p = 0.01$; linear model coefficients with 0 days as

intercept: 1 day, $t = 1.68$, $p = 0.09$; 5 day, $t = 2.73$, $p = 0.01$; 10 day, $t = 3.16$, $p = 0.003$; Figure 4.1). In the same trials, tadpoles that had been starved for 1, 5, or 10 days vibrated significantly more than tadpoles that had been starved 0 days (overall linear model: $R^2 = 0.57$, $F_{3,44} = 19.66$, $p < 0.001$; linear model coefficients with 0 days as intercept: 1 day, $t = 5.589$, $p < 0.001$; 5 day, $t = 7.14$, $p < 0.001$; 10 day, $t = 5.88$, $p < 0.001$; Figure 4.1). Only one of the 108 stimulus mother frogs laid eggs in the lab during trials, which occurred 2 minutes after the end of a trial with a vigorously begging tadpole (6.5 minutes spent vibrating).

In trials where tadpoles of different developmental stages were presented with adult frogs, tadpoles in the middle stages swam and vibrated more than those in early and late stages such that both behaviors were better predicted by a negative quadratic term in a generalized linear model than a simple linear model (Swimming GLM quadratic coefficient = -0.001 , $t = -2.23$, $p = 0.03$; Vibrating GLM quadratic coefficient: -0.002 , $t = -3.19$, $p = 0.002$; Figure 4.2). For both swimming and vibrating behaviors, GLM's with quadratic terms provided better model fit than non-quadratic models; GLM's with quadratic terms had lower Akaike's Information Criterion (AIC) values which are smaller with a better fit model (Swimming GLM: AIC with quadratic term = -70.48 , AIC without quadratic term = -67.45 ; Vibrating GLM: AIC with quadratic term = -16.99 , AIC without quadratic term = -9.11), and improved the model fit according to Chi-squared comparisons of the models themselves (Swimming GLM: Chi-squared test $df = 57$, $p = 0.02$; Vibrating GLM: Chi-squared test $df = 57$, $p = 0.001$).

Growth Study

Tadpoles encouraged to beg for 10 minutes before feeding bouts grew less in 2 weeks than those tadpoles fed after begging for only 10 seconds (Figure 4.3; $N = 12$, $U = 36.5$, $p = 0.043$). The shape of the growth curve of unmanipulated field tadpoles fit a quadratic GLM (coefficient of quadratic term = -0.07 , $t = -12.42$, $p < 0.001$; Figure 4.2), such that tadpoles in middle stages were larger than those in early or late stages.

Discussion

We found that tadpole begging signals in *Oophaga pumilio* varied according to both short-term need and developmental stage. Across 2 weeks of development, the action of begging was costly and reduced tadpole growth. Lastly, field observations suggest that mother frogs incorporate begging signals of tadpoles when making decisions about provisioning.

Videos of behavior obtained from field cameras allowed us to quantify the duration, frequency, and outcomes of mother-tadpole interactions in the natural habitat. Observations suggest that mother frogs account for levels of need of tadpoles when depositing eggs. In most cases, mother frogs fed tadpoles when they displayed vibration behavior. In a few cases, however, mother frogs did not feed tadpoles that vibrated, which may suggest either that mother frogs did not have the eggs available to feed tadpoles, or that they were allocating their eggs to siblings living in other cups or bromeliads. Although direct competition cannot affect begging behavior in individually reared *O. pumilio*, tadpoles still compete indirectly with siblings and this may affect parental investment with both short-term and long-term consequences for offspring.

Seasonal or habitat-based variation in the resources available to parents, the number of tadpoles transported to rearing sites from an egg clutch, and the number of eggs available in the ovaries of a mother frog may all play a role in whether a mother frog responds to a given tadpole's begging signal by provisioning eggs. Future experiments that manipulate tadpole need in the field would provide a more robust test of the degree to which maternal resource allocation is based on tadpole begging signals. Additionally, to gain an understanding of the role of begging signals on maternal resource allocation that is not coupled to offspring hunger (Searcy & Nowicki 2005), vibrating tadpole models could be used to stimulate mother frogs in a manner similar to acoustic playbacks in birds and mammals (Weary & Fraser 1995, Tarwater et al. 2009).

Tadpoles that ate a nutritive egg in the same day did not beg from adult frogs, whereas tadpoles that did not eat in the same day begged vigorously. Field videos showed that mother frogs typically deposit 1-2 eggs every other day, which suggests that tadpoles are fed slightly less than 1 egg per day. Thus, these results are consistent with the hypothesis that begging signals from tadpoles vary according to short-term need and developmental stage, and they concur with research in birds, mammals, and insects indicating that offspring begging signals are a reliable indicator of need (Royle et al. 2002, Mas & Kölliker 2011). During laboratory behavior trials in the current study, only one highly stimulated female *O. pumilio* laid eggs. The reluctance of mothers to provide eggs during trials may be explained by their use of specific spatial cues in the discrimination of offspring prior to provisioning in the natural habitat (Stynoski 2009).

By decoupling two components of the tadpole response to the presence of an adult frog, swimming and vibrating, we found that swimming behavior shows a graded

response to the level of short term need, whereas vibrating behavior is either displayed strongly or not at all. It may be that these two components differ in terms of costs via growth or predation risk, and there is differential selection on these behaviors made possible by the use of the integration of visual and tactile sensory cues by tadpoles (Stynoski & Noble 2012). While swimming may be a necessary behavior for tadpoles to physically locate the parent frog's body upon arrival, vibration is likely a specific and reliable signal that is only displayed when a hungry tadpole perceives a true potential egg provider.

Tadpole begging also varied according to developmental stage. Such variation in begging could indicate that tadpoles in mid-development have higher energy requirements than those in early and late stages. Measurements of unmanipulated tadpoles across development demonstrated that tadpoles in mid-development are also longer than tadpoles in early or late development. Lower levels of need could be due to stored yolk energy in the gut in tadpoles in early stages and energy derived from reabsorption of the tail in tadpoles in late stages (Gosner 1960). Equally, variation in begging across development could indicate that the sensory, neural, muscular, and/or mechanical ability of mid-stage tadpoles is more conducive to vigorous vibration behavior than either less developed tadpoles or nearly metamorphosed tadpoles (Gosner 1960, Crump 1984, Dudley et al. 1991, Brown & Taylor 1995). Although unexamined in the current study, parents may ignore signals from tadpoles in mid-development more frequently than tadpoles in other stages independently of whether mid-stage tadpole signals are honest or not (Royle et al. 2002). Regardless, the fact that variation exists in begging across developmental stages suggests that mother frogs may benefit from

perceiving differences in begging signals and modulating their allocation of eggs to tadpoles across 6 weeks of provisioning (Royle et al. 2002).

Tadpoles that begged for a biologically relevant period of time prior to being fed grew less than tadpoles that were fed but only begged for 10 seconds, suggesting that the energy used during rigorous begging bouts (that often total 5 to 6 minutes of full body vibration) presents a cost to tadpoles. That cost could serve to maintain honesty in the signal such that in the interest of conserving energy tadpoles would only choose to beg when they are both hungry and likely to receive food. Some studies of birds have shown that begging may reduce growth significantly enough to negatively impact long-term fitness (Rodríguez-Gironés et al. 2001), whereas other studies have not found such an effect (McCarty 1996, Bachman & Chappell 1998). In anurans, a cost that leads to reduced growth and size at metamorphosis is likely to have detrimental effects on long-term fitness (Smith 1987).

In *O. pumilio*, the risk of predation may also serve as a cost to begging that maintains signal honesty. Most tadpoles (67%) in this species are eaten prior to metamorphosis (Maple 2002). Also, tadpoles are able to visually discriminate between conspecific frogs and predators and respond to the presence of a predator by a reduction in movement (Stynoski & Noble 2012); presumably, movement such as swimming and vibrating in the presence of a predator would reduce a tadpole's chance of survival. It would be informative to examine whether tadpoles are less likely to reduce movement in the presence of a predator if they are hungry. The timing of mother feeding visits may represent a balance between the level of tadpole need and the risk of predation due to offspring activity, as well as the increased predation risk and energy cost to the mother

frog when traveling to visit and feed tadpoles (McDonald et al. 2009; Tarwater et al. 2009). The relative costs of begging behavior to parents and offspring via predation and offspring growth may vary widely across taxa (Smiseth & Parker 2008).

