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Toxicon 161 (2019) 40–43

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Short communication

Experimental evidence for maternal provisioning of alkaloid defenses in a dendrobatid frog

Ralph A. Saporito[a,*], Matthew W. Russell[a], Corinne L. Richards-Zawacki[b,1], Matthew B. Dugas[b,c]

[a] Department of Biology, John Carroll University, 1 John Carroll Boulevard, University Heights, Ohio 44118, USA
[b] Department of Ecology and Evolutionary Biology, Tulane University, 400 Lindy Boggs Building, New Orleans, LA 70118, USA
[c] School of Biological Sciences, Illinois State University, Normal, IL, 61790, USA

ARTICLE INFO

Keywords:
Chemical defense
Dendrobatid
Feeding experiment
Oophaga
Poison frog
Sequestration

ABSTRACT

Dendrobatid frogs sequester alkaloid defenses from dietary arthropods. Here, we provide experimental evidence that mother strawberry poison frogs (*Oophaga pumilio*) provision alkaloids to tadpoles. Captive-raised females were fed the synthetic alkaloid decahydroquinoline (DHQ), which we subsequently quantified in their skin, eggs, and developing tadpoles. DHQ quantity was positively associated with tadpole mass/development, suggesting high sequestration rates by tadpoles. These data confirm that tadpoles obtain nutrition and alkaloids by feeding exclusively on maternally provisioned eggs.

The use of defensive chemicals for protection from predators and pathogens is common and widespread in animals (Mebs, 2001). Most can biosynthesize defensive toxins, yet some must obtain these chemicals from their diet (Savitzky et al., 2012). Animals that sequester defenses usually specialize on toxin-rich prey that can vary in abundance in time and space, driving variation in how well organisms are defended (Nishida, 2002; Saporito et al., 2012). Some animals use chemicals to defend not only themselves, but also to protect their vulnerable offspring (Eisner et al., 2000; Hanifin et al., 2003; Hutchinson et al., 2007; Hayes et al., 2009; Williams et al., 2011; Stynoski et al., 2014).

Conspicuously colored poison frogs sequester alkaloid-based defenses from a diet largely consisting of terrestrial ants and mites, and as a result are protected from predators and microbial infections (Saporito et al., 2012, 2015; Bolton et al., 2017; Hovey et al., 2018). Stynoski et al. (2014) recently found evidence suggesting that females of the strawberry poison frog (*Oophaga pumilio*) provide their young alkaloid defenses by feeding them eggs containing alkaloids. Mothers of this terrestrial frog transport newly hatched tadpoles individually to small bodies of water (e.g., leaf axils). For about six weeks, mothers visit these nurseries every 1–2 days, supplying their tadpoles unfertilized (trophic) eggs with the nutrition required for growth (Dugas et al., 2016a,b) and alkaloids required for defense (Stynoski, 2009). Tadpoles entirely depend on trophic eggs for growth/development, and variation in tadpole size is thus explained primarily by consumption of maternally derived eggs (Brust, 1993; Dugas et al., 2016a,b). Stynoski et al. (2014) found that alkaloid quantity is positively associated with tadpole mass and developmental stage (Gosner, 1960), suggesting that tadpoles that eat more eggs accumulate more alkaloids. Stynoski et al. (2014) provided strong evidence that female *O. pumilio* provision their offspring with alkaloids, and here, we confirm experimentally that female *O. pumilio* provision developing tadpoles with alkaloid defenses.

Alkaloid uptake and maternal provisioning of tadpoles were studied in a captive *O. pumilio* breeding colony established at Tulane University (New Orleans, USA) in August 2009 with wild-caught frogs from the Bocas del Toro Region of Panama (details on populations and husbandry in Dugas et al., 2016a,b). Adult frogs were fed three times weekly with ca. 15 wingless *Drosophila melanogaster* per individual. Flies were dusted with either a (1) vitamin supplement, serving as a control (Nekton-Rep nutritional supplement powder, Nekton, Clearwater, FL, USA) or (2) 1% synthetic decahydroquinoline (DHQ) (MW 139; Acros Organics, New Jersey, USA) vitamin supplement mixture (following Hantak et al., 2013).

