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Evidence of maternal provisioning of alkaloid-based chemical defenses in the strawberry poison frog *Oophaga pumilio*

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Abstract. Many organisms use chemical defenses to reduce predation risk. Aposematic dendrobatid frogs sequester alkaloid-based chemical defenses from a diet of arthropods, but research on these defenses has been limited to adults. Herein, we investigate chemical defense across development in a dendrobatid frog, *Oophaga pumilio*. This species displays complex parental care: at hatching, mothers transport tadpoles to phytotelmata, and then return to supply them with an obligate diet of nutritive eggs for about six weeks. We collected eggs, tadpoles, juveniles, and adults of *O. pumilio*, and detected alkaloids in all life stages. The quantity and number of alkaloids increased with frog and tadpole size. We did not detect alkaloids in the earliest stage of tadpoles, but alkaloids were detected as trace quantities in nutritive eggs and as small quantities in ovarian eggs. Tadpoles hand-reared with eggs of an alkaloid-free heterospecific frog did not contain alkaloids. Alkaloids that are sequestered from terrestrial arthropods were detected in both adults and phytotelm-dwelling tadpoles that feed solely on nutritive eggs, suggesting that this frog may be the first animal known to actively provision post-hatch offspring with chemical defenses. Finally, we provide experimental evidence that maternally derived alkaloids deter predation of tadpoles by a predatory arthropod.

Key words: antipredator defense; chemical defense; *Dendrobatidae*; eggs; juveniles; *La Selva, Costa Rica*; maternal provisioning; *Oophaga pumilio*; parental care; phytotelmata; poison glands; tadpoles.

INTRODUCTION

Predation is a strong selective factor and has led to the evolution of a remarkable diversity of defensive adaptations aimed at reducing predation risk. For example, some animals use unpalatable, noxious, or poisonous chemicals that are sequestered from their diet as a defense against predators (Savitzky et al. 2012). The use of sequestered chemical defenses has independently evolved in flatworms, nudibranchs, arthropods, birds, snakes, and amphibians (for reviews, see Termonia et al. 2002, Saporito et al. 2012, Savitzky et al. 2012). The physiology, evolutionary significance, and ecological function of chemical sequestration have been well-studied in certain phytophagous arthropods (e.g., Nishida 2002, Hartmann et al. 2005), but remain poorly understood among vertebrates (Savitzky et al. 2012).

Organisms can use chemicals not only to defend themselves, but also as an antipredator mechanism to protect their offspring. Parents in a number of animal taxa use chemical defenses to reduce predation risk to their offspring by provisioning chemicals prior to hatching or birth (Williams et al. 2011). For example, the snake *Rhabdophis tigrinus* sequesters bufadienolides from toads and deposits these chemicals in its offspring via yolk and oviduct (Hutchinson et al. 2008). In some taxa, offspring produce or sequester their own chemical defenses (Nishida 2002, Williams et al. 2011), and in other cases chemical defenses are solely maternally derived (Hutchinson et al. 2008, Hayes et al. 2009).

Maternal deposition of chemical defenses prior to hatching or birth is well-documented in various species of amphibians (see Gunzburger and Travis 2005). Female rough-skinned newts (*Taricha granulosa*) invest tetrodotoxin (TTX) in their eggs, which protects offspring from predatory dragonflies (Gall et al. 2011). The harlequin frog (*Atelopus chiriquiensis*) also invests

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TTX in its eggs (Pavelka et al. 1977). Female cane toads (*Rhinella marina*) provision their eggs with bufadienolides, which renders the eggs and the hatched tadpoles toxic, but the quantity of the chemicals and their antipredator effectiveness decline across tadpole development (Hayes et al. 2009). Eggs, but not tadpoles, of boreal toads (*Anaxyrus* [=*Bufo*] *boreas*) are protected with bufadienolides (Benard and Fordyce 2003), and oocytes from redbelly toads (*Melanophryniscus simplex*) contain a variety of alkaloids (Grant et al. 2012). Many parental amphibians chemically arm their eggs, but the effectiveness of these predator defenses generally decreases with offspring development (Hayes et al. 2009, Santos and Cannatella 2011).