The current study demonstrates that the begging behavior of *O. pumilio* tadpoles varies predictably in intensity as a result of short-term level of need and developmental stage. It also shows that mother frogs appear to use the honest begging signals of tadpoles to appropriately match their level of provisioning to the level of need of tadpoles. Lastly, it shows that the production of begging signals in tadpoles presents a significant cost in metabolic energy and causes a reduction in tadpole growth. A cost to growth and perhaps a cost via predation risk along with provisioning that differentially benefits tadpoles with higher levels of need likely maintain the honesty of tadpole begging signals in *O. pumilio* and allow mother frogs to rely on those signals to allocate resources.

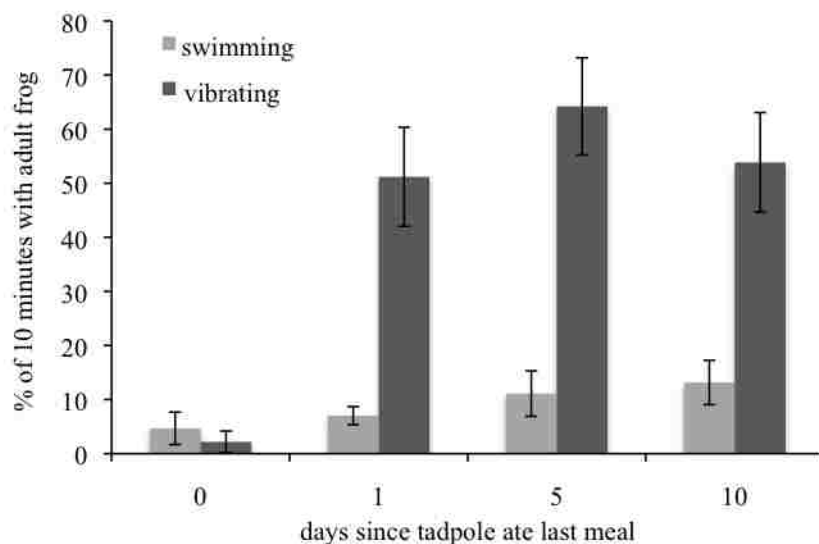


Figure 4.1 Behavioral response of *O. pumilio* tadpoles during 10-minute trials with adult frogs following different periods of starvation. Linear models show that in the presence of an adult frog, tadpoles starved for 5 or 10 days swam more than those starved 0 or 1 days and those starved for 1, 5 or 10 days vibrated more than those starved 0 days. Error bars represent SE.

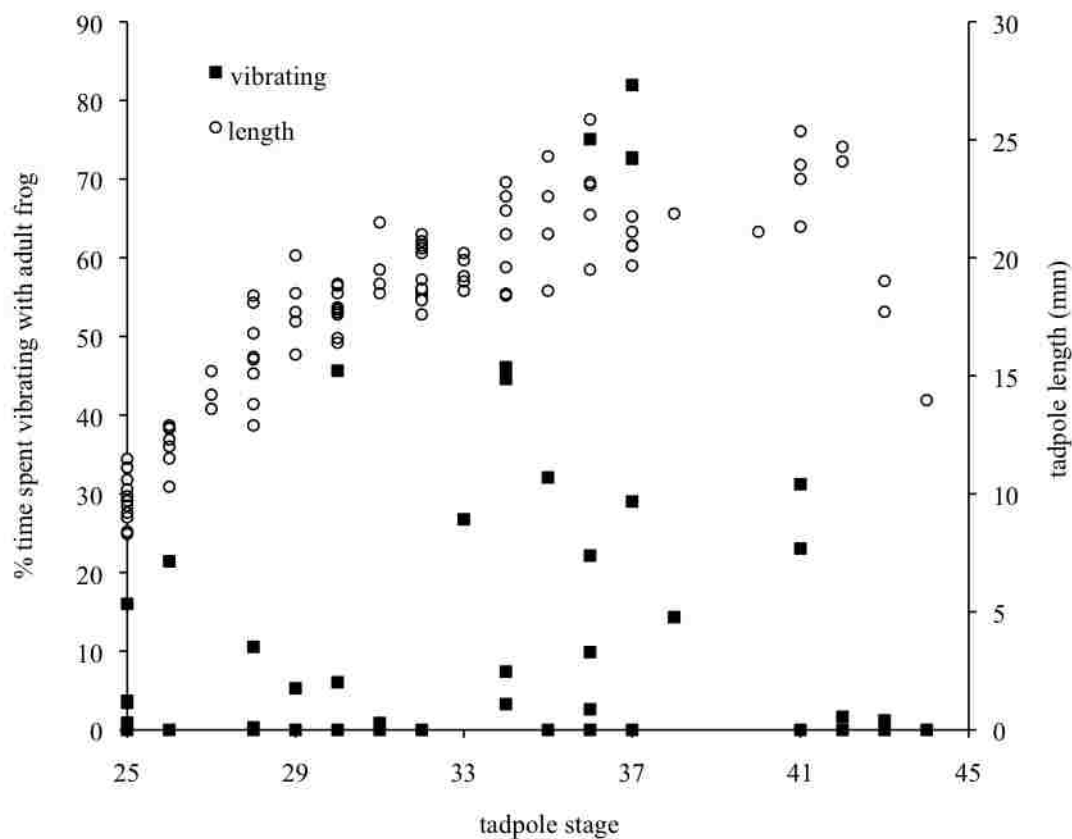


Figure 4.2 Left y-axis shows behavioral response of *O. pumilio* tadpoles in various stages of development during 10-minute trials with adult frogs and right y-axis shows length of unmanipulated *O. pumilio* tadpoles in the field at various stages of development. The intensity of begging behavior and tadpole length follow a similar pattern across development, and both are best demonstrated by a quadratic model.

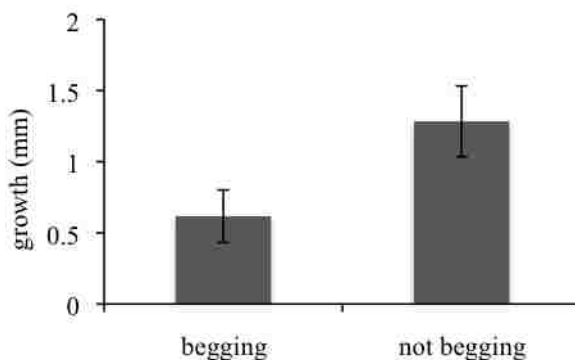


Figure 4.3 Growth of *O. pumilio* tadpoles fed every 48 hours following exposure to an adult male frog for either 10 minutes (begging) or 10 seconds (not begging). Tadpoles that begged grew less in two weeks than tadpoles that did not beg. Error bars represent SE.

Chapter 5: Ontogeny of alkaloid-based chemical defenses⁴

Summary

Many organisms use chemical defenses in various stages of life to reduce predation risk. Chemical defenses can be manufactured or sequestered, such as in some dendrobatid frogs that obtain alkaloid-based chemical defenses from an arthropod diet. A wealth of studies about alkaloids in dendrobatid frogs is thus far limited to adults. Here, we identified and quantified the alkaloids in all life stages of a dendrobatid frog, *Oophaga pumilio*, that displays complex parental care: at hatching, *O. pumilio* tadpoles are transported to water pools called phytotelmata, and then for at least 6 weeks, mother frogs return to supply tadpoles with an obligate diet of unfertilized nutritive eggs. We collected adults, juveniles, tadpoles, and nutritive and ovarian eggs, as well as early-stage tadpoles hand-reared with eggs of an alkaloid-free heterospecific frog. We detected alkaloids in mid- and late-stage tadpoles, juveniles, and adults, and found that the quantity and diversity of alkaloids increase with size. No alkaloids were detected in early-stage tadpoles or tadpoles fed with heterospecific eggs, perhaps because these tadpoles do not yet possess any form of granular glands. We also did not detect alkaloids in nutritive eggs, although alkaloids were detected in ovarian eggs in this and other studies. In frogs, alkaloid quantity increased dramatically at sexual maturity, which is ontogenetically timed with a similar increase in granular gland size. Because the same alkaloids derived from terrestrial arthropods were detected in adults and phytotelm-dwelling tadpoles, this

⁴ This chapter will be submitted for publication with authors Stynoski, J.L., Torres-Mendoza, Y., Sasa-Marin, M., and Saporito, R.A.

frog may be the first animal known to actively provision its post-hatch offspring with chemical defenses.