To study alkaloid uptake into skin and ovarian eggs, control and DHQ-supplement groups, each containing 4–7 females were established on 24 February 2014. A subsample of one control and two experimental females was sacrificed for alkaloid analysis after 88 days. During the experiment, mortality unrelated to the project occurred, and therefore a new set of control and DHQ-supplement groups was established. A subsample of two control and four experimental adult females from

* Corresponding author.
E-mail address: rsaporito@jcu.edu (R.A. Saporito).
[1] Present Address: Department of Biological Sciences, University of Pittsburgh, 4249 Fifth Avenue, Pittsburgh, Pennsylvania 15260 USA.

https://doi.org/10.1016/j.toxicon.2019.02.008
Received 18 December 2018; Received in revised form 23 January 2019; Accepted 7 February 2019
Available online 18 February 2019
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Table 1
Alkaloid quantity in adult female skins and maternally provisioned tadpoles of Oophaga pumilio.

	Samples	Mass (mg)	Synthetic Alkaloid DHQ (μg per skin/tadpole)	Natural Alkaloids DHQ 195A (μg per skin/tadpole)	5,8-I 205A	5,8-I 207A	5,6,8-I 223A	Total Quantity
Experimental frogs:								
	Feeding duration	**Skin**						
1	88 days	109.3	**14.7**	nd	nd	nd	nd	**14.7**
2	88 days	121.6	**7.0**	0.1	nd	nd	nd	**7.1**
3	40 days	133.9	**12.0**	0.4	0.3	0.9	nd	**13.6**
4	40 days	137.5	**16.7**	nd	nd	nd	nd	**16.7**
5	40 days	163.4	**7.6**	nd	nd	nd	nd	**7.6**
6	40 days	168.1	**20.2**	0.4	nd	nd	0.7	**21.3**
Control frogs:								
	Feeding duration	**Skin**						
1	88 days	143.1	**nd**	nd	nd	nd	0.2	**0.2**
2	88 days	155.6	**nd**	0.1	nd	nd	0.4	**0.5**
3	40 days	162.8	**nd**	nd	nd	nd	nd	**nd**
Experimental tadpoles:								
	Gosner stage	**Tadpole**						
1	30	45.7	**0.6**	nd	nd	nd	nd	**0.6**
2	30	47.8	**0.6**	nd	nd	nd	nd	**0.6**
3	31	63.4	**0.9**	nd	nd	nd	nd	**0.9**
4	34	81.4	**0.8**	nd	nd	nd	nd	**0.8**
5	36	108.9	**1.6**	nd	nd	nd	nd	**1.6**
6	42	178.7	**3.3**	nd	nd	nd	0.1	**3.4**
7	40	192.1	**1.8**	nd	nd	nd	nd	**1.8**
8	43	192.9	**7.0**	1.1	0.6	2.4	0.4	**11.5**
Control tadpoles:								
	Gosner stage	**Tadpole**						
1	35	67.1	**nd**	0.1	nd	nd	0.1	**0.2**
2	34	81.4	**nd**	0.1	nd	nd	nd	**0.1**
3	40	141.4	**nd**	0.5	nd	nd	nd	**0.5**

Note: Synthetic DHQ was not detected (nd) in control frogs or tadpoles; however, natural alkaloids were detected in 2 of 3 control frogs and 3 of 15 control tadpoles. Only the 3 control tadpoles containing natural alkaloids are presented in the table. The remaining 12 tadpoles ranged in Gosner stage from 26–38, and weight from 4.2–127.0 mg.
Alkaloid abbreviations: Synthetic DHQ (decahydroquinoline); DHQ (2,5-disubstituted decahydroquinoline); 5,8-I (5,8-disubstituted indolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine).

these new groups was sacrificed after 40 days. All adults were sacrificed by freezing at −80 C, and skin and ovarian eggs were collected immediately and stored in methanol (see below). To study maternal provisioning of tadpoles, breeding tanks containing a single male and female (hereafter, family) were established and reproduction monitored with regular censuses (details in Dugas et al., 2016a). Tadpoles from control and DHQ-supplement groups were sacrificed by direct immersion in methanol during several colony-wide “sweeps” intended to capture variation in tadpole size/development. Frog skins, ovarian eggs, and tadpoles were stored in glass vials with methanol (hereafter, methanol extracts) and sealed with Teflon-lined caps.

