Ontogenetic scaling of poison glands in a dendrobatid poison frog

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Abstract

The nature of chemical defenses in poison frogs has been explored in a variety of species, and most studies focus on the types of chemical defenses and their sources. The defensive compounds of frogs are stored in dermal granular glands that have been described for several species that are chemically protected from predators and/or microorganisms. Gland ultrastructure is known for some species of dendrobatoid frogs, but the relationship between body size and chemical defense has heretofore not been explored. It might be expected that the capacity for defensive protection increases as a function of body size, especially given the fact that juvenile poison frogs are known to have smaller quantities of alkaloids than adults. We examined poison glands histologically in a sample of the poison frog *Oophaga pumilio* to determine if the physical basis of the defensive system changes as a function of body size. We measured average gland size, estimated the number of glands, and calculated the density and percentage of skin area occupied by glands in a patch of dorsal skin for 25 individuals. For males and females, the size, number and percentage of skin area occupied by poison glands increased allometrically as a function of body size, whereas poison gland density decreased with body size. Adults have a larger capacity to store alkaloids and more of their dorsal skin is associated with poison glands as compared with juveniles, which may translate into greater protection from predators in adults and could explain why adults are more apparent (active above the leaf litter) than juveniles at our study site in north-eastern Costa Rica. Furthermore, juveniles and subadults may benefit from automimicry because they resemble adults in appearance.

Introduction

Avoiding predation is fundamental to the survival of animals, and has led to the development of a large diversity of defensive strategies (Edmunds, 1974). Chemical defenses are found in all major lineages of animals, and represent a common strategy used to avoid predation. Chemically defended animals either manufacture defensive compounds or are dependent on acquiring these compounds from external sources (e.g. dietary sources; Mebs, 2001). In such cases, animals generally possess specialized structures (e.g. poison glands in ants, opisthonotal glands in mites and granular glands in amphibians) to store defensive compounds (Neuwirth et al., 1979; Blum, 1981; Kuwuhara, 2004). Ontogenetic changes in body size can have a major influence on nearly all aspects of an organism (Peters, 1983; Schmidt-Nielsen, 1984; Dial, Greene & Irschick, 2008), including predator–prey interactions (e.g. Phillips & Shine, 2006; Hayes et al., 2009), yet little is known about how changes in body size are related to these specialized defensive structures (however, see Phillips & Shine, 2006).

Among anurans, granular glands (serous or poison glands) are largely responsible for the synthesis, storage and release of noxious or toxic chemicals that function as passive chemical defenses against predation and/or microorganisms (see Daly, Myers & Whittaker, 1987; Erspamer, 1994 for reviews). Granular glands are generally found as 'macroglands' (e.g. parotoid, paracnemic, lumbar glands) or are broadly distributed throughout the integument (see Toledo & Jared, 1995 for review), and their development begins during late larval stages and continues throughout the lifetime of the adult (Delfino, 1983; Fox, 1986, 1994). Most anurans appear capable of synthesizing the defensive chemicals present within their poison glands (see Daly, 1995 for review), and an ontogenetic increase in parotoid gland size has been reported for *Rhinella marina*, an anuran capable of synthesizing defensive bufadienolides (Phillips & Shine, 2006). Poison frogs represent a unique group of anurans that are dependent on acquiring their chemical defenses from dietary sources, yet virtually nothing is known about changes in gland morphology with frog size.