Background

Predation is a particularly strong selective factor that has led to the evolution of several mechanisms that reduce the risk of predation. For example, animals can use unpalatable, noxious, or poisonous chemical defenses that are either synthesized or sequestered from external sources (Mebis 2001). The use of sequestered chemical defenses to avoid predation has evolved in flatworms, nudibranchs, arthropods, birds, snakes, and amphibians (Termonia et al. 2002, Saporito et al. 2011). The physiological mechanisms of uptake and storage as well as the ecological function of chemical sequestration have been well studied in phytophagous arthropods (Bowers 1990, Bowers & Williams 1995) but are not as well understood in vertebrates. The use of chemical defenses can be accompanied by the use of aposematic coloration as a warning signal of unpalatability to potential predators (Ruxton et al. 2004).

Individuals can use chemicals not only to defend themselves but also as an antipredator mechanism to protect their offspring. Parents in diverse taxa use a wide range of chemical defenses to reduce predation risk by depositing chemicals within the bodies of young prior to hatching or birth. For example, before hatching, young can be provisioned with macrolides (Nudibranch, *Hexabranhus sanguineus*, Pawlik et al. 1988), miriamin (Slugs, *Arion sp.*, Schroeder et al. 1999), peptides (tunicates, Lindquist et al. 1992) and alkaloids including tetrodotoxin (TTX; Harlequin frog, *Atelopus chiriquiensis*, Pavelka et al. 1977; Rough-skinned newts, *Taricha granulosa*, Hanifin et

al. 2003, Gall et al. 2011; Pufferfish, *Fugu obscurus*, Wang & Fan 2010; Blue-ringed octopus, *Hapalochlaena lunulata*, Williams et al. 2011), pyrrolizidines (moths, *Utetheisa sp.*, Dussourd et al. 1988), bufadienolides (snake, *Rhabdophis tigrinus*, Hutchinson et al. 2008, Mori et al. 2011; toads, *Rhinella marina*, Hayes et al. 2009) and others (tunicates, Lindquist et al. 1992). *Rhabdophis tigrinus* is an Asian snake that sequesters bufadienolides obtained from eating toads and later deposits the chemicals in its offspring (Hutchinson et al. 2008). The quantity of alkaloids found in *R. tigrinus* offspring mirrors that found in the mother, and alkaloids are provisioned both by deposition in the yolk and again later via transfer through the oviduct (Hutchinson et al. 2008, Mori et al. 2011). In some taxa, offspring produce or sequester their own chemical defenses (Caterpillars, Nishida 2002; Blue-ringed octopus, *Hapalochlaena lunulata*, Williams et al. 2011) but in some cases chemical defenses are solely maternally derived (*Rhabdophis tigrinus*, Hutchinson et al. 2008; *Rhinella marina*, Crossland et al. 2011).

Maternal deposition of unpalatable chemical defenses prior to hatching or birth is well documented in various species of amphibians (see Gunzburger & Travis 2005). Mother rough-skinned newts (*Taricha granulosa*) invest TTX in the yolks of their eggs, and the quantity of TTX in the skin of a given mother correlates to the quantity of TTX found in her eggs (Hanifin et al. 2003, Gall et al. 2011). The Costa Rican Harlequin Frog (*Atelopus chiriquiensis*) also invests TTX in its eggs (Pavelka et al. 1977). Mother cane toads (*Rhinella marina*) provision their eggs with bufadienolides prior to hatching, which renders the eggs, and later the hatched tadpoles, lethal prey for *Litoria rothii* tadpoles, although the quantity of the chemicals and their antipredator effectiveness decrease across development (Brodie & Formanowicz 1987, Crossland & Alford 1998, Hayes et

al. 2009). Eggs of boreal toads (*Anaxyrus* [=*Bufo*] *boreas*) are also protected with bufodienolides, but only until the larval stage is reached (Benard & Fordyce 2003). Oocytes of redbelly toads (*Melanophryniscus* sp.) contain a variety of alkaloids (R.A. Saporito, unpublished data, but see Mebs et al. 2007). Evidence of chemical defenses was sought but not found in young *Leptobatrachum hazzelti* (Daly et al. 2004).

Dendrobatid frogs represent one of the best-studied examples of a vertebrate that sequesters its chemical defenses from dietary sources. Brightly colored members of the family Dendrobatidae possess alkaloid-based chemical defenses that are obtained from a diet of alkaloid-containing mites, ants, millipedes, and beetles (reviewed in Saporito et al. 2011). As a result of these defenses, some dendrobatids are unpalatable (and in some cases toxic) to a variety of invertebrate and vertebrate predators (Daly & Myers 1967, Daly et al. 1978, Brodie & Tumbarello 1978, Fritz et al. 1981, Szelistowski 1985). However, most studies of chemical defense in dendrobatids have focused on adults (e.g., Daly et al. 1987, Saporito et al. 2007c), resulting in relatively little information available on defense in early life-stages.

Daly et al. (1987) suggested that alkaloid-based chemical defenses might appear only in adult dendrobatids because the cutaneous granular glands that contain the alkaloids mature just prior to metamorphosis. These glands continue to enlarge later throughout the juvenile frog phase, especially at sexual maturity (Neuwirth et al. 1979, Angel et al. 2003, Delfino et al. 2010, Saporito et al. 2010a). However, trace amounts of an unknown alkaloid similar to batrachotoxin-homobatrachotoxin were present in eggs of *Phyllobates terribilis* (but not in a tadpole at stage 35 following Gosner 1960; Myers et al. 1978), and trace amounts of a pyrrolizidine alkaloid were identified in one juvenile *Dendrobates*

auratus that had been raised on alkaloid-free fruitflies since the tadpole phase (Daly et al. 1994). Recent studies of cutaneous granular glands in dendrobatid tadpoles have clarified their ontogeny. In *Phylllobates bicolor*, an early developmental form of granular glands is present in the skin of tadpoles by tadpole stage 38 and these early glands progress to the structure of mature glands by stage 43 (Delfino et al. 2010). In *Dendrobates auratus*, early structures and poison product are present by stages 38-40 while mature structure and product are visible in stages 44-45 (Angel et al. 2003). In *Oophaga pumilio*, the skin of recently metamorphosed juveniles already contains mature glands, albeit significantly smaller than in adults (Saporito et al. 2010a). Our current understanding of chemical defenses in pre-juvenile and juvenile stages of dendrobatid frogs is limited and anecdotal (Saporito et al. 2010a).

The adaptive radiation of dendrobatid frogs displays an exceptional diversity of forms of parental care (Grant et al. 2006). Parental behaviors can include guarding of a terrestrial egg clutch, dorsal transport of tadpoles at hatching to water pools in plants (“phytotelmata”), and even supplying tadpoles with nutritive eggs. These behaviors vary across species with regards to whether the mother, the father, or both parents perform a particular behavior (Grant et al. 2006). In the strawberry poison frog (*Oophaga pumilio*), egg clutches are guarded by father frogs in the leaf litter for 7-10 days until mother frogs return to transport tadpoles to individual phytotelmata (Weygoldt 1980, Brust 1993). Then, for approximately 6 weeks, mother frogs return to visit tadpoles in phytotelmata every 1-5 days and supply them with unfertilized eggs as food (Brust 1993, Stynoski 2009). Because *O. pumilio* tadpoles are known to eat only maternal eggs as opposed to alkaloid-containing arthropods (Brust 1993, Stynoski 2009), tadpoles might not contain

any alkaloid-based chemical defenses. However, it is possible that females provision eggs with alkaloids and thus provide developing tadpoles with chemical defenses. In this study, we determined the ontogeny of alkaloid-based chemical defenses in *O. pumilio* and tested for evidence that mother *O. pumilio* provision their tadpoles with alkaloid-based chemical defenses in nutritive eggs.

Methods

Sample Collection

To determine the diversity and amount of alkaloid-based chemical defenses that exist across development in *Oophaga pumilio*, we identified and quantified the alkaloids present in an age-series of wild-caught specimens. We collected specimens from the area known as the Huertos Plots at the La Selva Biological Station, Costa Rica in July 2010. Tadpoles and nutritive eggs were collected from an existing setup of artificial tadpole-rearing sites made from 15 ml plastic cups attached to trees. Mother frogs deposit and rear tadpoles in these cups just as they do in natural phytotelmata (Stynoski 2009, Stynoski & Noble 2012). From these cups, we collected 15 tadpoles in a range of developmental stages (25 to 45; Gosner 1960) as well as 3 samples of 5 unfertilized eggs that different mothers had supplied as nutrition for tadpoles. From the leaf litter, we collected 10 free-living juvenile frogs in a range of sizes (10 to 18 mm snout-to-vent length; SVL) as well as 5 adult males and 5 adult females (19 to 22 mm SVL). It is not known whether any of the collected individuals are genetically related.