Alkaloids were isolated from methanol extracts using acid-base extraction and analyzed 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 × 0.25 mm i.d. Varian Factor Four VF-5ms fused silica column (following Hovey et al., 2018). GC separation of alkaloids was achieved using a temperature program from 100 to 280 °C at a rate of 10 °C per minute with helium as the carrier gas (1 mL/min). Each alkaloid sample was analyzed with electron impact and chemical ionization MS. Alkaloids were identified via comparison of mass spectrometry properties and GC retention times to the DHQ standard and those of reported natural alkaloids in *O. pumilio* (Daly et al., 2005; Hovey et al., 2018 and references therein). Alkaloids were quantified with a nicotine internal standard, using a Varian MS Workstation v.6.9 SPI.

The synthetic alkaloid DHQ was detected in all six skin samples from adult females in the DHQ treatment, but not in the three control female skins (Fisher's Exact, $p = 0.012$). There was considerable variation in DHQ quantity among females (Table 1), but this was not obviously driven by duration of DHQ supplementation. Ovarian eggs from all six females supplemented with synthetic DHQ contained DHQ (mean ± SD: 0.11 ± 0.08 μg/egg), but no DHQ was detected in ovarian eggs from two control females (Fisher's Exact, $p = 0.036$); one control female did not have developed ovarian eggs. Similarly, DHQ was detected in all eight tadpoles from DHQ-supplemented families ($n = 6$ families), but not in any of the 15 control tadpoles ($n = 9$ families) (Fisher's Exact, $p < 0.001$). Total DHQ quantity was positively associated with tadpole mass ($r_s = 0.929$, $n = 8$, $p < 0.002$; Fig. 1a) and developmental stage (Gosner, 1960) ($r_s = 0.970$, $n = 8$, $p < 0.001$; Fig. 1b); however, whole body tissue DHQ concentration was no higher in heavier tadpoles ($r_s = 0.311$, $n = 8$, $p = 0.450$). Unexpectedly, four natural alkaloids (DHQ **195A**, 5,8-I **205A**, 5,8-I **207A**, and 5,6,8-I **223A**) that are not derived from synthetic DHQ were detected from some skin and tadpole samples, but not from ovarian eggs

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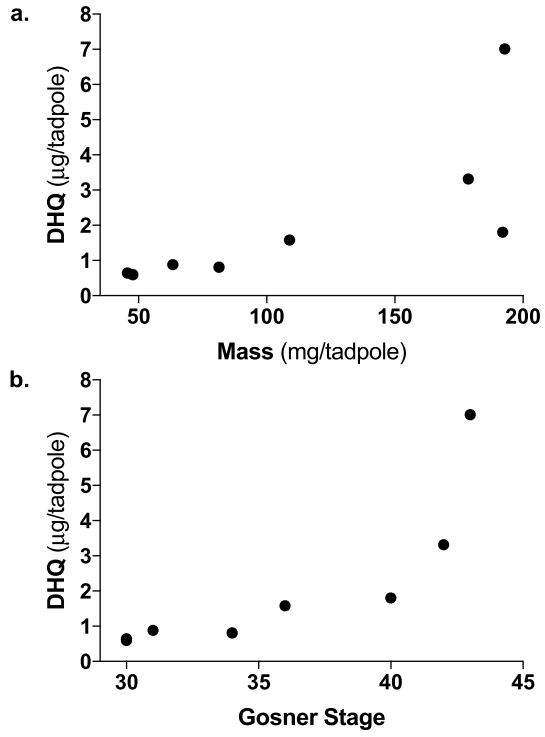

Fig. 1. The relationship between decahydroquinoline (DHQ) quantity and (a) total tadpole body mass and (b) Gosner developmental stage in maternally provisioned tadpoles of *Oophaga pumilio*. The DHQ present in tadpoles is derived from eggs provisioned by mothers fed experimentally with synthetic DHQ.

(Table 1). Most variables were normally distributed, but we used non-parametric tests throughout for consistency.