Dendrobatid frogs are well-studied vertebrates that sequester chemical defenses from dietary sources. Brightly colored members of the family Dendrobatidae obtain alkaloid-based chemical defenses from a diet of alkaloid-containing mites, ants, millipedes, and beetles (reviewed in Saporito et al. 2012). As a result of these defenses, some dendrobatids are unpalatable (and in some cases toxic) to many invertebrate and vertebrate predators (see the supplement of Santos and Cannatella 2011). However, studies of chemical defense in dendrobatids have focused almost entirely on adults (Daly et al. 1987, Saporito et al. 2012).

Daly et al. (1987) suggested that alkaloid-based chemical defenses might appear only in juvenile and adult dendrobatids because the cutaneous granular glands (the storage sites for alkaloids) mature just prior to metamorphosis. Glands enlarge throughout development, especially at sexual maturity (Angel et al. 2003, Delfino et al. 2010, Saporito et al. 2010b). However, trace amounts of alkaloid were detected in eggs of *Phyllobates terribilis* (but not in a tadpole at stage 35; Gosner 1960, Myers et al. 1978), and trace amounts of alkaloid were identified in one juvenile *Dendrobates auratus* that had been raised on alkaloid-free fruit flies since its tadpole stage (Daly et al. 1994). Recent studies of cutaneous granular glands in dendrobatid tadpoles have clarified their ontogeny. Early developmental forms of granular glands are visible in *P. bicolor* and *D. auratus* tadpoles by stages 38–40 and become mature glands by stages 43–45 (Angel et al. 2003, Delfino et al. 2010). In *Oophaga pumilio* (see Plate 1), the skin of recently metamorphosed juveniles contains mature glands, albeit significantly smaller glands than are present in adults (Saporito et al. 2010b). Our current understanding of chemical defenses in egg, tadpole, and juvenile stages of dendrobatid frogs is limited and anecdotal (Myers et al. 1978, Daly et al. 2002, Saporito et al. 2010b).

The adaptive radiation of dendrobatid frogs displays diverse parental behaviors, which 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 (Grant et al. 2006). In the strawberry poison frog (*Oophaga pumilio*),

fathers guard fertilized eggs in leaf litter for 7–10 days until mothers return to transport tadpoles to individual phytotelmata (Weygoldt 1980). Then, for approximately six weeks, mothers visit phytotelmata every 1–5 days and supply tadpoles with unfertilized (nutritive) eggs as food (Brust 1993, Stynoski 2009). Because *O. pumilio* tadpoles are known to eat only maternal eggs as opposed to alkaloid-containing arthropods (Daly et al. 1987, Brust 1993, Stynoski 2009), it has been assumed that tadpoles do 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. Herein, we provide the first evidence that female *O. pumilio* provide tadpoles with alkaloid-based chemical defenses in nutritive eggs. We also describe the ontogeny of alkaloid-based chemical defenses in *O. pumilio* from the egg to adult stage. Furthermore, we provide experimental evidence that maternally derived alkaloids in tadpoles may function as an effective deterrent against at least one arthropod predator.

