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VARIATION IN THE MATERNAL PROVISIONING OF ALKALOIDS IN THE STRAWBERRY POISON FROG OOPHAGA PUMILIO: THE RELATIONSHIP BETWEEN MOTHERS AND THEIR TADPOLE OFFSPRING

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Abstract

Within and among populations, alkaloid defenses of strawberry poison frogs (*Oophaga pumilio*) vary spatially, temporally, and with life history stage. Natural variation in defense has been implicated as a critical factor in determining the level of protection afforded to an individual from predators and pathogens. *Oophaga pumilio* tadpoles sequester defenses from nutritive eggs and are thus entirely dependent on their mothers for their alkaloids. However, it remains unclear how the alkaloid composition of a tadpole relates to that of its mother and if maternally provisioned defenses are effective against predators. Here, I demonstrate that natural variation in the alkaloid composition of mother frogs—even among individuals collected less than a few hundred meters apart—is reflected as variation in tadpole alkaloid composition. Mother frogs and their specific tadpoles were collected from La Selva Research Station in Costa Rica in order to make direct comparisons of the alkaloid profiles between mothers and their offspring. Additional tadpoles were collected for palatability assays to determine if maternally provisioned alkaloids provide meaningful protection from predators. Tadpoles, like mother frogs, varied widely in their alkaloid composition but contained the exact same types of alkaloids found in their mother. Late stage tadpole alkaloid quantity was highly correlated with the alkaloid quantity of the mother frog, and alkaloid quantity was the best predictor of tadpole palatability where tadpoles with higher alkaloid quantities were less palatable. Overall, the alkaloid profile of tadpoles are highly similar to that of their mother and variation in the alkaloid composition of mother frogs is translated as variation in the alkaloid composition of tadpoles. Mother frogs that provide greater quantities of alkaloids to their tadpoles likely ensure better protection for their offspring by providing defenses during one of the most vulnerable periods of life. Future studies

should examine how and if variation in alkaloid composition across the geographic range of *O. pumilio* translates to variation in tadpole alkaloid composition and the implications this has for protection from local predators and pathogens.

Introduction

The use of chemical defenses against predators, pathogens, and parasites is widespread in nature. Most organisms are able to biosynthesize defensive chemicals, whereas some organisms must obtain them secondarily through a specialized diet of chemically defended prey items (Nishida 2002; Saporito *et al.* 2012). The uptake, accumulation, and storage of secondarily derived defenses from diet is known as sequestration (Mebs 2001; Sanchez *et al.* 2019) and is a well-studied phenomenon among phytophagous arthropods (reviewed in Opitz & Muller 2009); however, sequestration has also evolved independently in several groups of vertebrates (reviewed in Savitzky *et al.* 2012) including snakes (Hutchinson *et al.* 2007), amphibians (Daly *et al.* 1994; Saporito *et al.* 2009), and likely birds (Dumbacher *et al.* 2004; Dumbacher *et al.* 2009).

Within and among species, organisms that sequester defensive chemicals can vary widely in their chemical composition, and this variability may have multiple points of origin (Speed *et al.* 2012). Spatiotemporal variation in the abundance and availability of prey appears to directly affect a predator's own chemical defenses (Saporito *et al.* 2007a; Hutchinson *et al.* 2013). For example, macrogeographic variation in prey defense (e.g., variation in availability of chemically defended prey across a species' range) may result in large-scale variation in the sequestered defenses of predators (Saporito *et al.* 2006; Triponez *et al.* 2007). Furthermore, microgeographic variation in prey defenses may result in small-scale variation in the sequestered defenses of predators within and among neighboring populations (Pasteels *et al.* 1995; Moranz & Brower 1998). Variation in sequestered chemical defenses may also originate from seasonal or long-term heterogeneity in prey availability (Malcolm & Brower 1989; Pasteels *et al.* 1995). Additionally,

chemical defenses within a species may vary with sex, age, and body size (Nishida & Fukami 1989; Alonso-Mejia & Brower 1994; Speed *et al.* 2012).

In many species, the presence of chemical defenses are dynamic with regard to life history stage. Some organisms may sequester defenses throughout their life cycle (Nishida & Fukami 1989; Eisner *et al.* 2000) whereas, in other organisms, defenses are temporary (Fordyce *et al.* 2005). The presence of defenses may be limited to one or a few life stages where compounds are sequestered, which can be early in development (Malcolm & Rothschild 1983) or as adults (Brown 1987). In many lepidopterans, defenses sequestered during larval stages are retained through metamorphosis and into adulthood (Malcolm & Brower 1989; Bowers & Williams 1995; Nishida 2002); however, the presence or effectiveness of these defenses may decrease with age (Alonso-Mejia & Brower 1994). In addition, some organisms are able to provision their offspring with chemical defenses prior to hatching or birth, though the effectiveness of these defenses can decrease with offspring growth and development (Hutchinson *et al.* 2008; Hayes *et al.* 2009; however see Williams *et al.* 2011).

The provisioning of chemical defenses to offspring is thought to serve as an antipredator (and possibly antimicrobial) mechanism during one of the most vulnerable periods of life (reviewed in Gunzburger & Travis 2005). For example, female ornate bella moths (*Utethesia ornatrix*) provision egg clutches with pyrrolizidine alkaloids, which in turn act as a deterrent to their primary predator—the larvae of green lacewings (*Ceraeochrysa cubana*) (Eisner *et al.* 2000). In some organisms, developing embryos absorb maternally provisioned chemical defenses and newly hatched offspring retain these defenses until they begin sequestering their own. In the Asian snake (*Rhabdophis tigrinus*), bufadienolides sequestered from a diet of toads are provisioned to embryos by gestating females. Neonates retain these chemical defenses post-

hatching until they begin independently feeding on toads and sequestering bufadienolides (Hutchinson *et al.* 2008). Many other organisms provision chemical defenses to their young, including fireflies in the genus *Photuris* (González *et al.* 1999), harlequin frogs (*Atelopus chiriquiensis*) (Pavelka *et al.* 1977), and the rough-skinned newt (*Taricha granulosa*) (Hanifin *et al.* 2003). The chemical defenses acquired by offspring via maternal provisioning are highly variable (Eisner *et al.* 2000; Hanifin *et al.* 2003) and several studies have suggested a positive correlation between mother and offspring defense quantities (Hanifin *et al.* 2003; Hutchinson *et al.* 2008; Williams *et al.* 2011).

A well-studied group of chemically defended vertebrates are the conspicuously colored poison frogs, which form a wide-ranging group of over 150 species with members in several families worldwide (reviewed in Saporito *et al.* 2012). Members of this group sequester their defensive chemicals from a diet of alkaloid-containing arthropods composed primarily of mites and ants (Saporito *et al.* 2004; Saporito *et al.* 2007b), but also millipedes and beetles (Daly *et al.* 2000; Saporito *et al*. 2003). As a result, poison frogs are unpalatable to certain predators (Hantak *et al.* 2016; Murray *et al.* 2016) and likely protected from microbial infection (Mina *et al.* 2016; Hovey *et al.* 2018). Alkaloid type, number, and quantity are highly variable within and among poison frog species. Populations of a single species may differ from one another across geographic space as well as through time (Saporito *et al.* 2007a). Within and among populations, alkaloid profiles may differ between sexes (Saporito *et al.* 2010), across life-history stages (Stynoski *et al.* 2014a) and may increase with age and body size (Jeckel *et al.* 2015). Variability in alkaloid defenses among poison frogs appears to play an important role in their effectiveness as deterrents against predators (Bolton *et al.* 2017) and pathogens (Hovey *et al.* 2018).

In the poison frog family Dendrobatidae, both sexes of the strawberry poison frog (*Oophaga pumilio*) are chemically defended and invest in parental care. Males moisten terrestrial egg clutches for seven to 10 days and upon hatching, tadpoles are singly transported by mothers to water-filled leaf axils of plants. Mothers then return every one to two days for a period of six to eight weeks to provision the obligatory oophagous tadpoles with unfertilized (nutritive) alkaloid-containing eggs—providing both nutrition and defense to developing tadpoles (Stynoski *et al.* 2014a, 2014b). Maternal provisioning of alkaloids is particularly important, given that tadpoles cannot access the terrestrial alkaloid-containing arthropods necessary for obtaining chemical defenses on their own. Although maternal provisioning of defenses is a described phenomenon, the provisioning of alkaloid-laden nutritive eggs to already hatched tadpoles is the first known example of provisioning in vertebrates to occur post-hatching or birth. Since the time of its discovery, maternal provisioning of alkaloids in *O. pumilio* has also been experimentally demonstrated (Saporito *et al.* 2019). Provisioned alkaloids provide defense to tadpoles from predators; however, this has only been demonstrated in late stage tadpoles (Stynoski *et al.* 2014b). Tadpole alkaloid quantity increases with both tadpole mass and developmental stage (Stynoski *et al.* 2014a; Saporito *et al.* 2019), suggesting that the level of defense against predators within a tadpole also increases over the course of development. Furthermore, alkaloids vary among females within and among populations, suggesting that these differences in alkaloid composition may be passed on to offspring (Saporito *et al.* 2007a; Saporito *et al.* 2010).

The present study aimed to further understand some of the fundamental ecological factors involved in this form of chemical defense—in particular, how variation in mother frog chemical defenses relate to tadpole chemical defenses and how variation in defenses translate to offspring defense. Therefore, the specific objectives of my study were (1) to determine if variation in

alkaloid number, type, and quantity within a population of adult *O. pumilio* translated to variation in the alkaloids of tadpoles via maternal provisioning, (2) to determine if tadpole alkaloid quantity is correlated with mother alkaloid quantity, and (3) to determine if variation in tadpole alkaloid quantity results in differences in protection from predators (palatability). Naturally occurring variation in the alkaloid defenses of adult poison frogs results in variation in palatability to predators (i.e., palatability spectrum) as well as variation in overall protection from pathogens and disease (Bolton *et al.* 2017; Hovey *et al.* 2018). Because tadpole chemical defenses are entirely dependent on the defenses of the mother frog, an individual tadpole's level of protection may also be entirely dependent on its mother. Therefore, variation in chemical defenses at the individual and population level may directly affect how well-protected an organism is from predators as well as from local parasites and pathogens.

Methods

Study site and frog collection. The present study was conducted at La Selva Research Station (10°25'52.33"N, 84° 0'12.74"W)—a private reserve located in Heredia Province, Costa Rica and managed by the Organization for Tropical Studies (OTS). The majority of the reserve is comprised of evergreen primary forest, but also includes selectively logged primary forest, pasture, and abandoned cacao plantations (McDade & Hartshorn 1994; Whitfield *et al.* 2007).

In order to determine the relationship in alkaloid profiles between mothers and their tadpoles, behavioral observations to identify mother/tadpole pairs were conducted within the Huertos Plots at La Selva (10°26' N, 84° 0'46.38" W). The Huertos Plots are the site of an abandoned cacao plantation and provide an ideal location to observe parental care and egg

provisioning because *O. pumilio* are abundant and reproductively active (Donnelly 1989b; Gade *et al.* 2016; DeMarchi *et al.* 2018). Mother *O. pumilio* deposit tadpoles into naturally occurring water-filled leaf axils of plants such as *Heliconia*, bromeliads, and bananas (*Musa*) (Donnelly 1989a; Haase & Pröhl 2002); however, mothers will also deposit tadpoles into cups (referred to as artificial tadpole-rearing sites), which mimic naturally occurring phytotelmata and allow for greater ease of access to tadpoles (Stynoski 2009; Stynoski *et al.* 2014a). Tadpole-rearing cups were constructed from 30 mL plastic polypropylene beakers each affixed to a single plastic knife with a zip-tie (Fig. 1). Two small holes were drilled in each cup to prevent excess rainwater from flushing tadpoles out of the top of the cup. A total of 786 cups were set up in transects in three separate arrays (hereafter plots) in the Huertos Plots with each set of cups affixed to a tree approximately 1.5 m off the ground (Fig. 2). Trees were selected at random every few meters to form the transects and the diameter at breast height (dbh) was recorded for every tree containing cups. Although tadpole-rearing cups were set up in transects in three separate plots, this was only to allow greater ease of access to each cup. The three plots represent a single field site comprising several hundred square meters and were not considered as independent entities. Tadpole-rearing cups were set out on 06 March 2019 and 07 March 2019 in order to allow mother frogs time to acclimate and begin using the artificial tadpole-rearing sites.