To test whether any alkaloids found in tadpoles would be of maternal origin, we collected 4 tadpoles from cups in the field within a few hours of the tadpole being

deposited by mother frogs. We reared these tadpoles in the water from which they were collected in glass vials in the laboratory. We fed them every 48 hours with the yolk of eggs of the Red-Eyed Treefrog, *Agalychnis callidryas*, which are not known to contain alkaloids. We also collected three samples of five *A. callidryas* eggs.

In the laboratory, we removed the skins from all metamorphosed specimens as well as oocytes from the ovaries of adult females. We placed each sample (skin, oocytes, eggs, or whole tadpole) in a 4 ml glass vial with a Teflon-lined cap in 4 ml of 100% methanol. Vials were weighed before and after adding the specimen and wrapped in parafilm before being stored in the freezer.

Chemical Analysis

We transported specimens to John Carroll University for chemical analysis with gas chromatography–mass spectrometry (GC-MS) on a Varian Saturn 2100T ion trap MS instrument coupled to a Varian 3900 GC with a 30 m x 0.25 mm i.d. Varian Factor Four VF-5ms fused silica column. The GC separation of alkaloids was achieved using a temperature program from 100 to 280°C at a rate of 10°C per minute with He as the carrier gas (1 ml/min). Alkaloid fractions were analyzed using electron impact MS and chemical ionization MS with methanol as the reagent gas.

Individual alkaloid fractions were prepared from methanol extracts of stored samples following methods in Saporito et al. (2006) and Saporito et al. (2010b). For each sample, 10 μg of nicotine ((-)-nicotine $\geq 99\%$, Sigma-Aldrich, Milwaukee, Wisconsin) and 50 μL of 1N HCl were added to 1 ml of the original MeOH extract. The combined MeOH extract was concentrated with N_2 to 100 μL and then diluted with 200 μL of water. This

solution was then extracted 4 times, each time with 300 μL of hexane. The HCl fraction was then basified with saturated NaHCO_3 , followed by extraction 3 times, each time with 300 μL of ethyl acetate. The combined ethyl acetate fraction was then dried with anhydrous Na_2SO_4 and concentrated to 100 microliters.

Individual alkaloids in each sample were identified by comparing the observed MS properties and GC retention times with those of previously reported anuran alkaloids (Daly et al. 2005). Anuran alkaloids have been assigned code names that consist of a bold-faced number corresponding to the nominal mass and a bold-faced letter to distinguish alkaloids of the same nominal mass (Daly et al. 2005). To determine the quantity of alkaloids in each sample, we compared the observed alkaloid peak area to the peak area of the nicotine internal standard, using a Varian MS Workstation v.6.9 SPI. Nicotine was chosen as an internal standard because its retention time is outside of the chromatographic range of most frog alkaloids, and has been used previously to quantify alkaloids (see Saporito et al. 2010b, Saporito et al. 2011).

Data Analysis

All data were tested for normality and equal variances before selecting parametric tests. To determine whether the quantity and diversity of alkaloids that we detected differed among samples from each life stage (tadpole, juvenile, and adult), we used ANOVAs with Tukey HSD posthoc tests. To determine whether the quantity of alkaloids and the number of unique alkaloids was correlated, we used a linear regression. To determine the influence of anuran size on alkaloid quantity and diversity, we used both individual linear regressions and ANCOVAs of SVL and skin weight in the case of frogs

and total length and total weight in the case of tadpoles. We used t-tests to determine whether we detected a higher quantity of alkaloids per unit mass of skin in either adult or juvenile frogs and to see whether we detected a higher total quantity of alkaloids in adult males or females.

Results

Chemical analysis revealed 117 unique alkaloids among all samples (including isomers; Table 5.1). We detected 62 unique alkaloids in adult females and 73 in adult males. All alkaloids seen in females were also seen in males. In juvenile frogs we detected 71 alkaloids, including 23 that we did not detect in adult frogs and 31 that we did not detect in tadpoles. In tadpoles, we detected 55 unique alkaloids, including 20 that we did not detect in adult frogs and 14 that we did not detect in juvenile frogs. We did not detect any alkaloids in the youngest (stage 25) tadpoles or in nutritive eggs, however alkaloids were detected in ovarian eggs.

On average for each of the samples that contained alkaloids, we detected a higher quantity and diversity of alkaloids in adult frogs (mean \pm SE quantity: $518 \pm 238 \mu\text{g}$; diversity: 37 ± 2 types) than in juveniles (quantity: $27 \pm 10 \mu\text{g}$; diversity: 25 ± 4 types) or tadpoles (quantity: $11 \pm 4 \mu\text{g}$; diversity: 13 ± 2 types; Figure 5.1; Table 5.1). The quantity of alkaloids was significantly different between these three life stages (ANOVA: $F_{2,33} = 57.47$, $p < 0.0001$), such that adult frogs contained more alkaloids than juveniles and tadpoles (Tukey: both $p < 0.0001$), but the quantity contained in juveniles did not differ from that in tadpoles (Tukey: $p = 0.94$). The number of unique alkaloids was significantly different between all life stages (ANOVA: $F_{2,33} = 23.68$, $p < 0.0001$; Tukey: Juvenile-

Adult $p = 0.01$; Tadpole-Adult $p < 0.0001$; Tadpole-Juvenile $p = 0.004$). Across all samples, the quantity of alkaloids and the number of unique alkaloids in each sample were linearly related ($R^2 = 0.47$, $F_{1,34} = 31.00$, $p < 0.0001$). In juvenile and adult frogs, both SVL and skin mass showed linear relationships with both alkaloid quantity and alkaloid diversity (Table 5.2). In tadpoles, both length and mass showed a linear relationship with alkaloid quantity, but no relationship existed between length or mass and alkaloid diversity (Table 5.2). Adult frogs contained a higher quantity of alkaloid per unit of skin mass ($5.1 \mu\text{g}/\text{mg}$) than juvenile frogs ($0.7 \mu\text{g}/\text{mg}$; $t = 6.66$, $df = 11.96$, $p < 0.0001$; Figure 5.2). We detected a higher total quantity of alkaloids in adult females ($629 \pm 95 \mu\text{g}$) than in adult males ($409 \pm 102 \mu\text{g}$) although this difference was not statistically significant ($t = 1.57$; $df = 8$, $p = 0.15$; Figure 5.3).

In general, alkaloid structural classes derived from mites, ants, and beetles were detected in adult male and female frogs, juvenile frogs, and tadpoles beyond stage 35 (Table 5.3). Spiropyrrolizidines were only detected in adult frogs, piperidines and lehmizidines were not detected in tadpoles, and pumiliotoxins were detected in juvenile frogs and tadpoles, but not in adult frogs.

Across all samples, the most common alkaloids based on quantity ($> 50 \mu\text{g}$ per sample) were histrionicotoxin (HTX) **291A** ($587 \mu\text{g}$), Unknown 1 ($187 \mu\text{g}$), Unknown 2 ($181 \mu\text{g}$), Unknown 3 ($170 \mu\text{g}$), Unclass **209G** ($142 \mu\text{g}$), 5,8-disubstituted indolizidine (5,8-I) **195G** ($123 \mu\text{g}$), 5,8-I **207A** ($54 \mu\text{g}$), and allopumiliotoxin (aPTX) **241H** ($53 \mu\text{g}$), and on the basis of frequency (detected in at least 30 of 40 samples) were 5,8-I **207A** (40), 5,8-I **195G** (36), 5,8-I **207A** (36), 5,6,8-trisubstituted indolizidine (5,6,8-I) **251S** (36), HTX **291A** (33), 5,6,8-I **223A** (33), 5,6,8-I **223A** (32), and 5,8-I **205A** (31). The

most common alkaloids detected in adult frogs were HTX **291A**, Unknown 2, Unknown 1, and Unknown 3 on the basis of quantity (average >50 μg per sample) and HTX **291A**, Unknown 1, 5,8-I **207A**, Unknown 4, pyrrolidine (Pyr) **277D**, 5,8-I **207A**, and 5,8-I **205A** on the basis of frequency (detected in all 10 samples). The most common alkaloid(s) in juvenile frogs was HTX **291A** on the basis of quantity (>35 μg per sample) and 5,8-I **195I**, 5,8-I **207A**, and 5,6,8-I **251S** on the basis of frequency (detected in at least 9 of 10 samples). The most common alkaloids in tadpoles were HTX **291A** and pumiliotoxin (PTX) **209F** on the basis of quantity (>10 μg per sample) and 5,8-I **195I**, 5,6,8-I **223A**, and 5,8-I **207A** on the basis of frequency (within at least 10 of 15 samples).