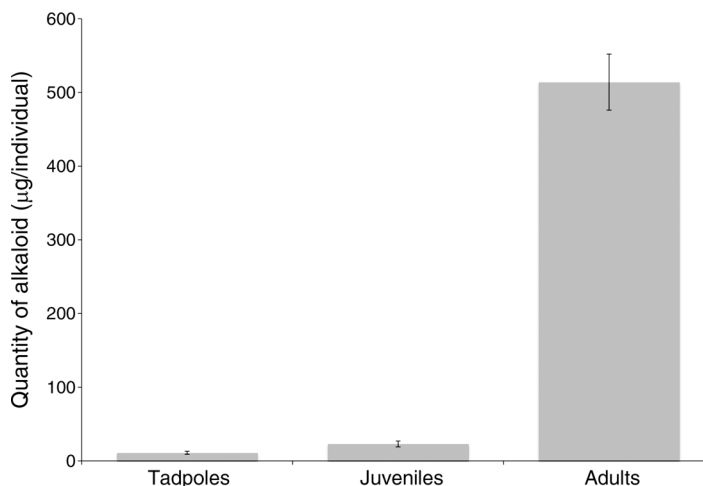
METHODS

Sample collection.—To determine the type and quantity of defenses that exist across development, we identified and quantified alkaloids in an age-series of wild-caught *Oophaga pumilio* from 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 in natural phytotelmata (Stynoski 2009, Stynoski and Noble 2012). From cups, we collected 15 tadpoles in a range of developmental stages (stage 25–45, Gosner 1960) as well as three samples of five unfertilized eggs that different mothers had supplied as nutrition for tadpoles. From leaf litter, we collected fertilized eggs ($N=3$; 5 per sample), 10 free-living juvenile frogs (10–18 mm snout-to-vent length; SVL), and 10 adults (5 males and 5 females; 19–22 mm SVL).

To investigate the origin of alkaloids in tadpoles, we hand-reared four wild-caught, newly hatched tadpoles in the water from which they were collected. We fed them every 48 hours for 37–42 days, which is approximately equivalent to the time period *O. pumilio* tadpoles are fed maternal eggs. We fed tadpoles with the yolk of eggs of the red-eyed treefrog, *Agalychnis callidryas*, which do not contain alkaloids. To ensure that *A. callidryas* eggs did not contain alkaloids, we also collected three samples of five wild-caught *A. callidryas* eggs (15 total).

Chemical analysis.—We removed skins from all metamorphosed specimens and oocytes from adult females. Samples (skin, oocytes, eggs, or whole tadpole) were stored in individual 4-mL glass vials containing 100% methanol. From each methanol extract, alkaloid fractions were prepared, identified, and quantified following methods in Saporito et al. (2010a), using gas

FIG. 1. Quantity (mean \pm SE) of alkaloids among tadpoles, juveniles, and adults of the dendrobatid strawberry poison frog, *Oophaga pumilio*.



chromatography–mass spectrometry (GC-MS) on a Varian Saturn 2100T ion trap MS instrument coupled to a Varian 3900 GC with a 30 m \times 0.25 mm diameter Varian Factor Four VF-5ms fused silica column (Varian, Palo Alto, California, USA). We identified alkaloids in each sample by comparing the observed MS properties and GC retention times with those of previously reported anuran alkaloids (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 a nicotine internal standard, using a Varian MS Workstation v.6.9 SPI (see Saporito et al. 2010a, 2012).

Experimental test of chemical defenses in tadpoles.—To gather initial evidence as to whether the presence of alkaloids in tadpoles would be an effective deterrent against invertebrate predators, we performed a simple predation assay. Following Fritz et al. (1981) and R. A. Saporito et al. (*unpublished data*), we used a generalist invertebrate predator, the bullet ant (*Paraponera clavata*), which nests at the bases of trees and actively forages in the surrounding area, returning to the nest to consume prey. We presented wild-caught late-stage *O. pumilio* tadpoles (stage 41–45; $N = 15$) and equivalent-sized *A. callidryas* tadpoles ($N = 15$) to actively foraging ants. We conducted randomized trials along colony foraging trails on tree trunks with a single tadpole of both species. Predator responses were categorized (as in Fritz et al. 1981) as (1) avoided/rejected or (2) attacked. Tadpoles that were attacked and not rejected were pulled into the nest by one or multiple ants, which we interpreted as an attempt by the ant(s) to feed upon the tadpole.

Survey of ants and mites in artificial phytotelmata.—To explore whether alkaloid-containing ants and mites could contribute directly to the presence of alkaloids in tadpoles, we collected the contents of artificial phytotelmata from which the tadpole samples were collected. We sampled all of the water and debris from 13 artificial phytotelmata holding tadpoles and 14 not holding

tadpoles. We counted the number of ants and mites in each sample using a dissecting scope, and when possible, classified ants and mites as genera that are known to contain alkaloids or not.