Artificial tadpole-rearing cups were surveyed daily from 04 June 2019 – 07 July 2019 in order to identify cups that contained tadpoles (presence/absence) and to record the developmental stage of each tadpole (Gosner staging; Gosner 1960). To avoid any possible effects of physically manipulating tadpoles, tadpoles were only qualitatively staged in the field and therefore, reported developmental stages are estimates. Tadpoles that were present during a

census but were missing during a subsequent census were classified into three categories, following Stynoski 2009: predation, metamorphosis, or splashed out by raindrops.

Cups containing tadpoles were targeted for behavioral observations to identify mother/tadpole pairs, and observations took place daily from 07 June 2019 – 05 July 2019 between the hours of 0500-1130 when mother frogs are known to actively provision their young (Haase & Pröhl 2002). Tadpoles were selected for observation based on estimated mass and stage in order to ensure that tadpoles representing a wide range of development were collected. Targeted cups containing tadpoles were observed from a distance of ~3 m until a female frog—presumably that tadpole's mother—returned to provision her offspring. Mother *O. pumilio* do not recognize their own offspring and instead use spatial cues to recognize and relocate where they left their tadpole(s) (Stynoski 2009). Therefore, when a mother is provisioning a tadpole, it can be assumed that it is her offspring. Mothers collected as part of a mother/tadpole pair were classified as tadpole-rearing, meaning that they were found in a cup containing a tadpole and were observed performing maternal provisioning behavior (e.g., delivering tadpoles to a tadpole-rearing cup, visiting a tadpole-containing cup, depositing nutritive eggs into a tadpole-containing cup).

Mother frogs were observed until they had climbed fully into the tadpole-containing cup and had at least partially submerged their body in the water. Once a mother frog climbed fully into a tadpole-containing cup, she was captured with an aquarium net, placed in a one-gallon Ziplock™ bag, and transported back to an ambient laboratory. Tadpoles were collected using disposable polyethylene transfer pipets and stored in a 20 mL glass vial with water from that tadpole's cup. A total of 13 mother/tadpole pairs were collected following this method. Additional tadpoles of varying developmental stages were collected from cups solely for use in

palatability assays. All tadpoles were collected from cups except for one tadpole that was collected directly from a mother's back and was subsequently placed in a vial of rainwater collected from a nearby cup that did not contain a tadpole. This tadpole did not contain any alkaloids further indicating that tadpoles only sequester alkaloids from nutritive eggs. There was one case in which two tadpoles were deposited into a single cup, and so each tadpole was stored in a separate 20 mL glass vial. Both tadpoles were collected solely for the purpose of palatability assays and were not examined as part of a mother/tadpole pair.

Following collection in the field, all mother frogs were weighed to the nearest 0.1 mg using a Pesola PPS200 digital pocket scale and measured for snout-to-vent length (adults; SVL 19-22 mm) (Donnelly 1989b) to the nearest 0.1 mm using a Traceable® Digital Calipers. Mother frogs were handled as little as possible to prevent alkaloid secretion. Mother frogs were euthanized via freezing, following which their skins were removed and stored in separate 4 mL glass vials with Teflon-lined caps containing 2 mL of 100% methanol. Tadpoles were also weighed to the nearest 0.1 mg using a Pesola PPS200 digital pocket scale and then euthanized via freezing and stored wholly in separate 4 mL glass vials with Teflon-lined caps containing 2 mL of 100% methanol.

Palatability assays. To examine how differences in tadpole alkaloid composition throughout development determines the level of protection from predators, ant palatability assays were conducted with *Ectatomma ruidum*, following the methods of Bolton et al. 2017. Ants were collected from the lab clearings and the arboretum at La Selva using Jolly Ranchers™ as bait. Individual *E. ruidum* were collected with pressure sensitive forceps between 1300-1700 hrs, stored in small plastic containers (~10 individuals per container) in an ambient laboratory, and

starved for a period of 48 hours prior to trials (Bolton *et al*. 2017). All ants collected within a 2 m radius of each other were assumed to be from the same nest (Lachaud 1990), and nests were not sampled more than three times throughout the study.

Palatability assays were performed using the methanol extracts from sixteen tadpoles that were selected to represent a range of developmental stages (stages 25-44) and masses (0-190 mg). Palatability assays consisted of feeding trials wherein ants were placed individually into the center of a small, glass petri dish or arena (~6 cm diameter) and allowed to choose between two sugar solutions: one containing alkaloids and one without alkaloids. To create the alkaloid sucrose solution, 1 mL of the original 2 mL methanol/tadpole solution was transferred to a separate vial and evaporated to dryness. Following evaporation, 250 µL of a sucrose solution (50% ethanol, 20% sucrose) was added to the vial to create an alkaloid/ethanol/sucrose solution. Each petri dish contained two coverslips, one with 10 µL of an alkaloid/ethanol/sucrose solution and one with 10 µL of a control solution (50% ethanol, 20% sucrose). The location of the solutions within the petri dish were randomized for each trial and ants were selected randomly with respect to nest location. Ants were allowed a five-minute period to detect and sample either solution, but ants were only considered to have successfully fed on a solution when the ant submerged its mandibles in a solution for more than three seconds (Bolton *et al*. 2017). If an ant did not feed on either solution within a five-minute period, the ant was removed and replaced with a second ant. If the second ant also did not feed on either solution within a five-minute period, the solutions were replaced, the arena cleaned with a 10% ethanol solution, and a new trial was initiated with a new ant. Fifteen trials were run per tadpole (n = 16 tadpoles) for a total of 240 trials. Arenas were cleaned with a 10% ethanol solution between trials. Tadpole palatability was quantified by assigning each tadpole a palatability score based on a palatability

index that ranged from -1 to 1. Individuals scoring closer to -1 were considered more unpalatable than individuals closer to 1. The palatability index was calculated as follows: (# ants that fed on the alkaloid solution) – (# ants that fed on the control solution) ÷ total number of ants. All palatability scores of 0 or greater were considered to represent a palatable prey source (Bolton *et al.* 2017), and because the intent of the palatability assays were to determine if tadpole alkaloids act as an effective deterrent against potential arthropod predators, no statistical distinction was made between neutral (0) and positive palatability scores. Therefore, all positive palatability scores were reduced to a value of 0 for statistical analyses (Dyer *et al.* 2003).

Alkaloid fractionation. Alkaloids from each of the 13 mother frog skins and 20 whole tadpoles were extracted using an acid-base technique (Saporito *et al.* 2010; Jeckel *et al.* 2015; Hovey *et al.* 2018). For each sample, 50 μL of 1 N HCL and an internal standard of nicotine were added to 1 mL of the original methanol extract. This solution was then concentrated to 100 μL using nitrogen gas, followed by the addition of 200 μL of deionized water. Four extractions were then performed, each time using 300 μL of hexane and the hexane layer was discarded each time. $NaHCO_3$ was added to the remaining solution in order to basify it. This solution was then extracted three times, each time using 300 μL of ethyl acetate. Excess water was removed from each ethyl acetate layer using anhydrous Na_2SO_4 and then blown down with nitrogen to dryness. Finally, 100 μL of methanol was added.

Alkaloid analysis. Gas Chromatography-Mass Spectrometry (GC-MS) was used to identify, characterize, and quantify alkaloids (Saporito *et al.* 2010; Jeckel *et al.* 2015; Hovey *et al.* 2018). The GC-MS is a Varian 3900 GC coupled with a Varian Saturn 2100 T ion trap MS using a 30 m

x 0.25 mm ID Varian Factor Four VF-5 ms fused silica column. A temperature program ran from 100 to 280 °C at a rate of 10 °C per minute using helium as a carrier gas (1 mL/min). Each alkaloid fraction was analyzed three times using electron impact-mass spectrometry (EI-MS) and once using chemical ionization-mass spectrometry (CI-MS). Alkaloids were identified by comparing GC retention times and mass spectral properties to already established dendrobatid alkaloid data (Daly *et al.* 2005; additional citations in Hovey *et al*. 2018), and were quantified by comparing alkaloid peaks to that of the nicotine internal standard using a Varian MS Workstation v.6.9 SPI (Hovey *et al.* 2018). Alkaloids detected in quantities below 0.5 µg in mother frogs and 0.01 µg in tadpoles were excluded from further analysis (Lawrence *et al.* 2019), except in cases where the alkaloids that a mother provisioned to her tadpole fell below the 0.5 µg threshold. Quantity thresholds were selected to represent a balance between the biological relevance of the alkaloids' quantity against predators and pathogens (Weldon *et al.* 2006) and also attempt to encompass the full range of alkaloid types present in mothers and their tadpoles.

Statistical analyses. Linear regressions were used to test for relationships between tadpole mass and alkaloid quantity (µg/tadpole) as well as the relationship between tadpole mass and number of alkaloids per tadpole. The relationships between tadpole Gosner stage and alkaloid quantity (µg/tadpole) as well as the relationship between tadpole Gosner stage and number of alkaloids per tadpole were also tested using linear regressions. In *O. pumilio* tadpoles, mass is considered a better predictor of alkaloid quantity than total length (Stynoski 2012); however, understanding how alkaloid sequestration in tadpoles coincides with the development of granular glands is also critical. Therefore, an additional linear regression was run to determine if tadpole mass is a reliable predictor of Gosner stage. For the palatability tests, additional linear regressions were

run to examine the relationship between tadpole palatability and Gosner stage, tadpole mass, tadpole alkaloid quantity, and number of alkaloids per tadpole respectively.

To gain a better understanding of how a tadpole's alkaloid profile relates to that of its mother, linear regressions were also used to determine the relationship between a mother frog's alkaloid quantity and her tadpole's alkaloid quantity. These data were examined by determining the relationships between late-stage tadpoles (41-44) only and their mothers (n = 6) and all alkaloid-containing tadpoles and their mothers (n = 9). Late stage tadpoles were analyzed separately from all other mother/tadpole pairs in order to avoid any potential confounding effects of tadpole gland development on sequestration capability. Alkaloids are present in tadpoles as young as stage 30 (Saporito *et al.* 2019), which approximately coincides with the early stages of granular (poison) gland development (stages 32-33, Stynoski & O'Connell 2017). Individuals appear to experience an exponential increase in alkaloid quantity in the stages shortly thereafter (ca. stages 30-35, Stynoski *et al.* 2014a; Saporito *et al.* 2019), suggesting that the process of gland development observed during this developmental period influences the capacity for alkaloid uptake. Late stage tadpoles (ca. stage 40 and up) are more likely to have developed granular (poison) glands (Stynoski & O'Connell 2017) and therefore the alkaloid quantity in those individuals is less likely to be influenced by the differentiation of glands, thus providing a more robust comparison between mother frogs and tadpoles. For the purposes of describing tadpole alkaloid composition with respect to development of granular glands, tadpoles were placed into one of three developmental age groups: early stage (pre-gland development; 25-29), middle stage (beginning of gland development; 30-32), and late stage (fully developed glands; 41-44). All mother frog/tadpole comparative analyses were corrected for both mother skin mass and tadpole total mass (Stynoski *et al.* 2014a).