The term poison frog is used to describe certain members of the anuran families Dendrobatidae, Mantellidae (*Mantella*), Bufonidae (*Melanophryniscus*), and Myobatrachidae (*Pseudophryne*), all of which contain alkaloid-based chemical defenses (see Daly, Spande & Garraffo, 2005 for review). Poison frogs do not produce alkaloids, but instead obtain them from a diet of alkaloid-containing arthropods, including mites, ants, millipedes and beetles (see Saporito *et al.*, 2009 for review). The only exception is seen in frogs of the genus *Pseudophryne*, which synthesize pseudophrynamine alkaloids, and sequester pumiliotoxin alkaloids from diet (Smith *et al.*, 2002), and members of the dendrobatid genera *Dendrobates* and *Adelphobates*, which are able to hydroxylate a pumiliotoxin alkaloid obtained from diet into a different alkaloid (Daly *et al.*, 2003). More than 800 alkaloids have been identified in this group of frogs (see Daly *et al.*, 2005 for review), all of which are stored in poison glands (Neuwirth *et al.*, 1979; Delfino *et al.*, 1998, 2010; Angel, Delfino & Parra, 2003). Numerous studies have focused on the number and diversity of alkaloids present in poison frogs, and have described patterns of variation in alkaloids within and among species (e.g. Daly *et al.*, 1987, 2008; Saporito *et al.*, 2007a). However, few studies have examined the structures responsible for the storage and release of alkaloids in poison frogs (i.e. poison glands). In the most comprehensive study to date, Neuwirth *et al.* (1979) described the structure of granular glands and morphology of the secretory cycle in nine adult dendrobatid species, and found strong similarities among these species. Delfino *et al.* (1998) reported on the presence of two different types of poison glands in the skin of the bufonid poison frog *Melanophryniscus stelzneri*. Angel *et al.* (2003) described patterns in ultrastructural features related to the maturation process of granular glands in larval and juvenile stages of the dendrobatid frog *Dendrobates auratus*, and most recently, a similar study was conducted with the dendrobatid frog *Phyllobates bicolor* (Delfino *et al.*, 2010).

Although it is established that poison frogs accumulate alkaloids into poison glands from dietary arthropods, and juvenile frogs have been shown to contain smaller quantities of alkaloids when compared with adults (Myers, Daly & Malkin, 1978; Daly *et al.*, 2002), relatively little is known about how gland morphology or distribution and the capacity for chemical defense changes in relation to body size. If gland number or size changes with frog body size, chemical defense might also vary as a function of morphological constraint. The purpose of our study was to examine changes in gland morphology in a size series of the dendrobatid poison frog *Oophaga pumilio* from north-eastern Costa Rica.

Materials and Methods

Samples and histological preparation

Oophaga pumilio were collected from La Selva Biological Research Station, Costa Rica (10°26′N, 83°59′W). All specimens were collected from an active cacao *Theobroma*

cacao plantation as part of a larger study on reproductive resource utilization in *O. pumilio* (see Donnelly, 1989a). Upon capture, all frogs were euthanized in chlorobutanol, preserved in a 10% buffered formalin solution, and stored in 70% ethanol. All specimens are currently located in the herpetological collection of M.A.D. at Florida International University.

For the present study, a sub sample of the above collection of *O. pumilio* was selected ($n = 25$), ranging in size from 10.7 to 22.3 mm in snout-to-vent length (SVL). This size series of frogs includes recent metamorphs and adults. Individuals were assigned to one of three age–sex classes (see Donnelly, 1989b): juvenile (SVL < 19 mm), adult male (SVL ≥ 19 mm) and adult female (SVL ≥ 19 mm). Frogs were selected based primarily on body size (SVL) and quality of preservation. A similar patch of skin from the dorsum of each frog was excised, which included the total area of dorsal skin between the forelegs and hind legs. The length and width of each skin patch was measured to determine its area (range: 17–106 mm^2). Each of the skin patches were individually embedded in paraffin using the techniques described by Presnell & Schreibman (1997), and were serially cross-sectioned transversely at 10 μm with a rotary microtome (Reichert & Jung, Model 2030). Cross-sections were mounted on pre-subbed microscope slides (Presnell & Schreibman, 1997), and stained with Reimer's modification of Mayer's hematoxylin and eosin (Jensen & Nielsen, 1968). Poison glands were viewed using bright-field microscopy (Nikon-Optiphot-2, Nikon Instruments Inc., Melville, NY, USA), and were identified based on gland characteristics described in Neuwirth *et al.* (1979) and Angel *et al.* (2003).

Null model of poison gland growth

Scaling (or allometry) is the study of relationships between body size and specific traits, and is fundamental to understanding the ecology and evolution of organisms (Peters, 1983; Schmidt-Nielsen, 1984; Brown & West, 2000). The simplest type of scaling relationship is isometric scaling (Schmidt-Nielsen, 1984), in which changes in one parameter are accompanied by equivalent changes in another, and can serve as a null model against which observed changes in growth can be compared. Allometric scaling refers to changes in one parameter that are not accompanied by equivalent changes in another (Schmidt-Nielsen, 1984). In this study, we constructed a simple isometric model (i.e. null model) to examine the growth of poison glands in *O. pumilio* as a function of overall growth in frog size (SVL).