Data analysis.—To determine whether the number and quantity of alkaloids differed among life stages (tadpole, juvenile, and adult), we used a one-way MANOVA, followed by two univariate ANOVAs and associated Tukey HSD post hoc tests. An alpha of 0.025 (Bonferroni correction) was used for these multiple comparisons, whereas alpha of 0.05 was used for all other statistical analyses. Correlation analysis was used to examine the relationship between quantity of alkaloids and the number of alkaloids. To determine the influence of anuran size on alkaloid quantity, we used both individual linear regressions and ANCOVAs of the independent variables SVL and skin mass in the case of frogs, and total length and total mass in the case of tadpoles. All assumptions of ANCOVAs were met. We used one *t* test to test for differences in quantity of alkaloids between adult males and females, and a second *t* test to test for differences in the quantity of alkaloids per unit mass of skin between adult and juvenile frogs. Nonmetric multidimensional scaling was used to graphically visualize patterns of alkaloid composition among age groups. Binary logistic regression was used to determine if tadpole species was a significant predictor of predation by bullet ants. All data analyses were performed using PRIMER-E v. 5 (PRIMER-E 2001) and SPSS v.18 (SPSS 2009). Variation is expressed as mean \pm SE.

RESULTS

We detected 72 types of alkaloids among samples from all life stages of *Oophaga pumilio*. We identified 62 alkaloids in adult females (10 not found in males) and 56 in adult males (four not found in females). In juvenile frogs, we identified 50 alkaloids (six not found in adults and 16 not found in tadpoles). In naturally fed tadpoles from stages 34–45, we identified 35 alkaloids (five not