Poison frog alkaloids can be classified into two main categories—those with branched and unbranched carbon skeletons—and inferences about dietary sources of alkaloids can be derived by examining these characteristics of a frog or tadpole's alkaloid composition. Broadly speaking, branched alkaloids are typically mite-derived whereas unbranched alkaloids are typically ant derived (reviewed in Saporito *et al.* 2012). Because *O. pumilio* alkaloid composition is currently thought to reflect the alkaloid composition of the sympatric arthropod community, the alkaloid composition (e.g., branched and unbranched alkaloids) of the adult frogs should reflect what dietary arthropods are available in the environment (Saporito *et al.* 2007a). To gain more insight into the arthropod sources of alkaloids in mother frogs and their tadpoles, two-tailed two-sample t-tests were performed to compare number of alkaloids and alkaloid quantity between branched and unbranched alkaloids for both mother frogs and tadpoles.

Adult *O. pumilio* alkaloid composition is known to vary among populations that are spatially separated on a large geographic scale and within populations on a smaller geographic scale (Saporito *et al.* 2007a; Hovey *et al.* 2018). Because large-scale geographic variation in alkaloid composition has been shown to be influential in providing protection against predators and pathogens (Bolton *et al.* 2017; Hovey *et al.* 2018), small-scale variation in the alkaloid composition of mother frogs may also be important in contributing to variation in tadpole alkaloid composition and therefore variation in protection within a population. Nonmetric multidimensional (NMDS) scaling was used to visualize differences in alkaloid composition among mother frogs.

Finally, mother *O. pumilio* should elect to deposit their tadpoles in artificial tadpole-rearing cups when naturally occurring bromeliad density is low because the cups serve as additional reproductive resources that allow mother frogs to raise tadpoles where they would

otherwise be unable (Donnelly 1989a). Therefore, bromeliad density within the study site may predict tadpole spatial deposition. A logistic regression was performed in order to assess if there was any relationship between bromeliad presence and the presence of tadpoles deposited in cups. Bromeliad density was collected on the basis of presence/absence for each tree within the study site equipped with a tadpole-rearing cup; bromeliads were only marked as present if they were of sufficient size to harbor developing tadpoles. Heatmaps depicting interpolated tadpole and bromeliad density were created using inverse distance weighting (IDW) in ArcMap® GIS version 10.7. Tadpole-rearing cups were spatially placed close together (within the range of error for most GPS units) and located under dense rainforest canopy. Therefore, transects in the study site were replicated by creating scatterplots using artificial GPS coordinates. Heatmaps created using ArcMap® GIS were then overlaid on a Google Earth image at their approximate locations. Predicted tadpole densities were created using known tadpole density (i.e., presence of tadpoles in cups) for all tadpoles documented in cups throughout the study period. Predicted bromeliad densities were extrapolated from presence/absence values of bromeliads on trees with cups. All statistical analyses were conducted in R (R Core Team 2018, v.3.5.1) and Primer 6 (v.6.1.6).

Results

Tadpole-rearing cup surveys. A total of 70 tadpoles were observed in artificial tadpole-rearing cups over the course of the collection period (9% occupancy rate). Tadpole cup occupancy and tadpole fate (metamorphosis, predation, splashed out by raindrops) are summarized in Table 1 and Table 2. A heatmap indicating tadpole cup occupancy for the study site is show in Figure 3. Tadpole density was highest in plot 3 and lowest in plot 1, and there was no relationship between

bromeliad presence and the spatial deposition of tadpoles into tadpole-rearing cups (odds ratio = 1.12, p = 0.71). Heatmaps comparing tadpole and bromeliad density for the study site are shown in Figure 4. The diameter at breast height for trees with tadpole-rearing cups spanned 8-180 cm and mother frogs did not appear to prefer any one tree size for tadpole deposition (Fig. 5). Mother *O. pumilio* were observed visiting, provisioning, and/or depositing tadpoles early in the morning, beginning at 0530 hr, with visits to cups tapering off (but not stopping completely) after 1000 hr (Fig. 6).

Mother frog and tadpole alkaloid composition. Of the 13 mother/tadpole pairs, alkaloids were identified in all 13 mother frogs and nine tadpoles (four tadpoles did not contain any alkaloids). A total of 172 alkaloid types (including isomers) in 21 structural classes were identified across all mother frogs and all alkaloid-containing tadpoles (Table 3). On average (mean±SE), mother frogs contained 523 ± 145 μg of alkaloids (range: 180-1,239 μg) and tadpoles contained 7 ± 2 μg (range: 0-25 μg). The relative variation in alkaloid composition among mother frogs (n = 13) is represented in Figure 7.

Relationship between mother frog and tadpole alkaloids. Tadpoles contained all of the same types of alkaloids as in their mothers even if present only in trace amounts (<0.01 μg). Major alkaloid structural classes shared between mother frogs and tadpoles included 5,8-disubstituted indolizidines, 5,6,8-trisubstituted indolizidines, piperidines, and pyrrolidines (Table 3). There was no relationship in alkaloid quantity between tadpoles and their mothers when all alkaloid-containing tadpoles were included in the analysis (R^2 = -0.2, n = 18, p = 0.40; Fig. 8); however, when examining the same relationship between only late-stage tadpoles (41-44) and their

mothers, a significant positive trend was detected between female and tadpole alkaloid quantity ($R^2 = 0.62$, n = 12, p = 0.03; Fig. 9).

Ontogenetic shifts in tadpole alkaloid composition and palatability. Both alkaloid quantity ($R^2 = 0.599$, n = 20, $p < 0.001$) and number of alkaloids per tadpole ($R^2 = 0.695$, n = 20, $p < 0.001$) significantly increased with tadpole mass (Fig. 10). Similarly, tadpole alkaloid quantity ($R^2 = 0.83$, n = 20, $p < 0.001$) and number of alkaloids per tadpole ($R^2 = 0.80$, n = 20, $p < 0.001$) significantly increased with developmental stage (Fig. 11). A visualization of the relationship between alkaloid composition, developmental stage, and tadpole mass is shown in Figure 12. There was also a significant relationship between tadpole mass and tadpole developmental stage ($R^2 = 0.85$, n = 20, $p < 0.001$; Fig. 13), and tadpoles appeared to begin sequestering alkaloids in the middle stages of development (stages 30-32, Fig. 14).

Tadpole alkaloid quantity was a significant predictor of palatability ($R^2 = 0.28$, n = 16, p = 0.02; Fig. 15a), but not the total number of alkaloids per tadpole ($R^2 = 0.12$, n = 16, p = 0.10; Fig. 15b). Tadpole mass was not a significant predictor of palatability ($R^2 = -0.0004$, n = 16, p = 0.33; Fig. 15c), and neither was Gosner stage ($R^2 = 0.02$, n = 16, p = 0.26; Fig. 15d). It is important to note that while tadpoles contained all of the alkaloids present in their mother, only alkaloids present in quantities greater than 0.01μg were used for tadpole alkaloid analyses.

Dietary origin of alkaloids. When comparing differences in alkaloid composition between branched and unbranched alkaloids in mother frogs and tadpoles, there was no significant difference in the number of alkaloids (t = -0.22, df = 24, p = 0.83). Additionally, there was no

significant difference for branched or unbranched alkaloid quantity for mother frogs ($t = -0.62$, $df = 24$, $p = 0.54$) or tadpoles ($t = -0.48$, $df = 16$, $p = 0.64$).

Discussion

Previous studies have demonstrated that *O. pumilio* tadpoles sequester alkaloid defenses from nutritive eggs (Stynoski *et al.* 2014a; Saporito *et al.* 2019), but it has remained unclear how tadpole alkaloid profiles relate to their mother's. In the present study, tadpoles were found to share very similar alkaloid profiles (type and quantity) to their mothers, suggesting that tadpole defenses in *O. pumilio* are largely a reflection of their mother's defenses. All tadpoles contained the same alkaloids as their mothers, providing the first direct evidence that the type of maternally provisioned alkaloids is identical between mother and offspring. The quantity of alkaloid defenses in a tadpole were also related to the amount of alkaloid in mother frogs but only for late-stage (older) tadpoles (stages 41-44). In general, older tadpoles that contained larger quantities of alkaloids tended to have mothers with proportionally higher quantities of alkaloid defenses. Although this was not the case for younger tadpoles, this is likely due to differences in poison glands between younger and older tadpoles, and in particular, their ability to store alkaloids. Late-stage tadpoles have fully developed poison glands (Stynoski & O'Connell 2017), which are physiologically mature and capable of storing alkaloids, whereas young tadpoles just beginning to develop glands may not have the same physiological ability or capacity to store alkaloids (see further discussion below). Therefore, it is not surprising that older tadpoles were more likely to reflect the actual differences in alkaloid quantities of their mothers when compared to younger tadpoles. Collectively, these findings suggest that both the type and

quantity of alkaloid defenses in mother *O. pumilio* are passed on directly to their offspring. Furthermore, from a physiological perspective, the high degree of similarity in alkaloid profiles suggests that mother frogs are passively provisioning alkaloid defenses to nutritive eggs, rather than actively modulating what is provisioned. In a similar system, mother Asian tiger snakes (*Rhabdophis tigrinius*) also appear to passively provision bufadienolide-defenses to their offspring (Hutchinson *et al.* 2008). However, mother *O. pumilio* are also known to vary in their provisioning behavior (Maple 2002; Dugas *et al.* 2016), and are more likely to provide larger meals to their older and more developed offspring (Dugas *et al.* 2016), suggesting that increases in alkaloid defenses in older tadpoles may also be attributed to differences in behavioral provisioning. Finally, adult *O. pumilio* are known to vary significantly in alkaloid defenses among populations (Saporito *et al.* 2007a), suggesting that the alkaloid composition of tadpoles also varies in a similar manner. Future studies should examine how natural variation in alkaloid composition as well as differences in provisioning behavior among mothers from different populations of *O. pumilio* influence the alkaloid composition of tadpoles and its implications for tadpole defense.

Provisioned alkaloid defenses are presumed to act as an effective deterrent against certain tadpole predators (Stynoski *et al.* 2014a, 2014b) and possibly microbes (Hovey *et al.* 2018), and variation in these defenses likely plays an important role in determining a tadpole's level of protection. In the present study, tadpole alkaloid quantity was the best predictor of palatability, where tadpoles with greater alkaloid quantities were less palatable to a model arthropod predator. Tadpole palatability, however, was not directly related to mass or developmental stage, suggesting that size and age alone are not good predictors of defense levels. Previous

experimental studies have demonstrated that late-stage *O. pumilio* tadpoles are chemically defended against bullet ants (*Paraponera clavata*) and ctenid spiders (*Cupiennius* sp.) (Stynoski *et al.* 2014a, 2014b); however, relatively little is known about natural tadpole predators in the wild (reviewed in Santos & Cannatella 2011). Anecdotal and experimental records of tadpole predation events suggest that tadpoles of all sizes and developmental stages are preyed upon by snakes and spiders (Maple 2002; Stynoski *et al.* 2014a, 2014b; Sellmeijer & van den Burg 2020), but none of these reports include a measure of tadpole alkaloid quantity. In the present study, 11% (8 of 70) of the tadpoles being reared by mothers in artificial cups—ranging in size from ca. stage 25-43—were predated upon. Although a specific predator was not identified, these findings suggest that predation risk is not based solely on the presence or absence of alkaloids. Although tadpoles with greater alkaloid quantities appear to be less palatable to an arthropod predator, the likelihood of a potential predator attacking and consuming an *O. pumilio* tadpole is also dependent on the specific predator and its physiology. Certainly, there is evidence of snakes that are immune to the alkaloid defenses of adult *O. pumilio* (and other alkaloid-defended poison frogs), which likely provides them similar immunity from defended tadpoles (Saporito *et al.* 2007c; Jovanovic *et al.* 2009; Lenger *et al.* 2014). Future research should explore predators of *O. pumilio* tadpoles in the wild, and the role maternally provisioned chemical defenses play in protecting tadpoles from a variety of predators. Additional work should address how and if predators are able to determine if tadpoles contain alkaloids (e.g., size, chemical cues, etc.), and if predators target young tadpoles that have not yet begun sequestering alkaloids.