In our model, *O. pumilio* is assumed to be cuboidal in shape and the number of glands is held constant with increases in body size (SVL). Therefore, poison gland growth only involves changes in gland size (hypertrophy), and not changes in gland number (hyperplasia). Since skin can be considered a planar object, our interest will be limited to changes in length and area (i.e. volume will be ignored; however, see 'Discussion'). If growth of poison glands in *O. pumilio* is isometric, and assuming no gland hyperplasia,

then our model predicts the following results: (1) increases in average poison gland diameter will be directly proportional to increases in SVL, and therefore a logarithmic plot of these two variables will produce a regression line with a slope of 1.0; (2) gland density will decrease with increases in SVL; (3) the percentage of skin area occupied by poison glands will remain constant with increases in SVL.

Poison gland morphological measurements

To investigate relationships between poison glands and body length (SVL) in *O. pumilio*, average poison gland size (diameter and area), the number of poison glands, poison gland density and the percentage of dorsal skin area occupied by poison glands were calculated for all individuals examined in this study.

Average poison gland diameter was calculated from measurements of 15 randomly selected poison glands from each frog skin patch, and was calculated by following individual glands through serially adjacent skin sections, summing the number of sections each gland spanned and multiplying this number by the section thickness of 10 μm. Average poison gland area was derived from the average poison gland diameter of each frog skin. Poison gland area was calculated as πr^2 (where r = average poison gland diameter $\div$ 2), assuming a spherical morphology for poison glands.

The total number of poison glands present in a single skin patch was counted for one individual (15 mm SVL), requiring *c*. 20 h of observation. As a result of this time-consuming process, the following method was devised to obtain an estimate of the number of poison glands present in a skin patch (hereafter referred to as 'estimated number of poison glands'). To minimize over counting glands that appeared in more than one skin section, serial sections from each frog were divided into *sampling strata* equal to the average poison gland diameter. *Sampling strata* were calculated by dividing the total number of sections present in an individual specimen by the average poison gland diameter for that same individual. Poison glands were counted per *stratum* by viewing the section from edge to edge and recording all glands observed. The width of each section was measured to the nearest 0.25 mm using an ocular micrometer (calibrated to 0.1 mm) at a magnification of $\times 400$. To control for variation in gland number associated with variation in section width, the width of the section was used to convert the raw gland counts to a frequency (calculated as the number of glands per mm). Plots of gland frequency for different *strata* from multiple individuals established that poison glands are evenly distributed across skin sections (data not shown). To estimate the number of poison glands present, the following formula was used: estimated number of poison glands = total number of poison glands counted/ [1/(number of strata counted/total number of sections)]. The estimated number of poison glands per frog was used to calculate poison gland density, as follows: poison gland density = estimated number of poison glands/original skin patch area (in mm²).

In an attempt to quantify changes in the quantity of alkaloid exposure to predators associated with changes in gland diameter and/or density, the percentage of dorsal skin area occupied by poison glands was calculated. Assuming predator avoidance of alkaloids is dependent on the amount of alkaloid present, this measure quantifies the exposure of alkaloids per unit measure of skin. To calculate the percentage of dorsal skin area occupied by poison glands, the following formula was used: percentage of dorsal skin area occupied by poison glands = [(estimated number of poison glands)(average poison gland area)/(adjusted patch area)] × 100. Embedding in paraffin has been shown to produce tissue shrinkage of up to 20% (Presnell & Schreibman, 1997), potentially altering estimates of skin area occupied by poison glands. Skin measurements prior and following embedding in the present study showed a linear reduction of approximately 12.5% (data not shown), and therefore the original skin patch was corrected as follows: adjusted patch area = (original length−12.5%)(original width−12.5%).

Statistical analyses

The relationships between poison gland size (diameter and area), the estimated number of poison glands, poison gland density and the percentage of dorsal skin area occupied by poison glands and body size (SVL) were examined using reduced major axis (RMA) regression analysis. RMA regression accounts for error in both the dependent and independent variables, and is considered a more appropriate method than ordinary least squares regression (OLS) when evaluating scaling relationships (LaBarbera, 1989). All data were logarithmically transformed (log$_{10}$) before analysis, and 95% confidence intervals for RMA slopes were calculated using the methods of Sokal & Rohlf (1981). To determine if certain scaling relationships were isometric or allometric, the 95% confidence intervals of the RMA slopes for each regression were compared to a null hypothesis of an isometric slope. A scaling relationship was considered allometric if the 95% confidence interval for its slope did not overlap with the slope predicted for isometry. An isometric slope of 1 was predicted for the regression of poison gland diameter and estimated number of poison glands on SVL, and an isometric slope of 2 was predicted for the regression of poison gland area and percentage of skin area occupied by poison glands on SVL. Statistical analyses were performed using spss (version 17.0 for Mac; SPSS Inc., Chicago, IL, USA) and RMA: software for Reduced Major Axis Regression for Java (Bohonak & van der Linde, 2004).