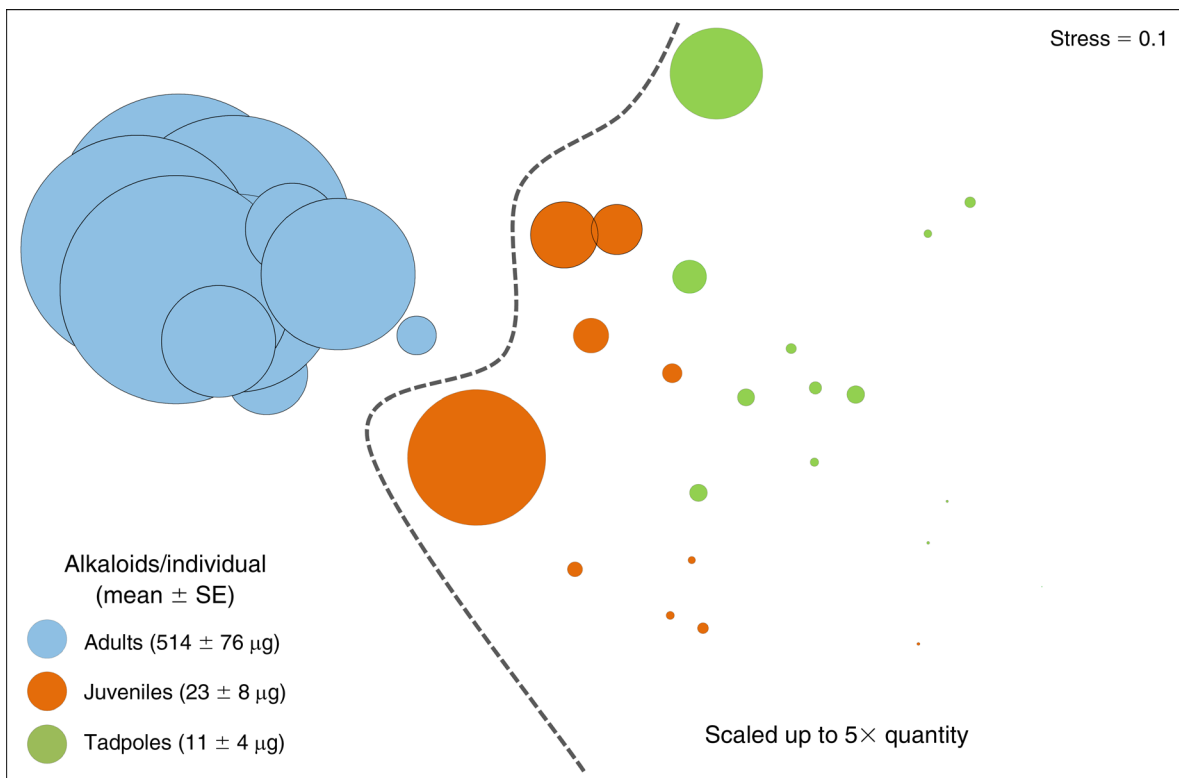


FIG. 2. Nonmetric multidimensional scaling (nMDS) plot of alkaloid composition among tadpoles, juveniles, and adults of *O. pumilio*. Each circle represents an individual, and the distance between symbols represents the difference in alkaloid composition. The diameter of each circle is directly equivalent to the quantity of alkaloids present in that individual. The circles are scaled up to $5 \times$ quantity for juveniles and tadpoles, due to the large quantities of alkaloids present in adults.

found in adults and one not found in juveniles). We did not detect alkaloids in stage 25 tadpoles or fertilized eggs, but we did detect trace quantities of alkaloids in nutritive eggs and small quantities in ovarian eggs.

On average, for the samples that contained alkaloids, we detected a higher quantity and number of alkaloids in adult frogs ($514 \pm 76 \mu\text{g}/\text{individual}$, mean \pm SE; 36 ± 2 types of alkaloids) than in juveniles ($23 \pm 8 \mu\text{g}$; 23 ± 3 types) or tadpoles ($11 \pm 4 \mu\text{g}$; 12 ± 2 types; Fig. 1). MANOVA revealed significant difference in the quantity and number of alkaloids among life stages ($F_{4,60} = 19.09$, Wilks' $\Lambda = 0.194$, $P < 0.001$). Univariate ANOVA indicated significant differences in the quantity of alkaloids among life stages ($F_{2,31} = 33.11$, $P < 0.001$), and Tukey HSD post hoc comparisons indicated that adults contained more alkaloids than juveniles or tadpoles ($P < 0.001$), but juveniles did not differ from tadpoles in quantity of alkaloids ($P = 0.989$). Similarly, univariate ANOVA indicated that the number of alkaloids differed among all life stages ($F_{2,31} = 36.39$, $P < 0.001$). Alkaloid composition differed among adults, juveniles, and tadpoles (see nMDS plot in Fig. 2). The quantity and number of alkaloids were positively correlated among tadpoles, juveniles, and adults ($r = 0.734$, $n = 34$, $P < 0.001$). Among juveniles and adults, the quantity of alkaloids was positively correlated with

SVL ($R^2 = 0.69$, $F_{1,18} = 40.23$, $P < 0.001$) and mass ($R^2 = 0.82$, $F_{1,18} = 80.12$, $P < 0.001$). Among tadpoles, the quantity of alkaloids was positively correlated with tadpole mass ($R^2 = 0.29$, $F_{1,13} = 5.40$, $P = 0.037$; Fig. 3). We identified a nonsignificant trend toward more alkaloids in adult females ($628 \pm 95 \mu\text{g}/\text{individual}$) than in adult males ($400 \pm 101 \mu\text{g}/\text{individual}$; $t_8 = 1.65$, $P = 0.14$). Adult frogs contained a higher quantity of

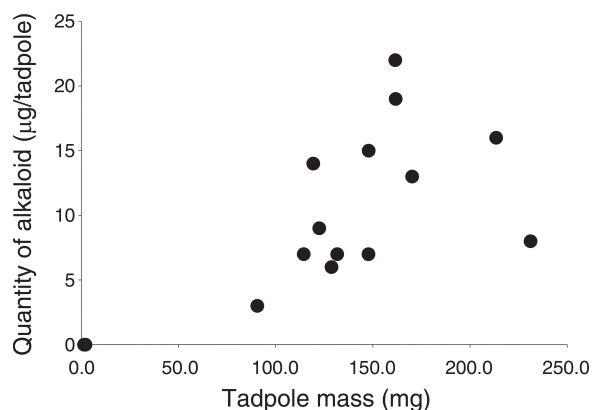


FIG. 3. Relationship between tadpole mass and quantity of alkaloids in *O. pumilio* tadpoles.