We fed 4 tadpoles at stage 25 with eggs of *A. callidryas* for 37 to 42 days. During that time, tadpoles grew 5.0 ± 0.7 mm and progressed to stage 28. At the time of collection, they weighed an average of 3.7 ± 0.005 mg. We did not detect alkaloids in either hand-reared tadpoles or unmanipulated samples of *A. callidryas* eggs.

Discussion

The present study demonstrates that alkaloid-based chemicals are present not only in adult *O. pumilio* but also in juvenile frogs and tadpoles, albeit in much smaller quantities. The quantity and diversity of alkaloids detected increased with SVL and skin weight of juvenile and adult frogs, and the quantity of alkaloids detected increased with length and mass of tadpoles. On average, older *O. pumilio* contained more alkaloids than younger *O. pumilio*, but this was not always the case among individuals as there was a high amount of variation that is likely associated with factors like differences in

arthropod availability, foraging behavior, and uptake efficiency of alkaloids encountered while foraging (reviewed in Saporito et al. 2011).

Both ant- and mite-derived alkaloids were widely detected in tadpoles, which is consistent with the hypothesis that mother frogs provision tadpoles with alkaloids because it suggests that the chemical defenses of aquatic tadpoles are derived from a terrestrial habitat. The most commonly detected alkaloids across all samples were an ant-derived histrionicotoxin (HTX **291A**), four mite-derived 5,8-disubstituted indolizidines (5,8-I **195G**, 5,8-I **205A**, and two isomers of 5,8-I **207A**), three mite-derived 5,6,8-trisubstituted indolizidines (5,6,8-I **251S**, and two isomers of 5,6,8-I, **223A**), a mite-derived allopumiliotoxin (aPTX **241H**) and four new or unclassified alkaloids (Unknown 1, two isomers of Unknown 2, and Unclass **209G**; Saporito et al. 2007a). Most unclassified alkaloids have been identified in mites (Saporito et al. 2011). The alkaloids on this list were also the most common alkaloids specifically within the tadpole, juvenile, and adult life stages. The alkaloids Unknown 4 and ant-derived Pyr **277D** were also especially common but only in adults, and a mite-derived pumiliotoxin, PTX **209F**, was especially common in tadpoles. Thus, with a few exceptions, the identities of individual alkaloids in samples from tadpoles through adults were similar, with no clear pattern to suggest separation among the common alkaloids found in different life stages.

Some patterns of variation emerged regarding alkaloid structural classes across life stages of *O. pumilio*. Spiropyrrrolizidines, which appear to be largely derived from siphonotid millipedes (but have also been found in one sample of oribatid mites; Saporito et al. 2007a), were only detected in adult frogs, perhaps indicating that juveniles are gape-limited and are unable to ingest millipedes. Although we detected numerous other

ant-derived alkaloids in tadpoles, ant-derived piperidines and lehmizidines were not detected. Piperidines may not have been present in tadpoles because they are not taken up as well as other alkaloids and were also detected only in small quantities in juveniles and adults (Daly et al. 1994). In addition to not being present in tadpoles, lehmizidines were present in much smaller amounts in juveniles than in adults and it could be that they are derived from larger ants that are more difficult for juveniles to consume. It could also be that the ants that contain piperidines and/or lehmizidines are rare, and that these alkaloids are seen in higher quantities in adults because adults have had more time to encounter those particular ants and accumulate the alkaloids. Pumiliotoxins are largely derived from mites and are a particularly toxic and common alkaloid class detected in *O. pumilio* adults in numerous studies (Saporito et al. 2007a). Here, they were detected in juvenile frogs and tadpoles but for an unknown reason were not found in adult frogs.

We did not detect alkaloids either in nutritive eggs or in recently transported stage 25 tadpoles. It is possible that we were not able to detect alkaloids that may be present in very small quantities in these samples. We detected small quantities of alkaloids in stage 34 tadpoles, which have likely already consumed 8-15 nutritive eggs and thus may have accumulated enough alkaloid to be detected with our methods. It could also be that tadpoles do not, in fact, acquire their alkaloids from the eggs supplied by mothers, but instead from other sources such as microinvertebrates present in the water of bromeliads or from passively transferred alkaloids that may leach from mother frogs' skin during provisioning visits. However, tadpoles reared to stage 28 on eggs of an alkaloid-free species of frog in water from their original phytotelm did not contain alkaloids, suggesting that microinvertebrates living in phytotelm water are not a source of alkaloids

for tadpoles. It is also possible that mother frogs can choose to withhold alkaloids from some nutritive eggs until tadpoles reach a later stage, although ovarian eggs both in this study and others do contain small amounts of alkaloids (Saporito et al. 2011). Lastly, it is possible that because of an unknown methodological issue we could not detect alkaloids that are actually present in nutritive eggs, but that tadpoles in stage 25 truly do not contain alkaloids and are not capable of sequestering the alkaloids in eggs provided to them by mother frogs as are tadpoles in stage 34. Although an early developmental form of granular glands is present in the dendrobatid *Phyllobates bicolor* at stage 38 and these mature by stage 43, there is no form of cutaneous glands present in stage 25 *P. bicolor* tadpoles (Delfino et al. 2010). However, tadpoles of dendrobatid species that display oophagy such as *O. pumilio* may follow a different structural ontogeny of granular glands than those species such as *P. bicolor* that do not receive eggs from parents and thus may not contain alkaloids (Grant et al. 2006). More information is needed regarding the ultrastructural ontogeny of granular glands in tadpoles of *O. pumilio* specifically to better interpret these findings.

We found that adult frogs (>19 mm SVL) contained approximately 7 times as much alkaloid per unit mass of skin as juvenile frogs (<18 mm SVL). In similar anecdotal studies, samples of adult *O. pumilio* contained approximately 5 times as much alkaloid as a sample of juveniles (based on peak area of published chromatograms measured with ImageJ [NIH] from Daly et al. 2002) and samples of adult *Phyllobates terribilis* contained approximately 5 times as much batrachotoxin-homobatrachotoxin as a sample of juveniles (Myers et al. 1978). Similarly, the granular glands of *O. pumilio* increase in size by 41% when frogs increase in SVL from 18 mm to 19 mm (Saporito et al. 2010a).

Thus, there is a correlation between gland size and alkaloid quantity that corresponds with sexual maturity in *O. pumilio*, which may indicate either that additional alkaloid sequestration increases the size of glands or that an increase in gland size allows for an increase in sequestration. Steroid hormones may play a role in the sudden increase of granular gland size and quantity of alkaloid chemical defenses at sexual maturity. In salamanders, exogenous testosterone induces an increase in the size of granular glands just prior to sexual maturation (Norris et al. 1989), and sex steroid hormones are widely known to play a role in stimulating increases in skin gland size and activity in a variety of vertebrates (Cerbón et al. 1990, Abell 1998, Pelletier & Ren 2004).

In the current study we detected a 53% higher quantity of alkaloids in adult females than in adult males on average, although given overlap in variation among individuals in the sexes and low sample size, this difference was not statistically significant. A sex difference in alkaloid quantity is consistent with a previous study of a population of *O. pumilio* in Bocas del Toro, Panama in which females contained a 71% higher quantity of alkaloids than males on average (statistically significant difference; Saporito et al. 2010b). The sex difference raises questions about the role of alkaloids contained in the skins of mother frogs in contrast to male frogs given that mother frogs might invest in both self-defense and offspring defense. Future studies should investigate if alkaloids fated for oocytes are first sequestered in the glands of the skin and then shuttled to either the ovary or the oviduct, or instead if there is a mechanism for alkaloids to be transferred directly to reproductive organs. Additionally, experiments might test whether the chemical defenses sequestered by mothers are diminished by the act of provisioning eggs with alkaloids during the parental phase of the reproductive cycle and whether mother

frogs target a particularly high alkaloid diet at this time. Lastly, studies should explore the relationship between the quantity and diversity of alkaloids present in a given mother frog with that of her own genetic offspring in order to determine how mothers might modulate provisioning of alkaloids by type and amount and to verify that mothers are indeed provisioning alkaloids to tadpoles via nutritive eggs.