In the present study, very small alkaloid quantities were detected in tadpoles as young as stage 27 (20 mg), yet tadpoles did not consistently demonstrate a capacity for sequestering

alkaloids until reaching stages 30-32 (ca. 80 mg) (Fig. 14). Poison glands in *O. pumilio* tadpoles begin their development around stages 32-33, which approximately coincides with the detection of maternally provisioned alkaloids in tadpoles (Stynoski *et al.* 2014a; Stynoski & O'Connell 2017; Saporito *et al.* 2019). The variable presence of alkaloids in early stage tadpoles (< stage 30) suggests that gland development largely controls when tadpoles are physiologically able to sequester (i.e., store) maternally derived alkaloid defenses. Therefore, the detection of alkaloids in tadpoles that have not yet developed glands, in the present study and Saporito *et al.* 2019, may not be the result of sequestration by those individuals, but may instead represent the presence of alkaloid-laden nutritive eggs passing through the digestive tract of these tadpoles. Further implicating the importance of gland development on the sequestration of alkaloids in tadpoles is the observation that tadpoles just beginning to develop glands only possessed minute quantities of alkaloids, whereas late-stage tadpoles (stages 41-44) possessed much larger quantities (Fig. 12). Although tadpoles begin to develop glands as young as stage 32, glands do not begin to mature until much later in development (ca. stage 40), suggesting that tadpoles are not physiologically capable of fully sequestering alkaloids until glands are more fully developed (Stynoski & O'Connell 2017). Furthermore, nothing is known about the location of alkaloids in nutritive eggs, which could also influence when tadpoles are able to begin accumulating alkaloids. It is known that young tadpoles only eat the inner yolk of nutritive eggs, and do not consume the entire nutritive eggs (yolk and outer jelly capsule) until later in development (Dugas *et al.* 2016). Therefore, it is possible that alkaloids are deposited into the jelly capsule of nutritive eggs, which would prevent (or reduce) access of alkaloids to younger tadpoles until they are able to consume eggs in their entirety. In other organisms that maternally provision, however, defensive chemicals are deposited primarily into egg yolks (Hanifin *et al.* 2003; Hutchinson *et*

al. 2008). Further research is needed to identify the location of alkaloid defenses in provisioned eggs and determine if mother frogs deposit alkaloids equally into clutches throughout the provisioning period. Independent of the location of alkaloids within eggs, the development and maturation of poison glands in *O. pumilio* tadpoles appears to be particularly important to alkaloid sequestration, which likely has consequences to how well protected a tadpole is from predators (and possibly microbes) throughout the course of its development.

Within the three plots of tadpole-rearing cups, there was no relationship between bromeliad presence and the spatial deposition of tadpoles, meaning that tadpoles were not more likely to be found in tadpole-rearing cups where bromeliad density was low (Fig. 4). *Oophaga pumilio* tadpoles are dependent on phytotelmata for growth and eventual metamorphosis, but bromeliads represent only one group of plants that provide this resource. Mother frogs may also elect to deposit their tadpoles into other naturally occurring phytotelmata such as the axils of banana (*Musa*) and *Dieffenbachia* plants (Haase & Pröhl 2002; Maple 2002). Because only the presence of bromeliads were recorded within the study site, it is possible that other phytotelmata were abundant and thus, tadpole-rearing sites were not a limited resource driving the deposition of tadpoles into cups. Additionally, tadpoles spend their entire developmental period within the confines of a single nursery and are therefore vulnerable to any associated predation or desiccation risks indicating that mother frogs should be selective in where they deposit their offspring (Maple 2002). Artificial tadpole-rearing cups can vary from naturally occurring phytotelmata in their temperature, water quantity, and possibly predation rates and light-levels (Maple 2002). Mother frogs may preferentially select nurseries that combine the most optimal of these characteristics—which could be either artificial tadpole-rearing cups or naturally occurring

phytotelmata—suggesting that bromeliad presence alone is not enough to determine the spatial deposition of tadpoles within a landscape.

Maternal provisioning of nutritive eggs is not unique to *Oophaga pumilio.* All members of the genus *Oophaga* are obligate egg-eaters, and recently, both *Oophaga granulifera* (Saporito *et al.*, unpublished data) and *Oophaga sylvatica* (Fischer *et al.* 2019) have been described providing alkaloid-laden nutritive eggs to tadpoles. Additionally, the mantellid poison frog, *Mantella laevigata*, provision their offspring with alkaloid-laden eggs suggesting the convergent evolution of maternal provisioning of alkaloids within poison frogs (Fischer *et al.* 2019). However, not all poison frogs that provision nutritive eggs also provision alkaloids. Dendrobatid poison frogs in the genus *Ranitomeya* are facultative egg-eaters, and mother frogs only provide nutritive eggs when food resources within a nursery are low (Brown *et al.* 2010), and alkaloids are absent in the facultative egg-eaters *Ranitomeya variabilis* and *Ranitomeya ventrimaculata* (Saporito *et al.*, unpublished data). Future research should explore the extent to which maternal provisioning of alkaloids is present among egg eating poison frogs and should also examine how natural alkaloid variation within and among species contributes to tadpole alkaloid composition and the implications for how alkaloid variation impacts protection from local predators and pathogens.

Oophaga pumilio is the first known organism to maternally provision chemical defenses to offspring post-hatching or birth. Tadpoles sequester maternally derived alkaloid defenses from nutritive eggs and as a result, share a similar alkaloid profile to their mother. All alkaloid types found in a mother frog were also found in her tadpole. Mother frogs with high alkaloid quantities also had tadpoles with high alkaloid quantities—a quality in tadpoles that was associated with a

decrease in palatability. Individual mother frogs varied in their alkaloid composition and because tadpoles depend solely on their mother for their alkaloid defenses, tadpoles also varied in their alkaloid composition. Variation in adult alkaloid composition has important implications not only for adult protection against predators and pathogens, but also how well-protected tadpoles are from similar threats.

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Tables

Table 1. Tadpole fate organized by plot. Percentages for tadpole fate reflect the number of tadpoles in each plot divided by the totals listed in the right-hand column.

	Plot 1	Plot 2	Plot 3	**Total**
Total number of tadpoles	12 (17%)	21 (30%)	37 (53%)	**70**
Predation	3 (38%)	3 (38%)	2 (25%)	**8**
Metamorphosis	2 (22%)	4 (44%)	3 (33%)	**9**
Splashed out by raindrops	1 (33%)	1 (33%)	1 (33%)	**3**

Table 2. Tadpole cup occupancy organized by plot. Except for the number of cups per plot, percentages for tadpole cup occupancy are a reflection of the number of cups occupied divided by the total number of cups present within that plot.

	Plot 1	Plot 2*	Plot 3	**Total**
Number of cups	248 (31%)	238 (30%)	300 (38%)	**786**
Number of cups occupied by tadpoles	12 (5%)	21 (9%)	34 (11%)	**67 (9%)**
Number of cups with multiple tadpoles	0 (0%)	0 (0%)	3 (1%)	**3 (0.4%)**
Number of trees with both cups occupied	2 (2%)	0 (0%)	8 (5%)	**10 (3%)**

*One tadpole-rearing cup was used twice by separate females to deposit tadpoles (i.e., once one female had completed raising her tadpole in the cup, another female deposited her tadpole into the same cup).

Table 3. Types of alkaloids (excluding isomers and unknown alkaloids) found in *Oophaga pumilio* mother frogs and tadpoles organized by structural class.

HTX	PTX	aPTX	deoxyPTX	deoxyhPTX	DHQ	3,5-P	3,5-I	5,8-I	5,6,8-I	1,4-Q	Lehm	Izidine	Pyr	Pip	Tri	CPQ	Spiro	Polyzonimine	Unclass
291A	**209F**	**225E**	**281B**	**193F**	**221C**	**195F**	**223AB**	**195I**	**207C**	**219B**	**277A**	**205G**	**183B**	**211I**	**193L**	**251J**	**222**	**151B**	**181C**
	225F			**207O**	**251A**	**223H**		**203A**	**209E**	**233A**	**275G**	**211B**	**197B**	**211J**	**207GH**		**236**		**197D**
					275B	**251K**		**205A**	**223A**	**279E**		**211F**	**211T**	**225B**			**252A**		**209G**
						265J		**207A**	**231B**			**225A**	**225C**	**225I**					**269E**
								209I	**237C**			**233B**	**225H**	**239L**					**271E**
								223D	**237L**				**253I**	**239O**					
								225M	**249U**					**241D**					
								233M	**251S**					**241G**					
								235B	**253H**					**253U**					
								247E											
								249O											
								271A											
								273B											
								275F											

HTX, histrionicotoxin; PTX, pumiliotoxin; aPTX, allopumiliotoxin; deoxyPTX, deoxypumiliotoxin; deoxyhPTX, deoxyhomopumiliotoxin; DHQ, decahydroquinoline; 3,5-P-disubstituted pyrrolizidine; 3,5-I, 3,5-disubstituted indolizidine; 5,8-I, 5,8-disubstituted indolizidine; 5,6,8-I, 5,6,8-trisubstitutedindolizidine; 1,4-Q, 1,4-disubstituted quinolizidine; Lehm, lehmizidine; Pyr, pyrrolidine; Pip, piperidine; Tri, Tricyclic; CPQ,cyclopentaquinazoline; SpiroP, spiropyrrolizidine; Unclass, unclassified.

Figures

Figure 1. (a) Artificial tadpole rearing cups. Two cups were affixed to each tree; (b) tadpole deposited into a cup by a mother *O. pumilio*.