PLATE 1. Female *Oophaga pumilio* in a bromeliad (La Selva Biological Station, Coasta Rica). Photo: R. A. Saporito.

alkaloid per unit skin ($5.0 \pm 0.6 \mu\text{g}/\text{mg}$) than juvenile frogs ($0.6 \pm 0.2 \mu\text{g}/\text{mg}$; $t = 6.96$, $\text{df} = 18$, $P < 0.001$). No alkaloids were detected in fertilized eggs, trace quantities of alkaloids were detected in one of the three samples of unfertilized eggs, and ovarian eggs contained, on average, $41 \pm 20 \text{ ng}$ of alkaloids per egg.

Tadpoles fed with *A. callidryas* eggs grew to $5.0 \pm 0.7 \text{ mm}$ and reached stage 28. We did not detect alkaloids in *A. callidryas* eggs or in *O. pumilio* tadpoles that were fed with *A. callidryas* eggs.

In predator tests, tadpole species significantly predicted attack ($P = 0.014$; $\text{Exp(B)} = 9.8$), and *P. clavata* were over nine times more likely to attack *A. callidryas* tadpoles than *O. pumilio* tadpoles.

In the contents of phytotelmata that held tadpoles, we found an average of 0.9 ants (range 0–4) and 2.1 mites (range 0–6). In the contents of phytotelmata that did not hold tadpoles, we found an average of 1.4 ants (range 0–10) and 1.2 mites (range 0–4). No beetles or millipedes were found in the phytotelmata, and only three ants (*Solenopsis* sp.) and one mite (*Schelorbates* sp.) were identified as genera known to contain alkaloids.

DISCUSSION

We demonstrate for the first time that alkaloids are present in all life stages of *Oophaga pumilio*, including oocytes, nutritive eggs, and tadpoles. Alkaloid quantity

and number increased with the size and age of frogs and tadpoles. The presence and increase in quantity of alkaloids in tadpoles suggests that females are providing chemical defenses to their vulnerable young via nutritive eggs. Only ant- and mite-derived alkaloids were detected in eggs and tadpoles, indicating that chemical defenses of phytotelm-dwelling tadpoles are probably derived from a terrestrial habitat by way of maternal provisioning. Furthermore, alkaloids were absent in tadpoles reared in water from phytotelmata on alkaloid-free eggs. Although provisioning of chemical defenses to embryos is known in many taxa (Gunzburger and Travis 2005, Williams et al. 2011), this is the first example to our knowledge of provisioning that occurs after hatching.

We detected alkaloids in stage 34–45 tadpoles that had already consumed 10 or more maternal eggs, but not in stage 25 tadpoles. The absence of alkaloids in fertilized eggs and stage 25 tadpoles might indicate that female frogs withhold alkaloids from eggs until they begin feeding their young nutritive eggs. Ovarian eggs (oocytes) in this and other studies contained small amounts of alkaloids (Saporito et al. 2012), suggesting that mothers were feeding their young unfertilized eggs provisioned with alkaloids. Tadpole gland ontogeny is unknown in *O. pumilio* and other egg-eating dendrobatid tadpoles, but granular glands first appear in related species at tadpole stage 38 (Angel et al. 2003, Delfino et al. 2010). Very young *O. pumilio* tadpoles may not yet be

able to sequester alkaloids provided in maternal nutritive eggs. However, it remains possible that alkaloids are present in stage 25 tadpoles, but in quantities too small to detect.

Adult frogs (>19 mm SVL) contained approximately seven times as much alkaloid per unit mass of skin as juvenile frogs (<18 mm SVL). In other anecdotal reports, adults contained about five times as much alkaloid as a juvenile (*Phyllobates terribilis*, Myers et al. 1978; *O. pumilio*, Daly et al. 2002). When frogs increase from 18 to 19 mm at sexual maturity, their granular glands become notably enlarged (average increase in area of 41%; Saporito et al. 2010b) and their diet shifts (Saporito et al. 2012). Presumably, either alkaloid sequestration enlarges the granular glands or gland enlargement enhances sequestration capability. Sex steroid hormones might play a role in the sudden increase in gland size and quantity of alkaloids at sexual maturity, as they have been shown to stimulate skin gland size and activity in many vertebrates (Norris et al. 1989).