Results

Average poison gland diameter and area increased with increasing body size (Fig. 1). Poison gland diameter increased from a mean of 36.7 μm at 10.7 mm SVL to 119.3 μm at 23 mm SVL (Fig. 2; $r^2 = 0.90$). The slope of the RMA regression line was 1.67 (95% CI = 1.44–1.90), indicating a positive allometric relationship between poison
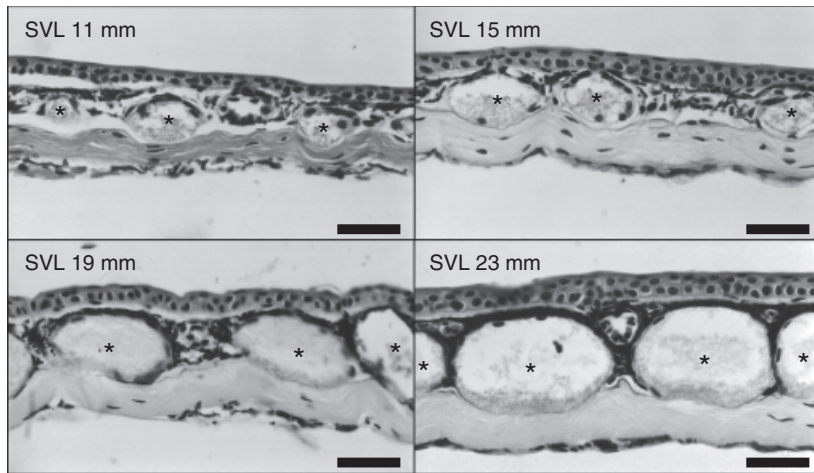
Figure 1 Microphotographic images of representative skin sections at 11, 15, 19 and 23 mm snout-to-vent length, illustrating an increase in poison gland size with an increase in body size in *Oophaga pumilio*. Poison glands are indicated with asterisks. Skins were cross-sectioned transversely at 10 μm; scale bars measure 50 μm.
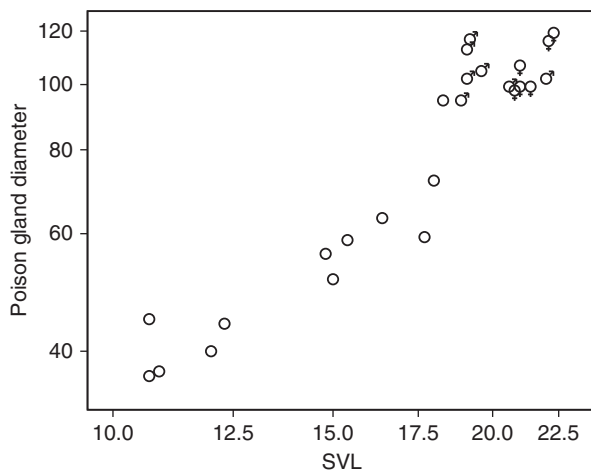

Figure 2 The relationship between average poison gland diameter and body size in *Oophaga pumilio*. Juveniles are indicated by circles, and males & females are indicated by their respective symbols. Graph axes are $\log_{10}$ scaled.
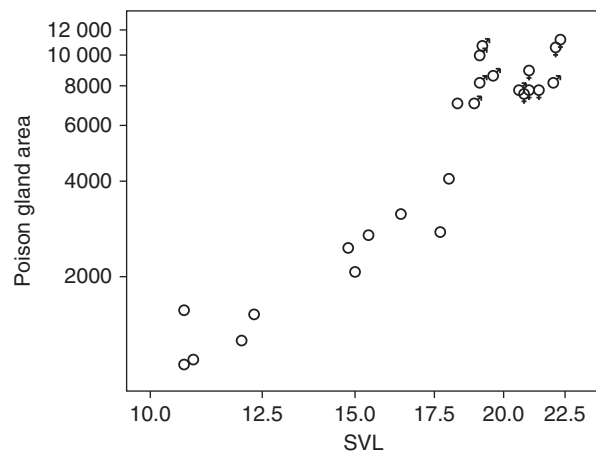