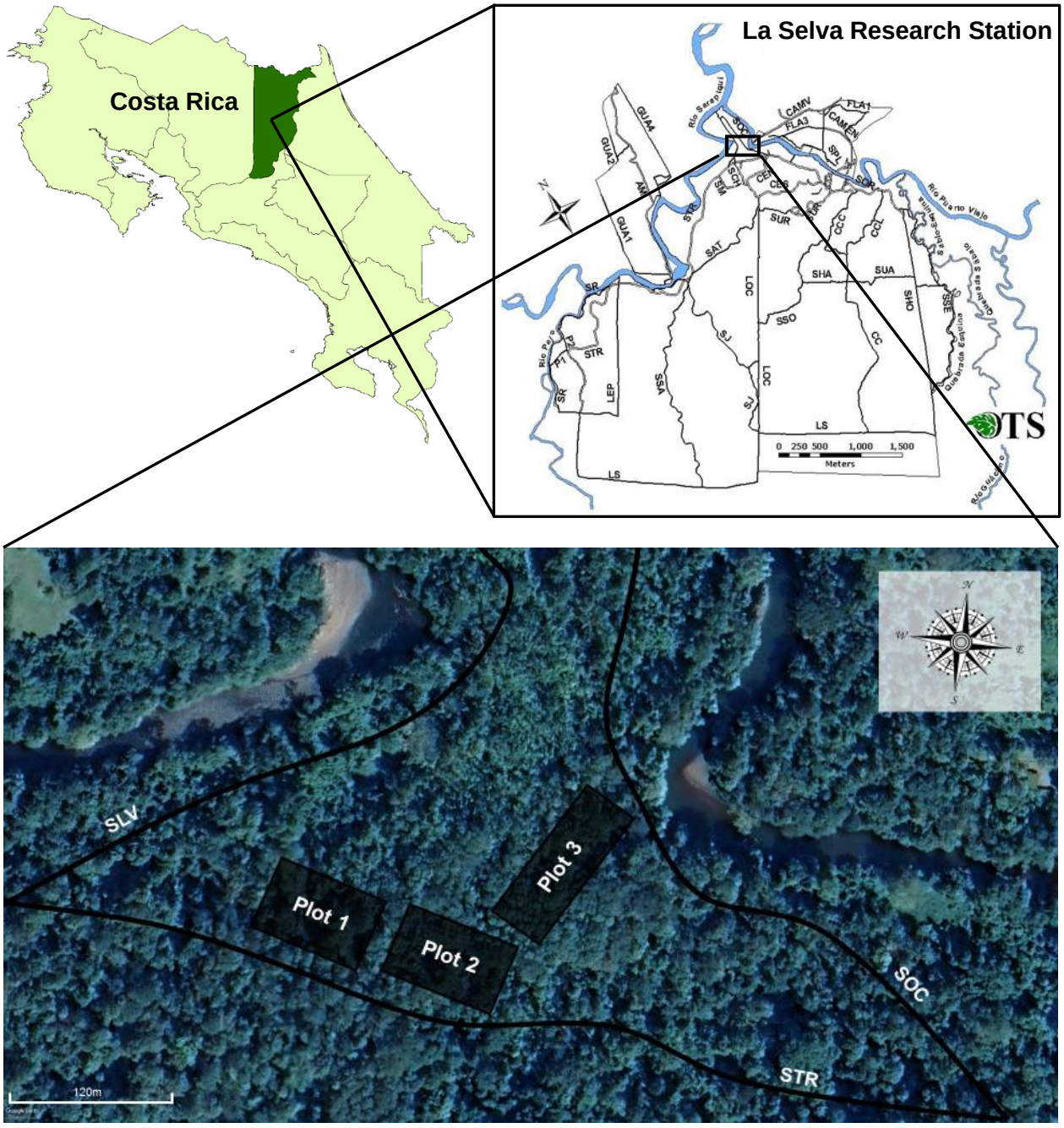


Figure 2. Aerial view of the river and trail systems and the three plots containing transects of tadpole-rearing cups at La Selva Research Station, Costa Rica. The black lines mark the trail system at La Selva. SOC = Sendero occidental, SLV = Sendero Las Vegas, STR = Sendero Tres Rios. Plot locations are approximate.

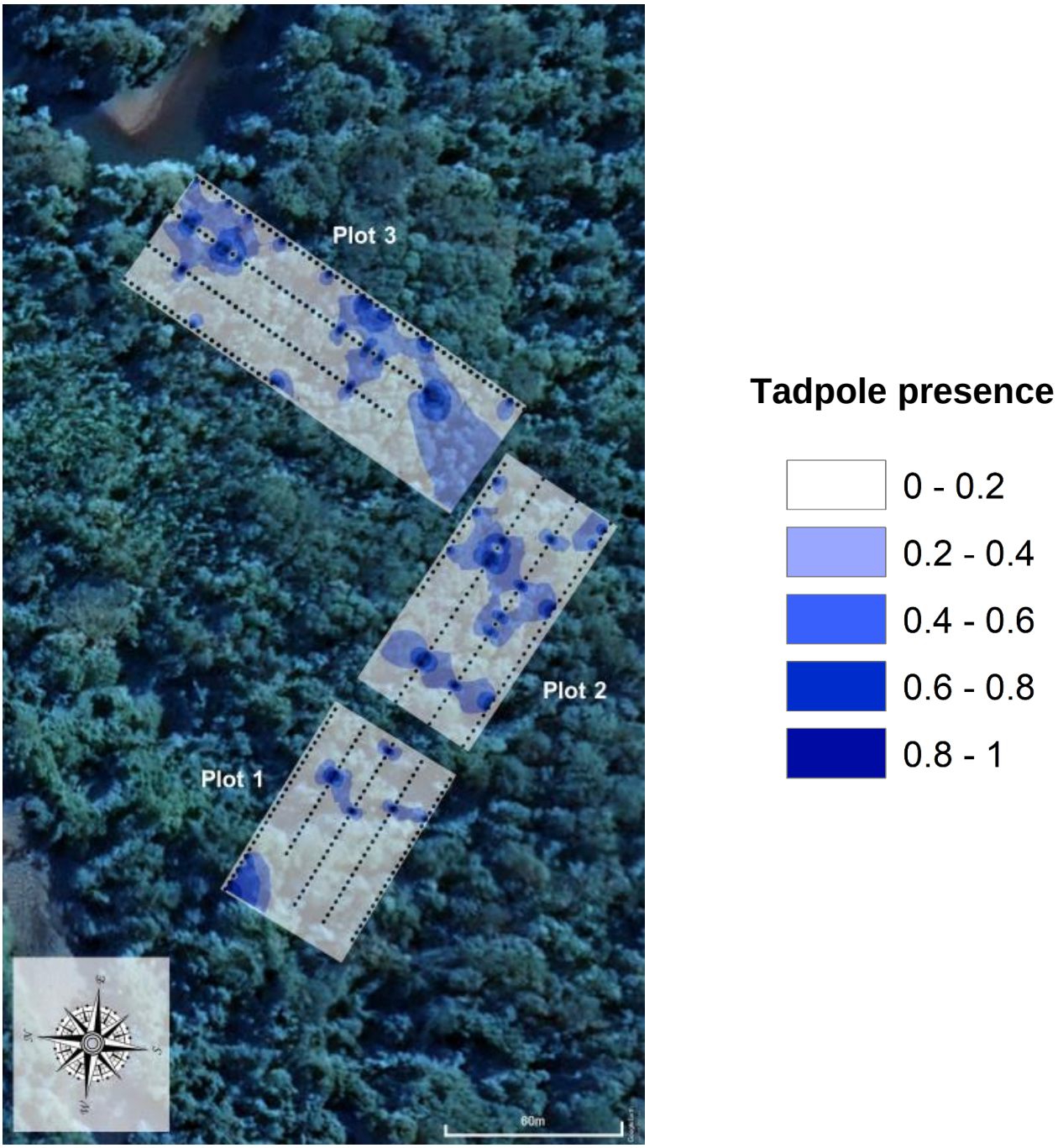
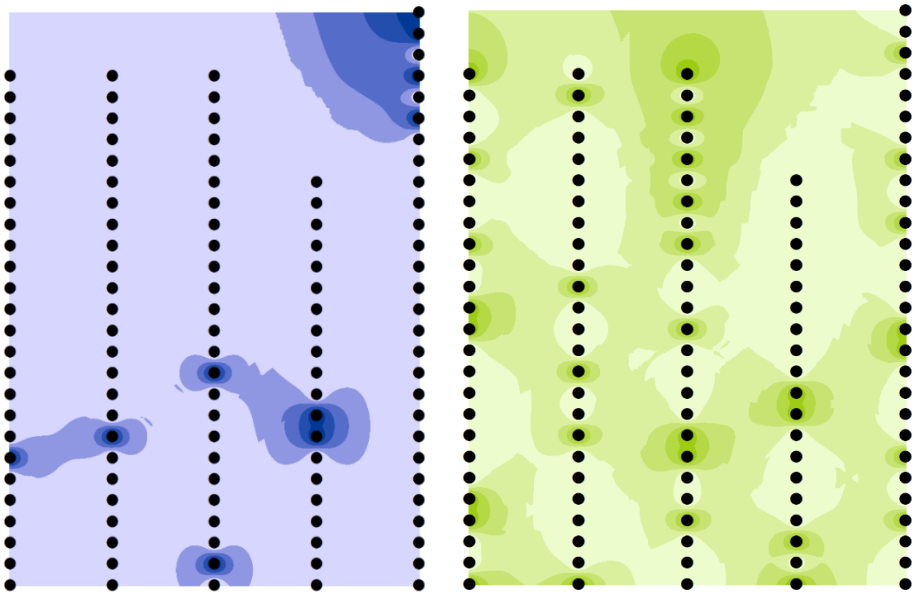


Figure 3. Aerial view of the three plots containing transects of tadpole-rearing cups at La Selva Research Station, Costa Rica**.** Each black dot represents two tadpole-rearing cups attached to a single tree. Predicted occupancy of cups by tadpoles (density) is shown for each plot where a value of 1 indicates a definitive tadpole presence. Plot locations are approximate.