Adult females contained 53% more alkaloids (quantity) than adult males, which agrees with the findings of Saporito et al. (2010a), in which females contained 71% greater quantity of alkaloids. Mothers, but not fathers, might need to invest simultaneously in chemical defense of self and offspring. Future studies should explore whether egg provisioning diminishes females' own chemical defenses and if they target a high-alkaloid diet while provisioning. Lastly, future studies should explore the relationship between the quantity and diversity of alkaloids present in a mother frog and her offspring to determine if mothers modulate the amount and type of alkaloids that they provision.

Experimental evidence suggests that bright coloration in adult *O. pumilio* is aposemantic (Saporito et al. 2007), and coloration first appears during the tadpole phase (stage 42–43; J. L. Stynoski, *personal observation*). Considering that tadpoles stage 42 and above contain alkaloids, the emergence of dorsal coloration might be ontogenetically timed with the onset of unpalatability or toxicity. *O. pumilio* tadpoles contain an average of $11 \pm 4 \mu\text{g}$ of alkaloid and juveniles contain an average of $23 \pm 8 \mu\text{g}$. Eggs of *Utethesia* moths containing $0.8 \mu\text{g}$ of alkaloid per egg were defended against ants, beetles, and chrypsid larvae (Dussourd et al. 1988), and PTX 251D, a pumiliotoxin alkaloid like those in tadpoles in the current study, is lethal to mosquitoes at $0.1 \mu\text{g}/\text{cm}^2$ (Weldon et al. 2006). The quantity of alkaloids present in *O. pumilio* tadpoles and juveniles is likely to be an effective defense against at least some potential predators. In the current study, the predatory ant *P. clavata* avoided late-stage *O. pumilio* tadpoles but not nontoxic *A. callidryas* tadpoles, suggesting that alkaloids in *O. pumilio* tadpoles may be an effective predator deterrent, particularly against invertebrate predators. The capacity of female frogs to arm their tadpoles with defensive alkaloids may have conferred a selective advantage to

members of the genus *Oophaga*, which may have led to the evolution of egg feeding as an obligate, rather than facultative, maternal behavior in this genus of dendrobatid frogs (Grant et al. 2006).

A small number of ants and mites were present in our artificial phytotelmata. Although most of these ants and mites are not known to contain alkaloids, it is possible that some of the alkaloids in *O. pumilio* tadpoles were derived from feeding (i.e., scavenging) on these dead arthropods. This scavenging, however, is unlikely to account for the observed amount of alkaloids present in tadpoles. Most of the alkaloids (78%) present in tadpoles are derived from oribatid mites, which represent a major dietary source for alkaloids in adult frogs (Saporito et al. 2007). Relatively little is known about the quantity of alkaloids in mites, but the oribatid *Scheloriobates laevigatus* contains an average of 17 ng of alkaloid per individual (Saporito et al. 2011). In the present study, the earliest staged tadpoles contained an average of $2.5 \mu\text{g}$ (equivalent to ~ 145 mites) and the latest staged tadpoles contained an average of $14 \mu\text{g}$ (equivalent to ~ 825 mites). Considering that *O. pumilio* tadpoles are obligately oophagous (Brust 1993), it is unlikely that they are consuming such large numbers of mites. Thus, maternal provisioning is the most plausible explanation for the presence of such large amounts of alkaloids in *O. pumilio* tadpoles.

This study is the first to show that alkaloid-based chemical defenses are present not only in adults, but also in all life stages of a dendrobatid frog, and that alkaloid quantity and diversity increase with size. Also, we provide evidence of a novel provisioning system in which female frogs supply post-hatch offspring with predator-deterrent alkaloids initially derived from terrestrial arthropods.

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