The presence of the supplemental synthetic DHQ in female *O. pumilio* and their ovarian eggs, coupled with positive associations between DHQ quantity and tadpole mass and developmental stage, provide experimental evidence that females provision their tadpoles with alkaloid defenses. Higher DHQ quantity in larger and older tadpoles almost certainly arises because they have cumulatively consumed more trophic eggs than smaller and younger tadpoles (Dugas et al., 2016a,b). Mature ovarian eggs, prior to being deposited as trophic eggs for tadpoles, contained an average of 0.1 µg of DHQ per egg in the present study. Thus, a late-stage tadpole that consumed 1 egg/day over 6 weeks of development (the mean in this colony: Dugas et al., 2016a) could contain ca. 4.2 µg of DHQ. The late-stage tadpoles in our study (stages 40–43) contained an average of 4.0 µg of DHQ per tadpole, suggesting efficient DHQ sequestration.

Granular gland development in *O. pumilio* tadpoles has been described and hypothesized to be associated with alkaloid sequestration (Stynoski and O'Connell, 2017). In the present study, alkaloids were detected in tadpoles younger (stage 30) than those previously examined for the presence of granular glands (stage 32), perhaps suggesting slightly earlier development of these glands than currently reported. Furthermore, the large increase in DHQ quantity in our late-stage tadpoles (Fig. 1) may suggest an increase in the efficiency of alkaloid sequestration associated with the presence of mature granular glands (Stynoski and O'Connell, 2017).

The quantity of DHQ in skin varied among female *O. pumilio*, suggesting differences in the feeding on and/or sequestration of DHQ among individuals in our study. No matter the origin of this variation, it is likely that difference in DHQ quantity among females explains some, if not all, of the variation in DHQ quantity in tadpoles. Natural variation in the quantity of alkaloids among female *O. pumilio* is common within and among populations (Hovey et al., 2018), and it is possible that mothers pass (via genetic and/or non-genetic mechanisms) on these differences to developing offspring. It remains unclear whether a female has physiological control over the type or quantity of alkaloids she allocates to eggs (however, see below), but future studies should test explicitly for co-variation between female and offspring alkaloid defenses and for the possibility that brood size limits and/or is limited by alkaloid availability (Dugas et al., 2016a).

Interestingly, small quantities of four natural alkaloids were present in some female *O. pumilio* and their tadpoles (Table 1). All adult females used in our study were captive-born offspring of wild-caught frogs, which contain these same natural alkaloids (Saporito et al., 2007). This suggests that wild-caught mothers originally provisioned these alkaloids to their offspring (adult females used here) that, in turn, provisioned these alkaloids, along with DHQ in experimental frogs, to some of the tadpoles in our experiment. Although the quantity of these natural alkaloids were very small, all alkaloid types present in females were also present in tadpoles, suggesting that alkaloid provisioning may not be selective with respect to alkaloid type (or that females are not selectively retaining these low-abundance alkaloids for themselves).

Oophaga pumilio is the first animal known to provision free-living offspring with chemical defenses (Stynoski et al., 2014), yet virtually nothing is known about the taxonomic distribution of this strategy. Among dendrobatids, trace amounts of alkaloid have been detected in eggs of *Phyllobates terribilis*, but not tadpoles (Myers and Daly, 1978), and no alkaloids have been detected in tadpoles of the facultative egg-feeding species *Ranitomeya variabilis* (L.M. Schulte and R.A. Saporito, unpub. data) or in tadpoles of the non egg-feeding species *Dendrobates auratus* (R.A. Saporito, unpub. data); however, alkaloids have recently been detected in tadpoles of *Oophaga granulifera* (S.K. Bolton and R.A. Saporito, unpub. data). All nine members of *Oophaga* are obligate egg-feeders (Grant et al., 2006; Frost, 2018), which may suggest that maternal alkaloid provisioning is unique to members of this genus. Documenting variation in allocation of chemical defenses to eggs and tadpoles will offer insights into the selective pressures and physiological innovations/constraints that explain this unique defensive strategy.

Acknowledgements

We thank the Panama for providing collecting (SE/A-112-08, SE/A-27-09) and export permission (SEX/A-58-09) for frogs used to establish the colony. Several Tulane undergraduate students, in particular Allison Killius, assisted with frog care, and M.A. Nichols assisted in maintaining the GC-MS. Funding for this project was partially supported by a Kresge Challenge Grant awarded to JCU. Tulane University's IACUC (0382; 0382R) approved the methods used in this study.

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