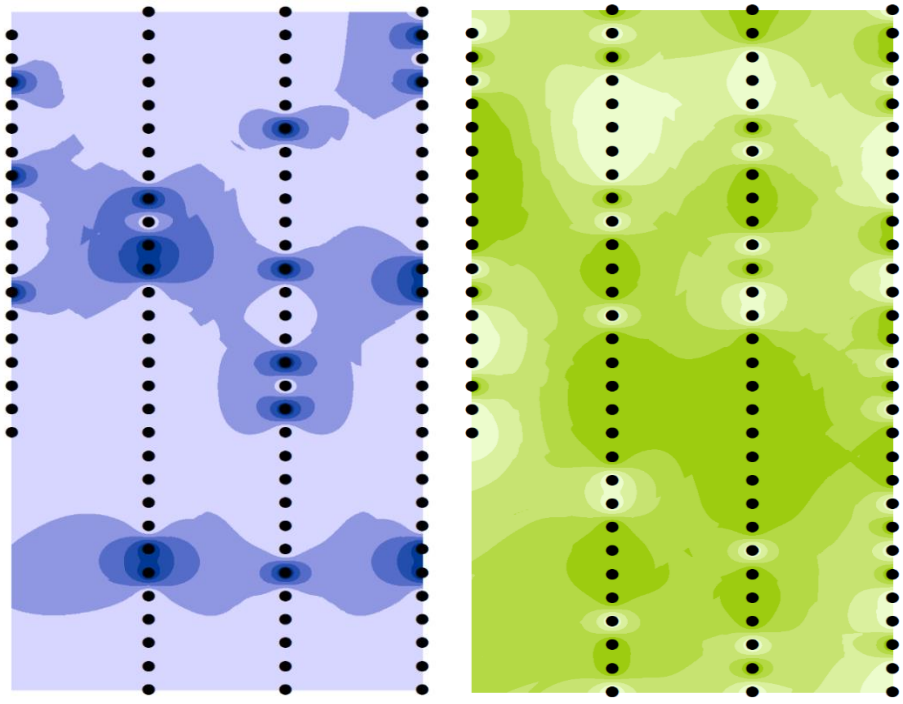
Plot 1



Tadpole presence

- 0 - 0.2
- 0.2 - 0.4
- 0.4 - 0.6
- 0.6 - 0.8
- 0.8 - 1

Plot 2



Bromeliad presence

- 0 - 0.2
- 0.2 - 0.4
- 0.4 - 0.6
- 0.6 - 0.8
- 0.8 - 1

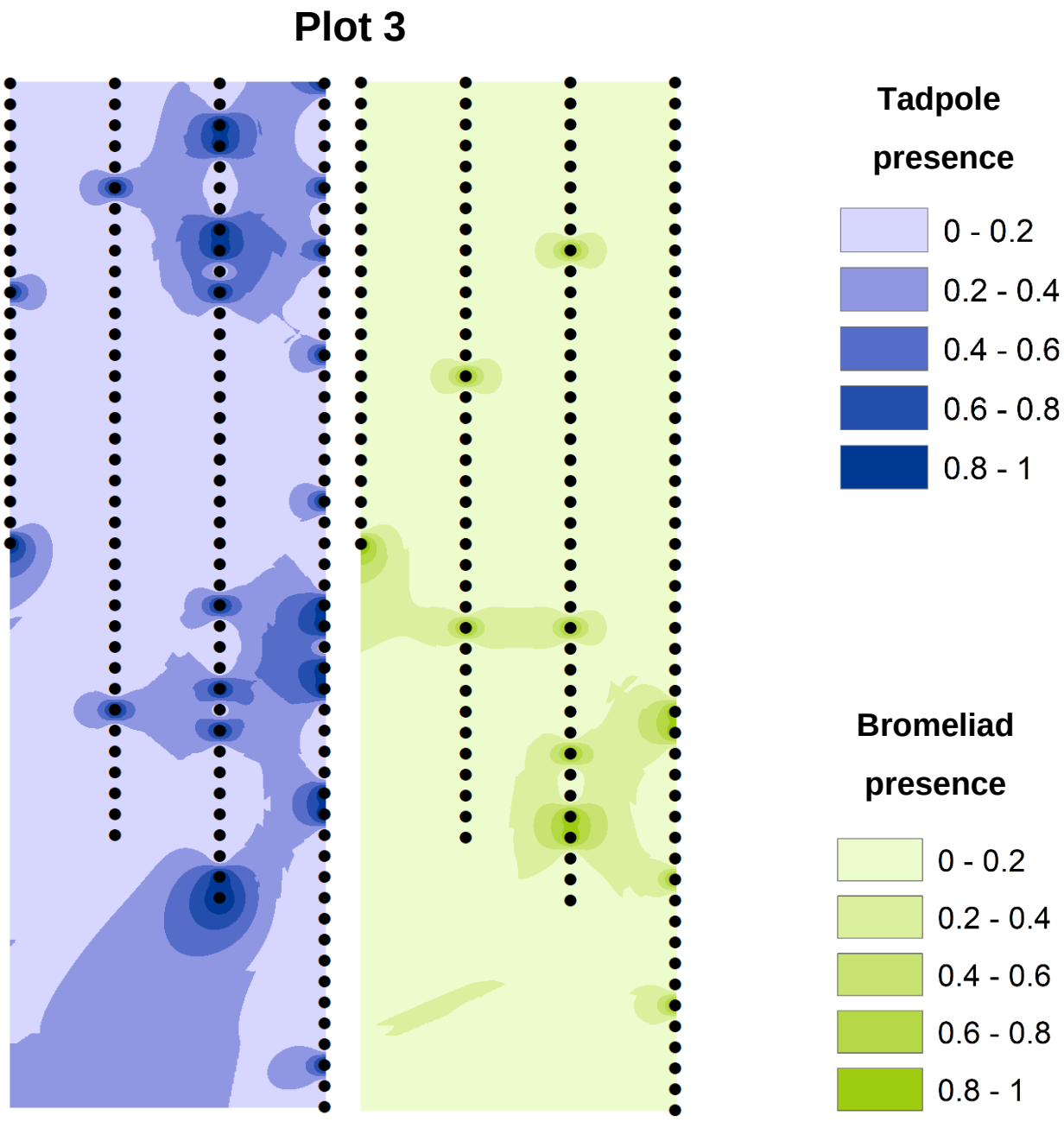


Figure 4. Heatmaps representing the interpolated density of tadpoles (blue) occupying tadpole-rearing cups and the interpolated density of naturally occurring bromeliads (green) within each of the three plots. Each black dot represents two tadpole-rearing cups attached to a single tree. For both tadpole density and bromeliad density, a value of 1 indicates a definitive tadpole or bromeliad presence.

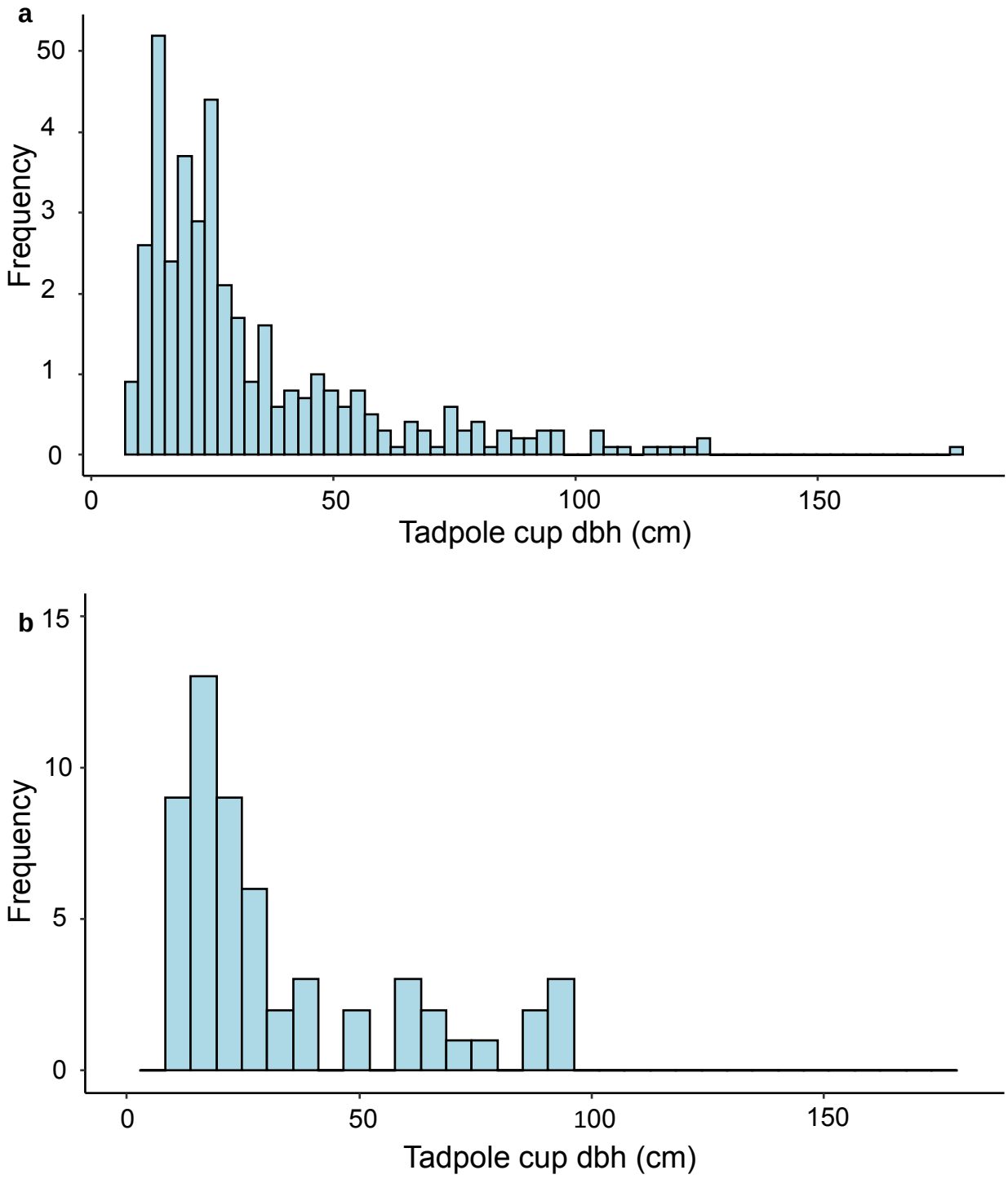


Figure 5. Frequency distribution of tadpole-cup containing trees diameter at breast height (dbh) for (a) all trees containing tadpole cups (n = 393) and (b) trees containing tadpole cups occupied by at least one tadpole during the course of the study.

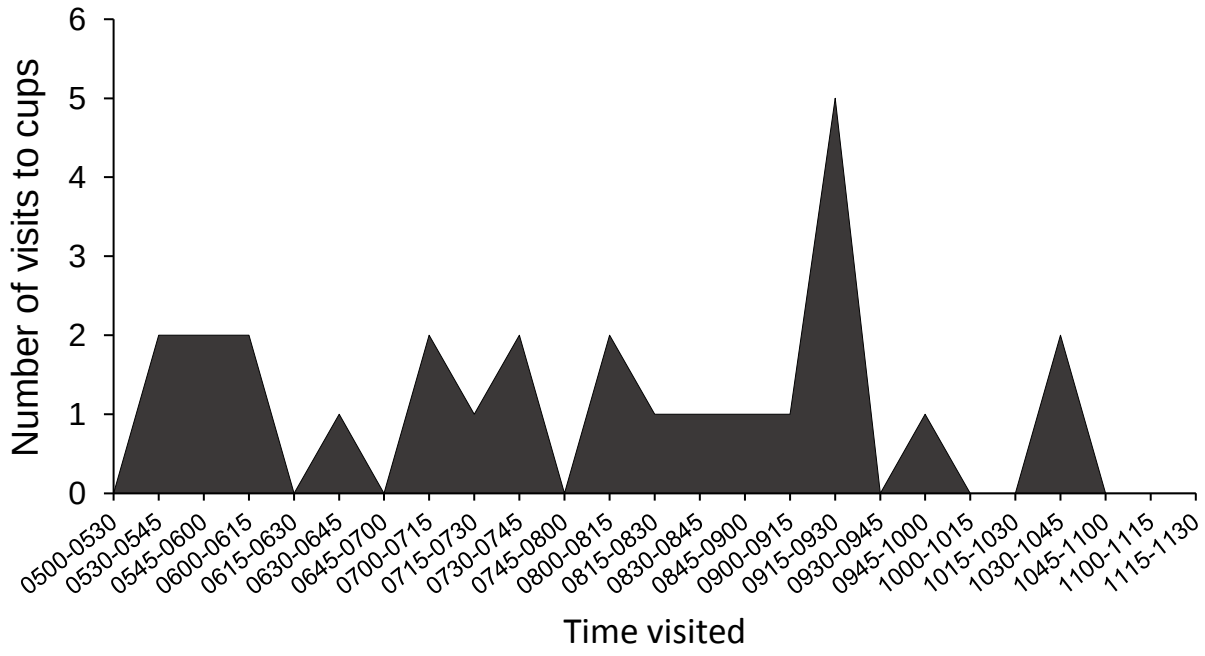


Figure 6. Observed activity period for mother *Oophaga pumilio* visits to tadpole-rearing cups. For each 15-minute time interval, the number of visits to cups represents the total number of (different) mother frogs that visited a tadpole-rearing cup containing a tadpole. Twenty-five mother frogs were observed visiting tadpole cups between 0500 and 1130 from June 7, 2019 to July 5, 2019. All observations of visits to tadpole-rearing cups were conducted with mother frogs as a component of the present study.