Figure 3 The relationship between average poison gland area and body size in *Oophaga pumilio*. Juveniles are indicated by circles, and males & females are indicated by their respective symbols. Graph axes are $\log_{10}$ scaled.

gland diameter and SVL. The greatest period of increase in poison gland growth was between individuals of 18 mm and 19 mm SVL, and on average there was a 31.2 μm (41%) increase in gland size. Poison gland area was calculated directly from measurements of poison gland diameter, and therefore the scaling patterns are identical. Poison gland area increased from a mean of 1055.9 μm² at 10.7 mm SVL to 11 184.4 μm² at 22.3 mm SVL (Fig. 3; $r^2 = 0.90$). The slope of the RMA regression line was 3.34 (95% CI = 2.88–3.80), indicating a positive allometric relationship between poison gland area and SVL.

The estimated number of poison glands per skin patch increased from *c*. 987 glands at 10.7 mm SVL to 4673 glands at 22.1 mm SVL (Fig. 4; $r^2 = 0.74$). The slope of the RMA regression line was 1.56 (95% CI = 1.22–1.91), indicating a positive allometric relationship between the estimated number of poison glands and SVL. Poison gland density decreased from 60.9 glands per mm² at 10.7 mm SVL to

43.3 glands per mm² at 22.3 mm SVL (Fig. 5; $r^2 = 0.27$). The slope of the RMA regression line was −1.12 (95% CI = −1.54 to −0.71), indicating a negative relationship between poison gland density and SVL. The percentage of dorsal skin area occupied by poison glands increased from 6.9% at 10.7 mm SVL to 71.6% at 22.1 mm SVL (Fig. 6; $r^2 = 0.83$). The slope of the RMA regression line was 3.01 (95% CI = 2.48–3.54), indicating a positive allometric relationship between the percentage of dorsal skin area occupied by poison glands and SVL.

Discussion

During post-metamorphic growth, poison gland size (diameter and area) and the percentage of dorsal skin area occupied by poison glands increased with increasing body size. The overall increase was greater than that predicted by the isometric model of gland growth, suggesting that poison
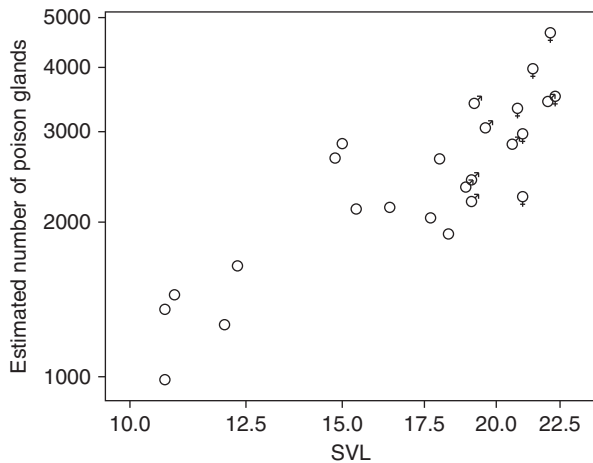
Figure 4 The relationship between the estimated number of poison glands and body size in *Oophaga pumilio*. Juveniles are indicated by circles, and males & females are indicated by their respective symbols. Graph axes are log$_{10}$ scaled.
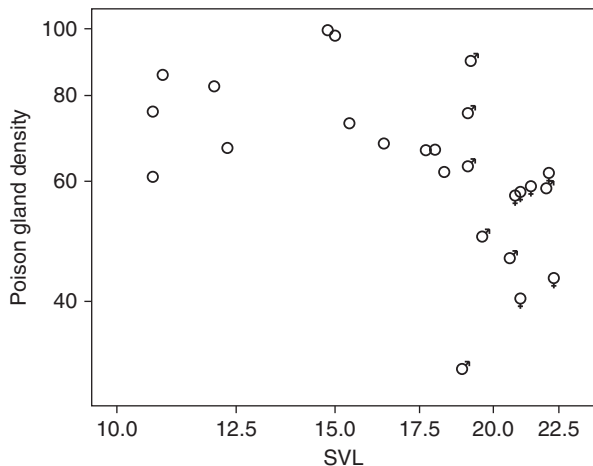

Figure 5 The relationship between average poison gland density and body size in *Oophaga pumilio*. Juveniles are indicated by circles, and males & females are indicated by their respective symbols. Graph axes are log$_{10}$ scaled.

gland growth in *O. pumilio* is allometric, and indicating that poison glands are increasing in size at a rate faster than body size. The number of poison glands also increased with increasing body size, however, gland density, which accounts for changes in skin area, showed a slight decline with increasing body size. The overall decrease in gland density suggests that poison gland hyperplasia is minimal during growth, and that increases in gland size during growth are mainly the result of gland hypertrophy.