Experimental evidence suggests that bright coloration in adult *O. pumilio* is aposematic (Saporito et al. 2007b), but coloration in this species first appears during the tadpole phase (stage 42-43; J.L. Stynoski, pers. obs.). All tadpoles above stage 42 in this study contained alkaloids (up to 60 μg in one stage 44 tadpole), suggesting that the emergence of dorsal coloration might be ontogenetically timed with the onset of unpalatability or toxicity. However, more work is necessary to determine whether the quantity of alkaloids seen in late stage tadpoles and/or young juvenile frogs acts as an effective defense against tadpole predators in phytotelmata or juvenile frog predators in the leaf litter. If this quantity of alkaloids did not deter predators, the appearance of dorsal coloration in tadpoles could be considered a form of intraspecific mimicry that relies on the unpalatability of older individuals to prevent predation by imitating their aposematic coloration (Saporito et al. 2010a). Eggs of *Utethesia* moths containing 0.8 μg of alkaloid per egg were defended against ants, coccinellid beetles, and chryopsid larvae (Dussourd et al. 1988) and PTX **251D** is lethal to mosquitoes at 0.1 $\mu\text{g}/\text{cm}^2$ (Weldon et al. 2006), suggesting that the quantity of alkaloids present in *O. pumilio* tadpoles and juveniles would be an effective defense at least against some potential predators.

In conclusion, we found that alkaloids are present in tadpole, juvenile, and adult life stages of *O. pumilio* and that alkaloid quantity and diversity increase with size, especially

at sexual maturity. Given that we detected a similar suite of alkaloids across life stages that are derived from a variety of terrestrial sources, it is likely that mother frogs provision alkaloids to tadpoles in phytotelmata.

Table 5.1 Quantity and number of unique alkaloids detected in *Oophaga pumilio* samples in various life stages at La Selva, Costa Rica.

Life Stage	Length or SVL (mm)	Sample Mass (mg)	Alkaloid Quantity (μ g)	Number of Alkaloids
Nutritive Eggs	NA	NA	0	0
Nutritive Eggs	NA	NA	0	0
Nutritive Eggs	NA	NA	0	0
Tadpole Stage 25	10.2	2.1	0	0
Tadpole Stage 25	12.0	1.4	0	0
Tadpole Stage 34	19.3	128.7	2	6
Tadpole Stage 34	22.4	131.6	3	7
Tadpole Stage 37	20.4	90.5	1	3
Tadpole Stage 37	22.1	122.4	6	9
Tadpole Stage 38	22.7	114.4	3	7
Tadpole Stage 39	23.1	147.7	12	7
Tadpole Stage 41	23.5	231.1	8	8
Tadpole Stage 41	25.0	170.2	12	15
Tadpole Stage 42	20.1	147.8	9	18
Tadpole Stage 42	23.2	161.5	12	26
Tadpole Stage 44	12.2	161.7	7	21
Tadpole Stage 44	10.5	213.4	60	21
Tadpole Stage 45	10.7	NA	22	25
Tadpole Stage 45	9.4	119.3	6	14
Juvenile	11.3	16.7	0.6	2
Juvenile	11.9	23.1	5	18
Juvenile	11.9	23.8	6	21
Juvenile	13.6	36.0	13	23
Juvenile	14.4	42.6	87	21
Juvenile	14.6	33.7	35	24
Juvenile	15	42.2	8	22
Juvenile	15.6	31.6	27	43
Juvenile	16.6	49.2	13	29
Juvenile	16.9	38.7	83	44
Adult male	19.0	108.7	486	26
Adult male	19.9	92.4	128	32
Adult male	20.2	101.0	320	41
Adult male	20.6	99.2	748	43
Adult male	21.4	76.4	363	37
Adult female	19.2	66.8	260	26
Adult female	20.0	103.1	635	43
Adult female	21.1	115.5	773	44
Adult female	21.6	108.9	743	37
Adult female	23.6	127.5	732	43

Table 5.2 Results of linear regressions (LR) and ANCOVAs (AN) used to determine the influence of size on alkaloid quantity and diversity among *Oophaga pumilio* frogs and tadpoles. Asterisk (*) indicates $p < 0.05$.

Test	Factor	R ²	F	df	p
<i>Frogs: Alkaloid quantity</i>					
LR	SVL	0.70	42.02	1,18	<0.0001*
LR	Skin mass	0.82	81.05	1,18	<0.0001*
AN	SVL * Skin mass	0.85	29.42	3,16	<0.0001*
	SVL		72.98	1,16	<0.0001*
	Skin Mass		12.34	1,16	0.002*
	SVL:Skin Mass		2.93	1,16	0.11
<i>Frogs: Alkaloid diversity</i>					
LR	SVL	0.60	27.30	1,18	<0.0001*
LR	Skin mass	0.42	13.49	1,18	0.002*
AN	SVL * Skin mass	0.64	9.53	3,16	0.0007*
	SVL		19.10	1,16	0.0005*
	Skin Mass		9.28	1,16	0.008*
	SVL:Skin Mass		0.21	1,16	0.66
<i>Tadpoles: Alkaloid quantity</i>					
LR	Length	0.24	3.70	1,12	0.08
LR	Mass	0.36	6.25	1,11	0.03*
AN	Length * Mass	0.85	16.99	3,9	0.0004*
	Length		12.28	1,9	0.007*
	Mass		19.70	1,9	0.002*
	Length:Mass		18.98	1,9	0.002*
<i>Tadpoles: Alkaloid diversity</i>					
LR	Length	0.25	3.90	1,12	0.07
LR	Mass	0.20	2.80	1,11	0.12
AN	Length * Mass	0.33	1.51	3,9	0.28
	Length		2.75	1,9	0.13
	Mass		1.67	1,9	0.23
	Length:Mass		0.10	1,9	0.76

Table 5.3 Quantity, structural class, and insect origin of alkaloids detected in male, female, and juvenile frogs as well as tadpoles of *Oophaga pumilio* at La Selva, Costa Rica.

Alkaloid	Alkaloid Structural Class (insect origin)	Average Quantity of Alkaloid (μg)			
		Male	Female	Juvenile	Tadpole
HTX 291A	HTX (ant)	175	37	26	587
Unknown 1		59	3	3	185
Unknown 2		60	0	0	182
Unknown 3		51	0	0	171
Unclass 209G Iso 2		48	0	0	143
5,8-I 195G	5,8-I (mite)	35	0	0	124
5,8-I 207A Iso	5,8-I (mite)	17	1	2	54
aPTX 241H Iso	aPTX (mite)	17	2	1	53
5,8-I 195I	5,8-I (mite)	14	1	2	45
5,6,8-I 223A	5,6,8-I (mite)	15	0	1	45
3,5-P 251K Iso	3,5-P (ant)	13	0	0	42
Lehm 275A	Lehm (ant)	14	1	0	38
Pyr 253I	Pyr (ant)	11	1	0	35
Unclass 209G		11	0	0	33
Unknown 4		11	0	0	33
Unknown 5		15	0	0	29
Unknown 6		14	0	0	28
Unknown 7		14	0	0	28
Unclass 209G Iso		8	1	0	26
Pyr 277D	Pyr (ant)	8	1	0	26
5,8-I-271A	5,8-I (mite)	9	0	0	26
Spiro 252A	Spiro (millipede)	10	0	0	24
Unknown 8		12	0	0	23
5,6,8-I 251S Iso	5,6,8-I (mite)	8	0	0	23
1,4-Q 279E	1,4-Q (mite)	11	0	0	23
Pip 267K	Pip (ant)	11	0	0	22
Pyr 277D Iso	Pyr (ant)	6	1	0	19
Pyr 197B	Pyr (ant)	6	2	0	19
Tri 207GH	Tri (beetle/mite)	6	0	0	18
3,5-P 251K	3,5-P (ant)	5	0	0	17
Spiro 222	Spiro (millipede)	6	0	0	16
Unknown 9		8	0	0	16
Unknown 10		5	0	0	16
5,8-I 209B	5,8-I (mite)	5	0	0	15
3,5-I 223AB	3,5-I (mite)	5	0	0	15
DHQ 275B	DHQ (ant)	4	0	0	14
Pyr 279G	Pyr (ant)	4	0	0	13
Unknown 11		4	0	0	13
5,8-I 205A	5,8-I (mite)	4	0	0	12
5,8-I 207A	5,8-I (mite)	4	0	0	12
Unclass 225G		4	0	0	11
PTX 209F	PTX (ant/mite)	0	3	9	11
3,5-P 223H	3,5-P (ant)	4	0	0	11
5,8-I 275F	5,8-I (mite)	3	1	1	11
Unknown 12		5	0	0	11
5,6,8-I 223A Iso	5,6,8-I (mite)	3	0	0	11
Unknown 13		4	0	0	9
Unknown 14		3	0	0	9
5,8-I 271A	5,8-I (mite)	4	1	0	8
Unknown 15		2	1	0	8
Unknown 16					