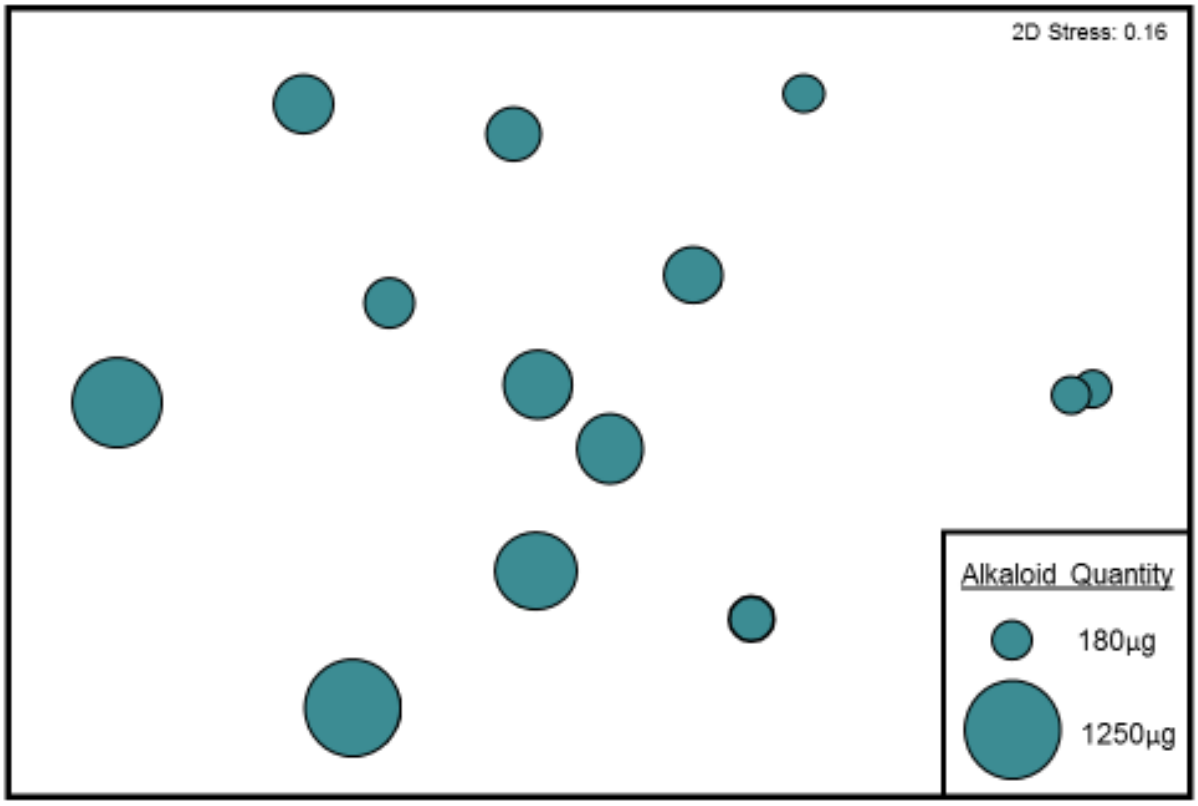


Figure 7. Nonmetric multidimensional scaling (NMDS) plot of alkaloid composition of *Oophaga pumilio* mother frogs (n = 13). Each circle represents a different mother frog and the distance between each circle represents the relative difference in alkaloid composition. Each circle is scaled for the quantity of alkaloid present in that individual.

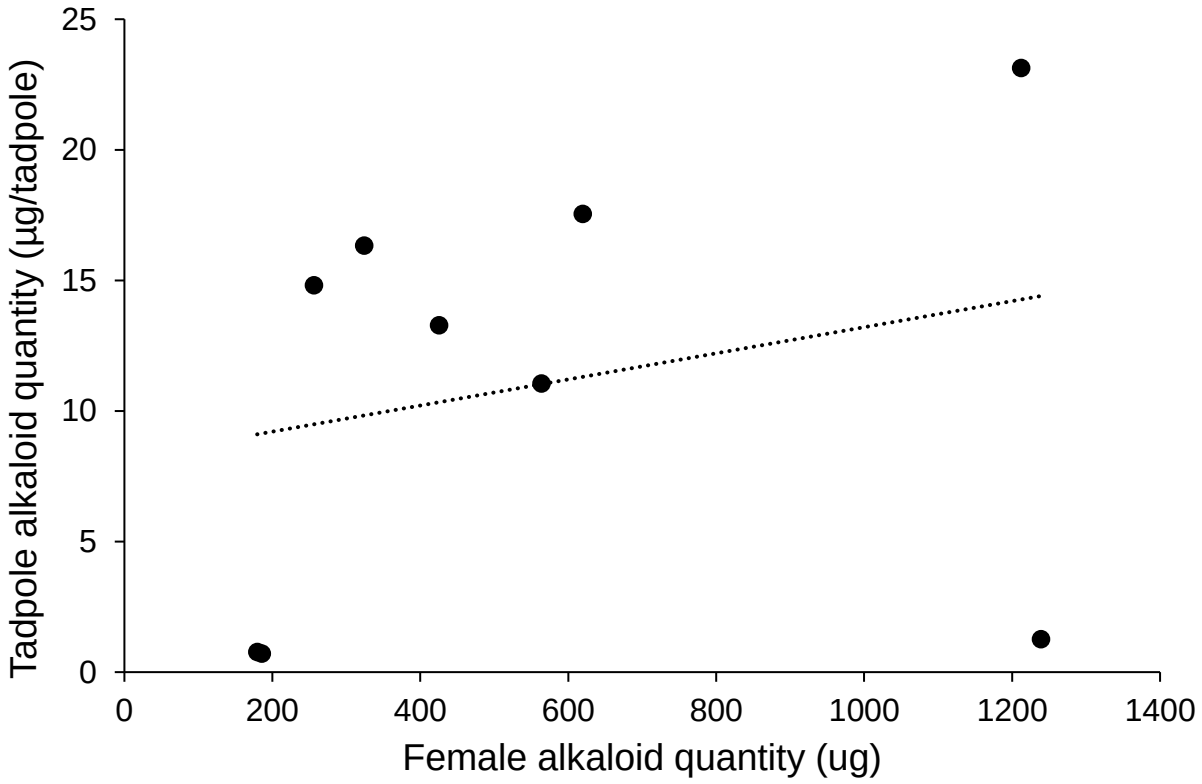


Figure 8. The relationship between mother *Oophaga pumilio* alkaloid quantity and her tadpole's alkaloid quantity. Four tadpoles of the total 13 mother/tadpole pairs did not contain alkaloids and so are excluded here.

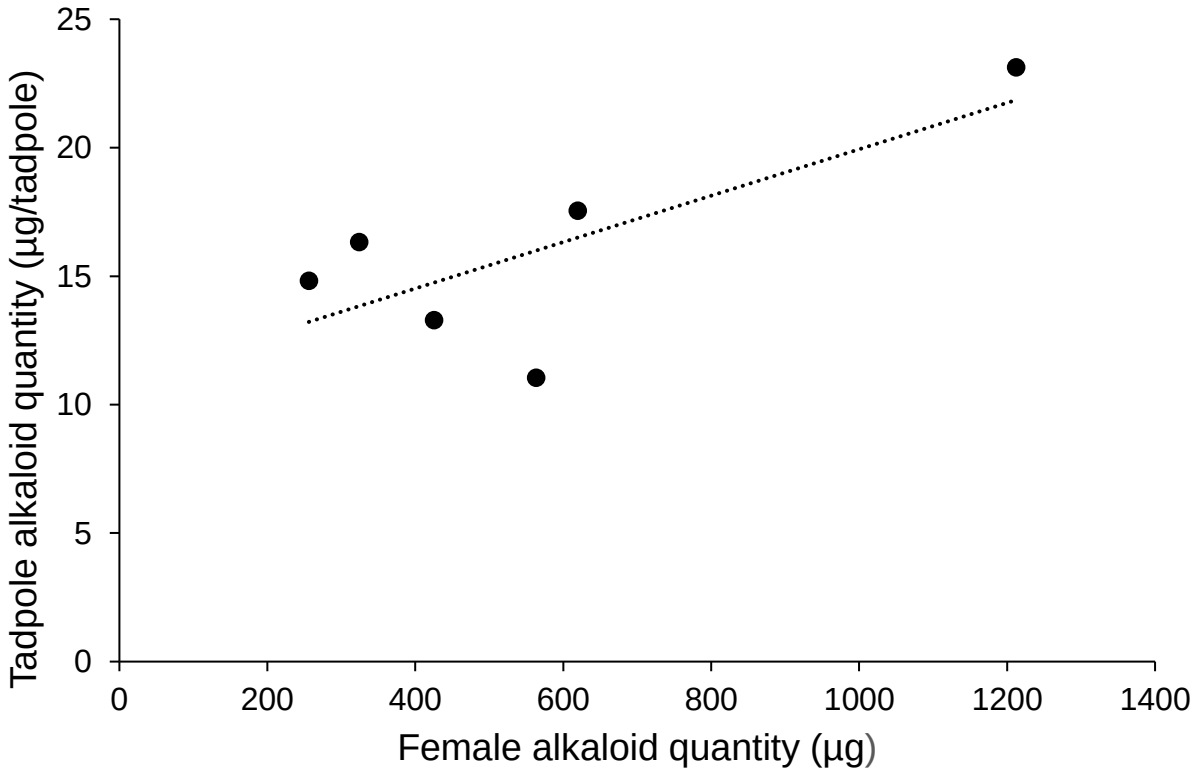


Figure 9. The relationship between mother *Oophaga pumilio* alkaloid quantity and her tadpole's alkaloid quantity. The mother/tadpole pairs selected for this comparison comprises six late-stage tadpoles (stages 41-44) and their respective mothers.

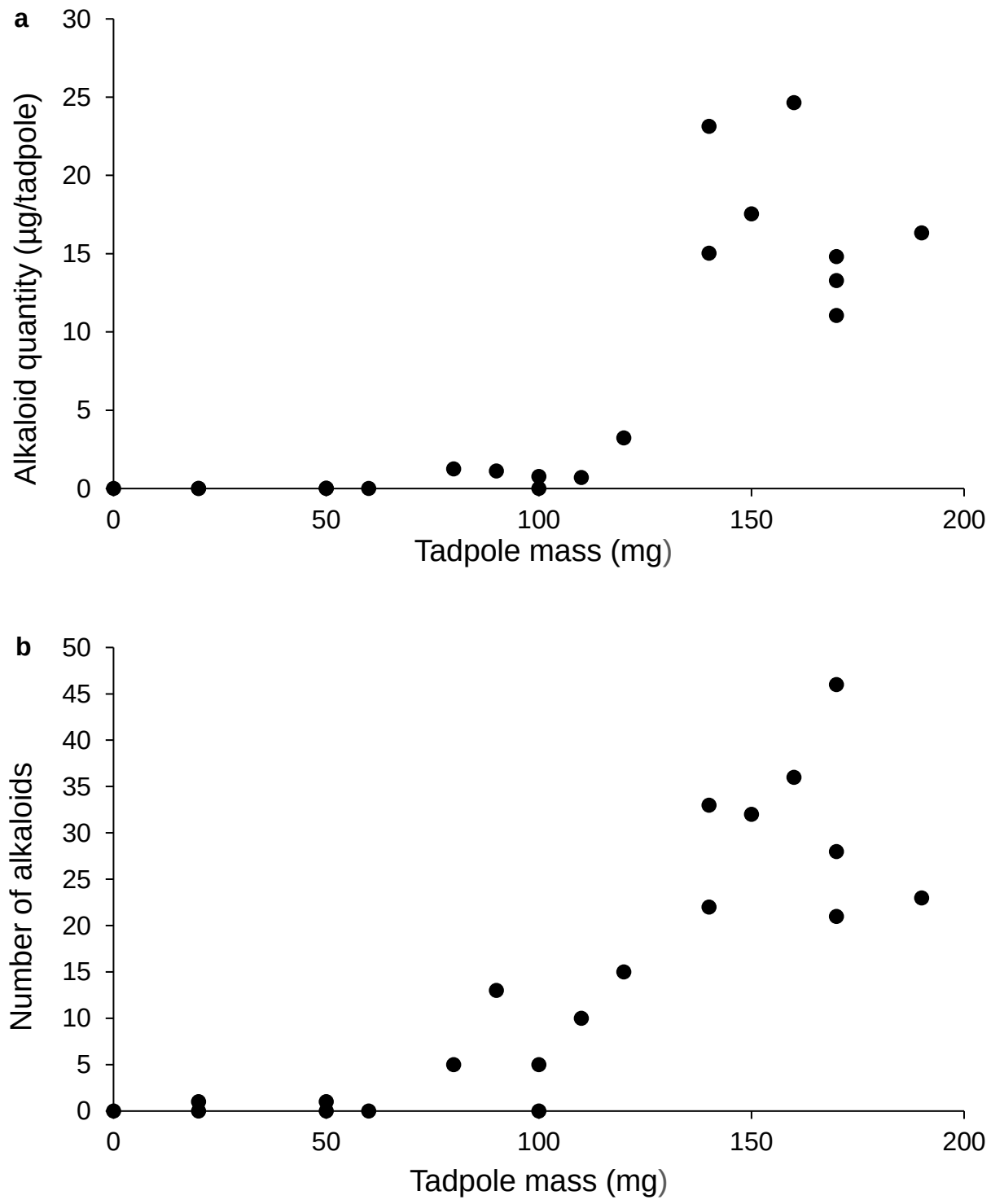


Figure 10. The relationship between *Oophaga pumilio* tadpole (n = 20) (a) mass and alkaloid quantity and (b) mass and number of alkaloids.