The presence of alkaloids in poison glands of *O. pumilio* (and other poison frogs) is the result of a lifetime accumulation from dietary arthropods (Saporito *et al.*, 2009), and alkaloids in certain species of wild-caught dendrobatids raised in captivity have been maintained for years (e.g.,
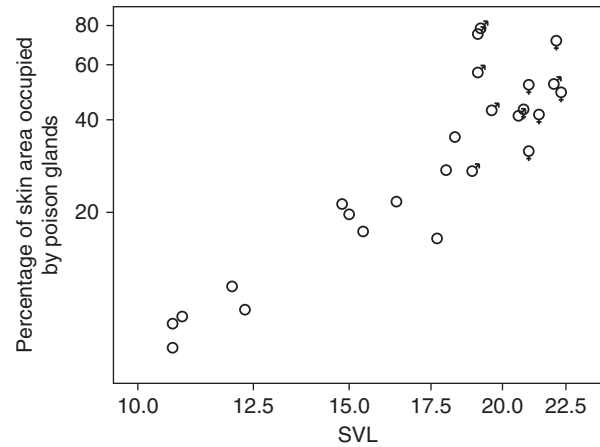

Figure 6 The relationship between the percentage of skin area occupied by poison glands and body size in *Oophaga pumilio*. Juveniles are indicated by circles, and males & females are indicated by their respective symbols. Graph axes are log$_{10}$ scaled.

Myers *et al.*, 1978; Daly *et al.*, 1992, 1994). Juvenile dendrobatid frogs (including *O. pumilio*) contain smaller quantities of alkaloids than adults (e.g. Myers *et al.*, 1978; Daly *et al.*, 2002), and a positive relationship between body size and the number/quantity of alkaloids has been reported for certain species of mantellid poison frogs (Daly *et al.*, 2008). Our findings that poison glands increase in size with *O. pumilio* body size supports these findings, and further suggests that larger frogs have the capacity to accumulate and store larger quantities of alkaloids as compared to smaller frogs. Although gland volume was not measured in the present study, it should be noted that the supposed increased capacity to accumulate and store alkaloids in adult frogs appears to be associated with an increase in gland volume. As a result of the increase in poison gland size, the percentage of dorsal skin area occupied by poison glands also increases with *O. pumilio* body size, suggesting that larger frogs are capable of exposing these higher quantities of alkaloids to potential predators. Whether or not exposure to higher quantities of alkaloids results in increased avoidance by natural predators is not currently known, however differences in the quantity of alkaloids among adult *O. pumilio* (Daly & Myers, 1967) and between adult and juvenile *Phyllobates terribilis* (Myers *et al.*, 1978) have corresponded to differences in 'toxicity,' as measured by injection into standard 20 g NIH mice. Although some poison frog alkaloids are 'toxic' (e.g. Daly & Myers, 1967; Myers *et al.*, 1978; Daly *et al.*, 2003), many are considered unpalatable because of their unpleasant and/or bitter taste (e.g. Fritz, Rand & Depamphilis, 1981; Szelistowski, 1985; Saporito *et al.*, 2007a). The bitter nature of alkaloids can act as a warning to predators if they taste the prey item before ingestion (Brower, 1984; Hartmann, 1991; Nishida, 2002), and it is therefore reasonable to expect that increased exposure to alkaloids will result in increased predator avoidance. Therefore, an increase in the surface area

exposure of poison glands in *O. pumilio* may translate into greater predator avoidance of large frogs as compared with small frogs.