5,6,8-I 251S	5,6,8-I (mite)	3	0	0	8
Pip 253U	Pip (ant)	3	0	0	8
Spiro 236	Spiro (millipede)	3	0	0	7
5,8-I 247E	5,8-I (mite)	3	0	1	7
Pyr 277D Iso2	Pyr (ant)	2	0	0	7
aPTX 241H	aPTX (mite)	3	0	0	6
Pyr 279D	Pyr (ant)	3	0	0	5
Unknown 17		3	0	0	5
Unknown 18		2	0	0	5
5,8-I 203A Iso	5,8-I (mite)	2	0	0	4
Unknown 19		2	0	0	4
Unknown 20		2	0	0	4
Unknown 21		1	0	0	4
Unknown 22		2	0	0	3
5,8-I 217B	5,8-I (mite)	1	0	0	3
Unknown 23		1	0	0	3
Unknown 24		1	0	0	3
Pip 239L	Pip (ant)	1	0	0	3
Pip NEW	Pip (ant)	1	0	0	3
5,8-I 203A	5,8-I (mite)	1	0	0	3
5,8-I 239G Iso	5,8-I (mite)	1	0	0	3
Unknown 27		0	3	0	3
Unknown 28		1	0	0	2
Unknown 29		0	2	0	2
PTX 225F	PTX (ant/mite)	0	1	2	2
Pip 225I	Pip (ant)	1	0	0	2
PTX 225F Iso	PTX (ant/mite)	0	0	2	2
Pip 225I Iso	Pip (ant)	0	0	0	2
Unknown 30		0	2	0	2
Unknown 31		1	0	0	1
Unknown 32		1	0	0	1
dehydro-5,8-I NEW	D-5,8-I (mite)	0	0	0	1
Unclass 225G Iso		1	0	0	1
Unknown 31		1	0	0	1
3,5-P 223H Iso	3,5-P (ant)	0	0	0	1
Unknown 32		0	0	1	1
5,8-I 239G	5,8-I (mite)	0	0	0	1
Unknown 33		0	1	0	1
Unknown 34		0	0	0	1
5,8-I 223J	5,8-I (mite)	0	0	0	1
Unknown 35		0	0	0	1
5,8-I 271A Iso	5,8-I (mite)	0	0	0	0
Unknown 36		0	0	0	0
Unknown 37		0	0	0	0
PTX 251D	PTX (ant/mite)	0	0	0	0
Unknown 38		0	0	0	0
5,8-I 195I Iso	5,8-I (mite)	0	0	0	0
Unknown 39		0	0	0	0
Unknown 40		0	0	0	0
Unknown 41		0	0	0	0
Unknown 42		0	0	0	0
5,8-I 221I	5,8-I (mite)	0	0	0	0
Unknown 43		0	0	0	0
Unknown 44		0	0	0	0
Unknown 45		0	0	0	0
Unknown 46		0	0	0	0

Unknown 48		0	0	0	0
Unknown 49		0	0	0	0
Unknown 50		0	0	0	0
Unknown 51		0	0	0	0
Unknown 52		0	0	0	0
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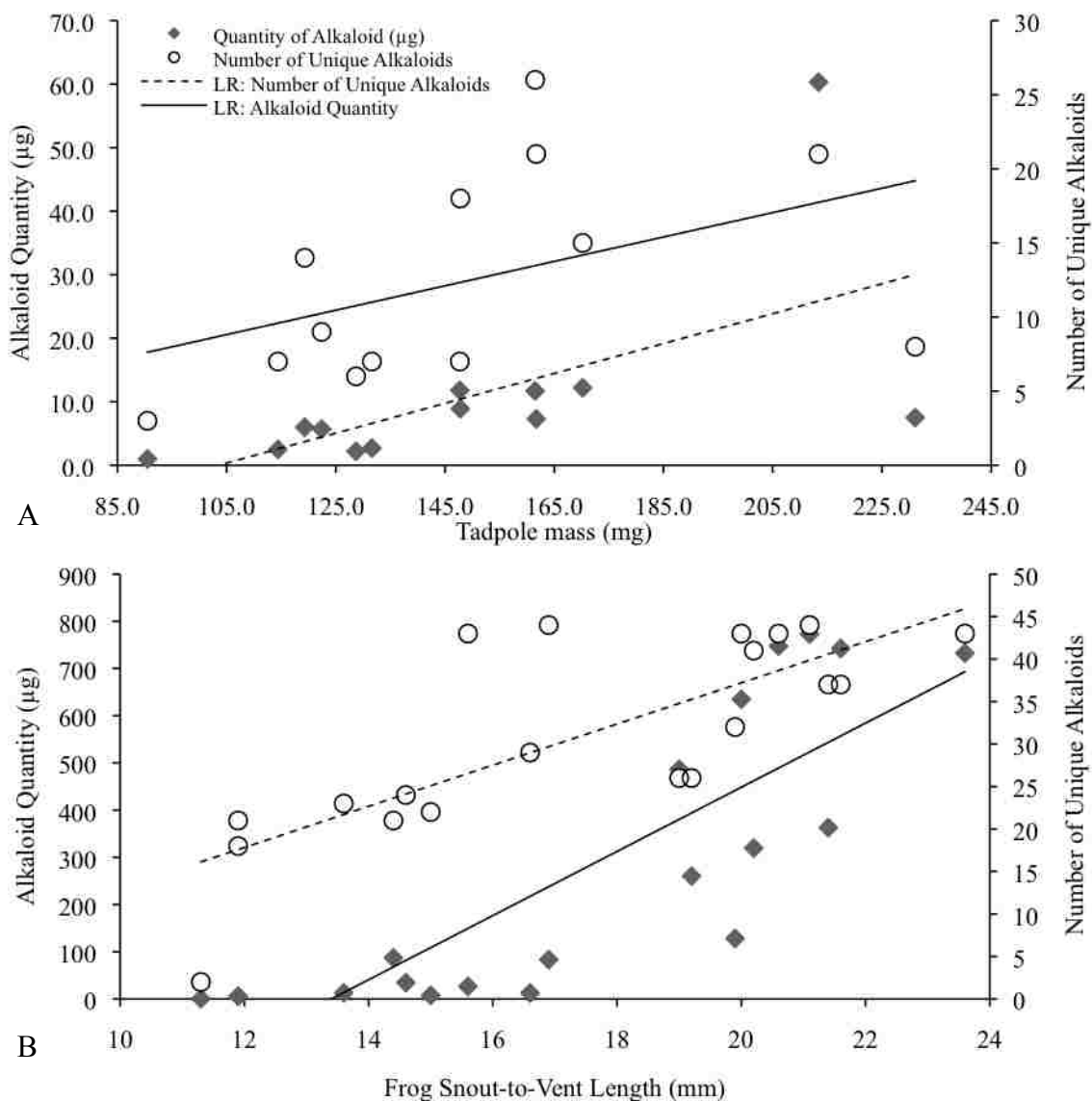


Figure 5.1 Scatterplot showing the relationship between the quantity and diversity of alkaloids and the (A) mass of tadpoles and (B) snout-to-vent length of juvenile and adult frogs in samples of *Oophaga pumilio* from La Selva, Costa Rica with linear regressions (LR). No alkaloids were detected in early stage tadpoles.