The sequestration of alkaloids in *O. pumilio* (and other poison frogs) from dietary arthropods raises the possibility that ontogenetic changes in feeding ecology could result in changes in the scaling of poison glands. Ontogenetic shifts in diet are common among dendrobatids, and larger frogs tend to consume different sizes and types of prey (e.g. Donnelly, 1991; Lima & Moreira, 1993; Lima & Magnusson, 2000). Donnelly (1991) described an ontogenetic change in the diet of *O. pumilio* that was temporally correlated with sexual maturity (19 mm SVL; Donnelly, 1989b), in which the number of prey consumed increased with frog size, and the diet of juveniles consisted largely of mites, whereas the diet of adults consisted largely of ants. Incidentally, the frogs analyzed by Donnelly (1991) are from the same location as the present study. In the present study, the greatest increase in poison gland size was observed between individuals of 18 and 19 mm snout-to-vent length (Fig. 2), which corresponds to the ontogenetic shift in diet of *O. pumilio*. On average there was a 41% increase in gland diameter between frogs of 18 and 19 mm snout-to-vent length, suggesting a marked increase in the capacity to accumulate and store alkaloids in adult *O. pumilio*. Both mites and ants represent the two largest groups of dietary sources for alkaloids in *O. pumilio* (Saporito *et al.*, 2007b, 2009), and it is possible that the observed increase in gland diameter between juveniles and adults may be the result of a change in the consumption of these alkaloid-containing arthropods. Given that poison glands act as storage cells for alkaloids, then it is conceivable that changes in overall gland size are directly related to changes in the quantity of alkaloids accumulated. The diet of juveniles consists largely of mites, whereas adults consume almost twice as many ants as juveniles (Donnelly, 1991), suggesting that an increase in gland size could be the result of an increased accumulation of ant-derived alkaloids in adults. R. A. Saporito *et al.* (unpubl. data) found that alkaloids in adult *O. pumilio* from this population consist largely of ant-derived alkaloids, and Saporito *et al.* (2007a) reported that populations of adult *O. pumilio* from nearby collection sites also contained mainly ant-derived alkaloids. These data suggest that alkaloids in adult *O. pumilio* from the La Selva population and this region are largely ant-derived, which may be a result of an increased consumption of alkaloid-containing ants by adults. Currently, there are no alkaloid data available for juveniles from this population, but if increases in poison gland size between juveniles and adults are the result of increases in consumption of alkaloid-containing ants, then we might expect juveniles to contain fewer ant-derived alkaloids when compared to adults.

The overall increase in poison gland size with body size and sexual maturity in *O. pumilio* suggests that larger frogs have the capacity to accumulate and store larger quantities of alkaloids, which may confer greater protection from predation for large and/or sexually mature frogs. Although relatively little information is available on the behavior and activity of juvenile *O. pumilio*, adults are diurnally active leaf-litter inhabitants that are characterized by complex courtship, parental care, and territoriality (Guyer & Donnelly, 2005). Furthermore, *O. pumilio* are aposematically colored (Saporito *et al.*, 2007c) and exhibit a reduction in predator escape behaviors when compared with palatable frogs in the genus *Craugastor* (Cooper Jr, Caldwell & Vitt, 2009). All of these behaviors presumably increase the vulnerability of adults to predation, and therefore an ability to accumulate and store larger quantities of alkaloids in adult *O. pumilio* may be associated with an increased risk in predation at this life stage. Although juveniles contain smaller glands and may not be chemically defended as well as adults, they are aposematically colored and may still gain protection from predators by resembling adults (i.e. automimicry; Brower, Pough & Meck, 1970). Automimicry has been described in phytophagous arthropods that sequester chemical defenses from plants (see Ruxton, Sherratt & Speed, 2004 for review), but to the best of our knowledge has not been described among vertebrates that sequester defenses. Future studies aimed at examining natural predation between juvenile and adult frogs are needed to further test the hypothesis of automimicry in *O. pumilio*.

Poison gland size and area occupied by these glands increases during post-metamorphic growth in the dendrobatid frog *O. pumilio* from north-eastern Costa Rica, suggesting that chemical defense in this poison frog species also increases with body size. The increase in poison gland growth is allometric, and appears to be the result of gland hypertrophy. Poison glands in *O. pumilio* act as storage sites for the alkaloid-based chemical defenses that are sequestered from dietary arthropods. The largest increase in poison gland size appears to be temporally correlated with the onset of sexual maturity, which may result from an ontogenetic diet shift and/or increases in steroid hormones that accompany sexual maturity. Here we demonstrate that the physical basis of chemical defense in one dendrobatid species is allometric, which may suggest that all poison frogs have a similar pattern in gland maturation and development.

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