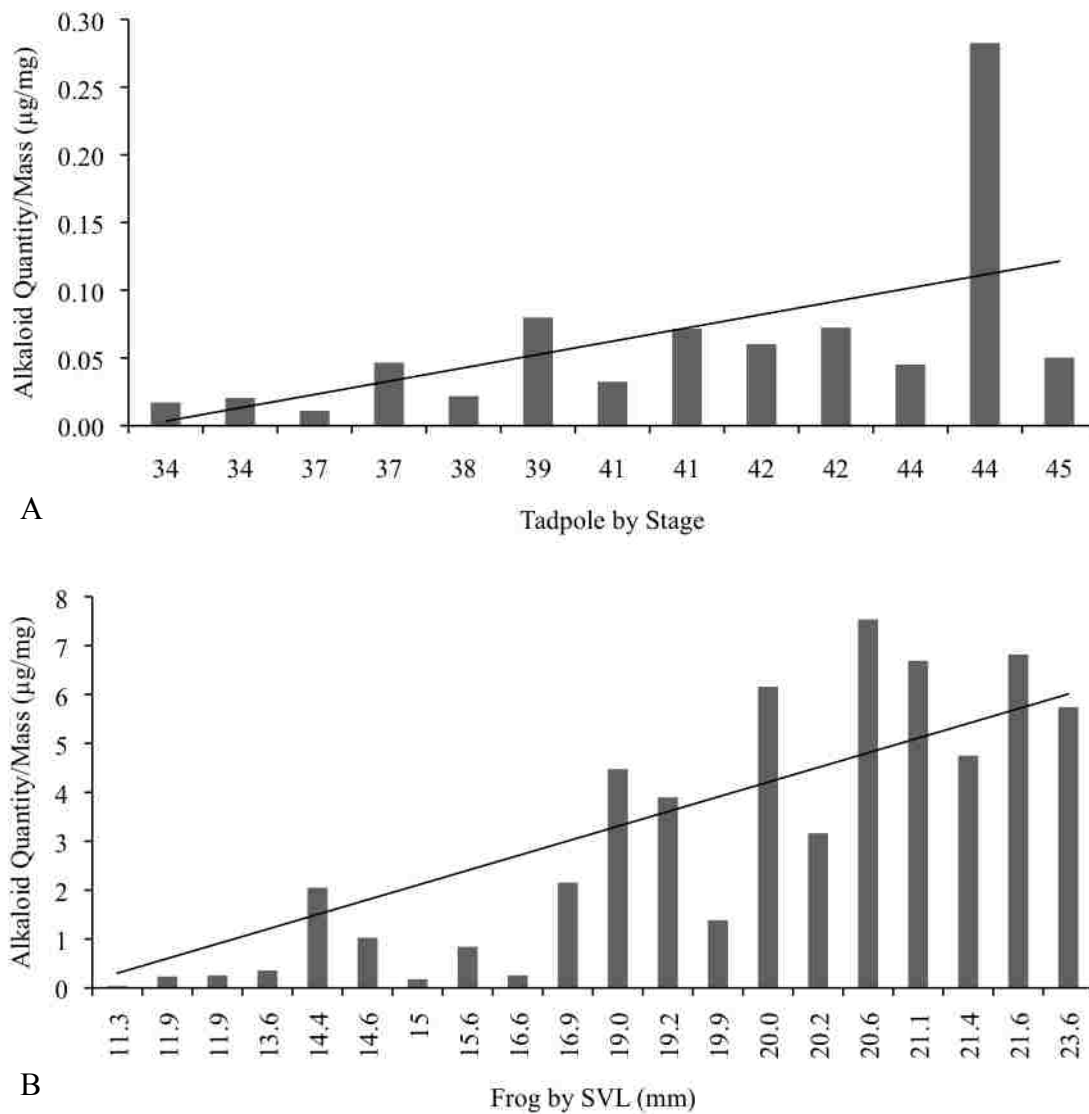


Figure 5.2 Quantity of alkaloids per unit mass in *Oophaga pumilio* (A) tadpoles and (B) juvenile and adult frogs (B) ordered by sample according to (A) tadpole stage or (B) Snout-to-Vent Length (SVL).

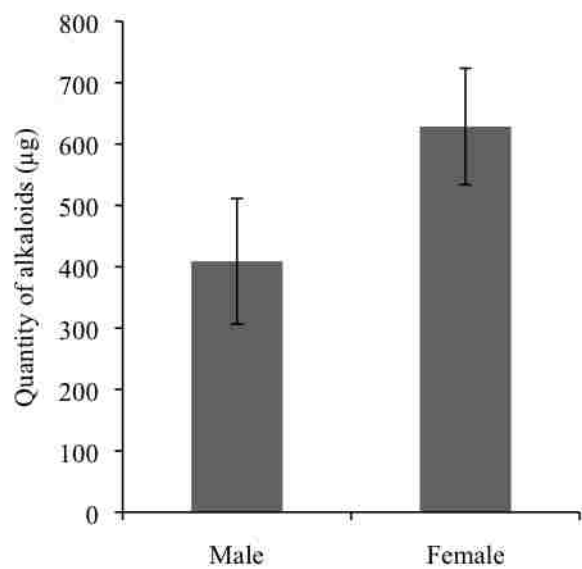


Figure 5.3 Quantity of alkaloids in male and female adult *Oophaga pumilio* from La Selva, Costa Rica. Difference not statistically significant. Error bars represent SE.

Chapter 6: Conclusions

The findings of this dissertation provide a stronger understanding of how an amphibian species performs complex parental care. Mother *Oophaga pumilio* cannot recognize their offspring but instead use features of offspring location to discriminate between their offspring and unrelated young when supplying nutritive eggs. Tadpoles are able to combine visual and tactile cues to modulate their behavior in the presence of mother frogs, potential predators, and harmless heterospecific frogs. When mother frogs return to the site of their tadpole and the tadpole establishes that it is indeed a potential egg-providing frog, tadpoles vibrate to communicate. Tadpoles only perform this vibration behavior at mothers when they have not eaten in the last 24 hours, suggesting that this signal is an honest indication of tadpole hunger. Mothers only provide eggs to tadpoles when tadpoles are in the exact site where they deposited the tadpole and when the tadpole vibrates to indicate that it needs eggs to eat. The eggs that the mother provides may contain alkaloids, which appear to accumulate in tadpoles as they age. It remains to be seen whether the alkaloids in tadpoles are an effective defense against predators, but the quantity of alkaloids is similar to that of other organisms that provide chemical defenses to their offspring prior to hatching or birth and are an effective defense. Thus, this dissertation provides novel evidence to suggest that mother *Oophaga pumilio* use indirect recognition in combination with a reliable signal of hunger from their tadpole to provide an optimal number of eggs that are provisioned with alkaloid chemicals. Behavioral modulation by tadpoles may act along with chemical defenses from mother frogs to reduce the risk of predation to offspring. So, it seems that mothers and tadpoles work together to keep the kids well fed and safe.

Many of the findings in this dissertation are novel for the class Amphibia. Aside from taxonomic novelty, however, the uniqueness and accessibility of this system as a research model allowed for new ways to explore parental care. For example, we easily manipulated offspring hunger and used cups and motion activated video to see if parents allocate resources according to offspring signals of need. Easily observed tadpole begging behavior allowed for isolation of sensory cues in a parent-offspring communication system, and revealed a clear example of multimodal integration. Direct observation of parental behavior, easy manipulation of rearing habitat in La Selva's ideal infrastructure, an extended parental phase, solitary offspring, low adult predation due to aposematism, and chemical defenses all contributed to an exceptional ability to test interesting hypotheses about parental care and chemical defenses in fresh ways.

This project broadens our comparative understanding of the behavioral mechanisms behind complex parental care at the class level. The information acquired will serve as a foundation for future studies that address ultimate questions about the significance and evolution of parental care within the adaptive radiation of dendrobatid frogs, which displays an impressive range of derived parental behaviors (Grant et al. 2006). Also, aspects of this research have important conservation implications in the face of global amphibian declines (Stuart et al. 2004). Other topics that arose during the research (predators of tadpoles, tadpoles in phytotelm communities, habitat fragmentation and frog reproductive behavior, etc.) will stimulate my own future studies in this system for years to come. With hope, these findings will also encourage studies by other researchers in comparative behavioral ecology and in amphibian conservation.

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