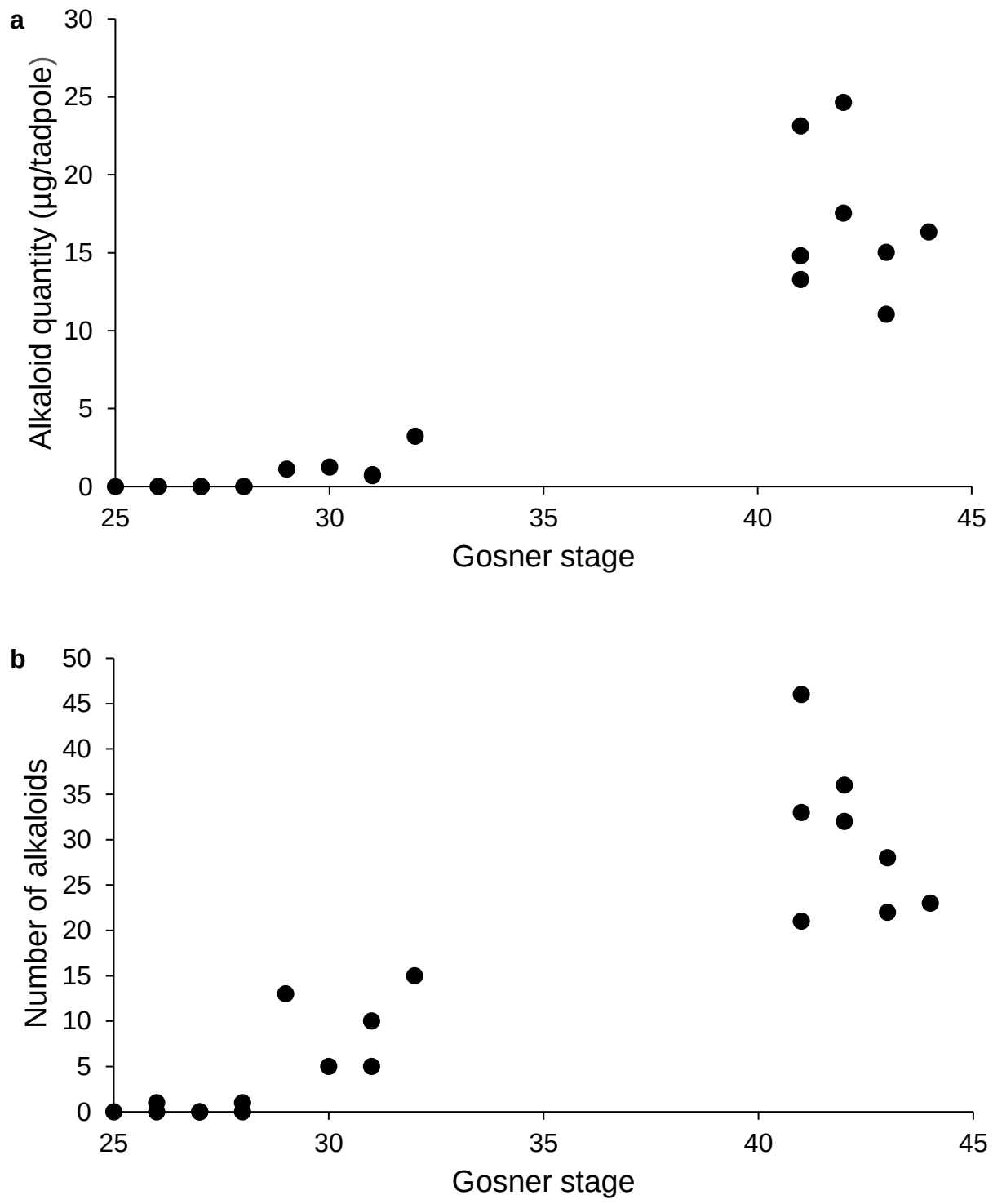


Figure 11. The relationship between *Oophaga pumilio* tadpole (n = 20) (a) Gosner stage and alkaloid quantity and (b) Gosner stage and number of alkaloids.

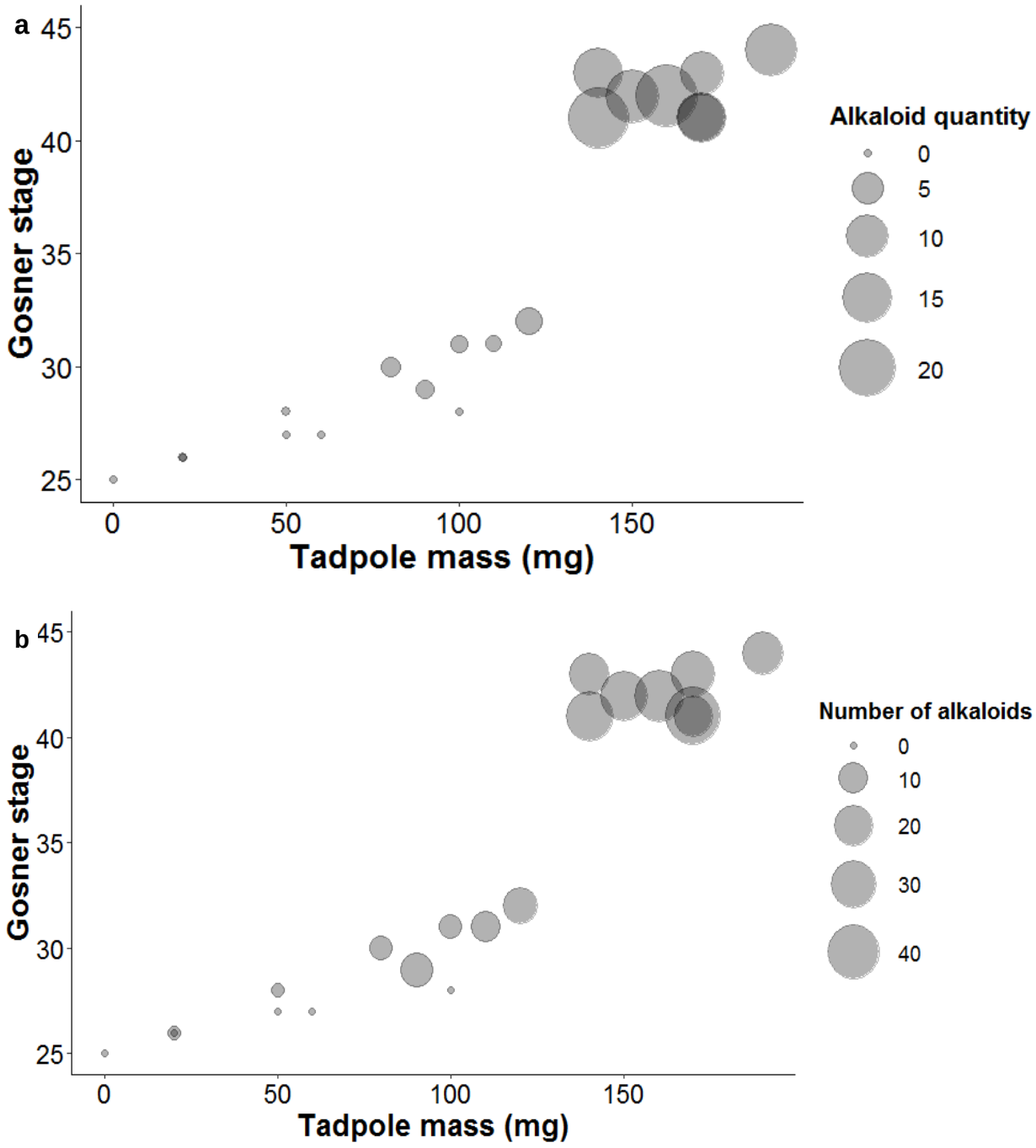


Figure 12. The relationship between *Oophaga pumilio* tadpole mass and Gosner stage as a function of both (a) alkaloid quantity (μg/tadpole) and (b) total number of alkaloids. Each circle (n = 20) represents a different tadpole and each circle is scaled to the quantity of alkaloid in an individual or the number of alkaloids respectively.

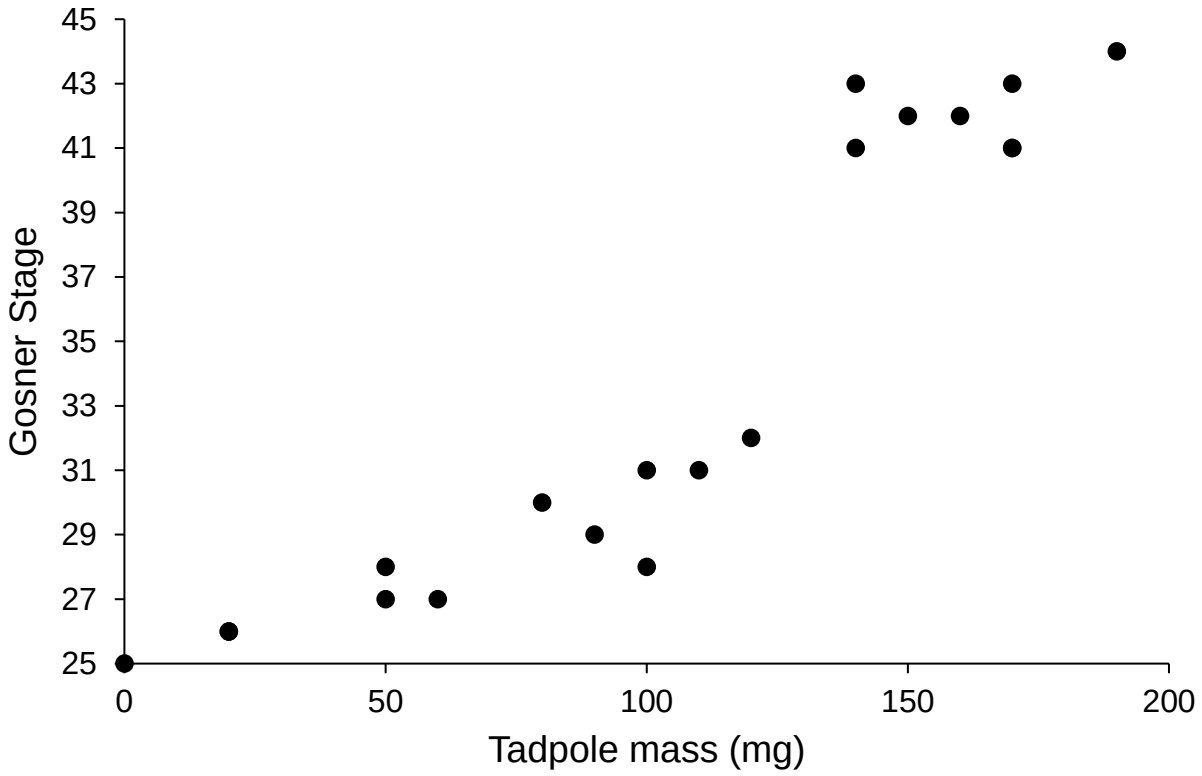


Figure 13. The relationship between *Oophaga pumilio* tadpole (n = 20) Gosner stage and tadpole mass.

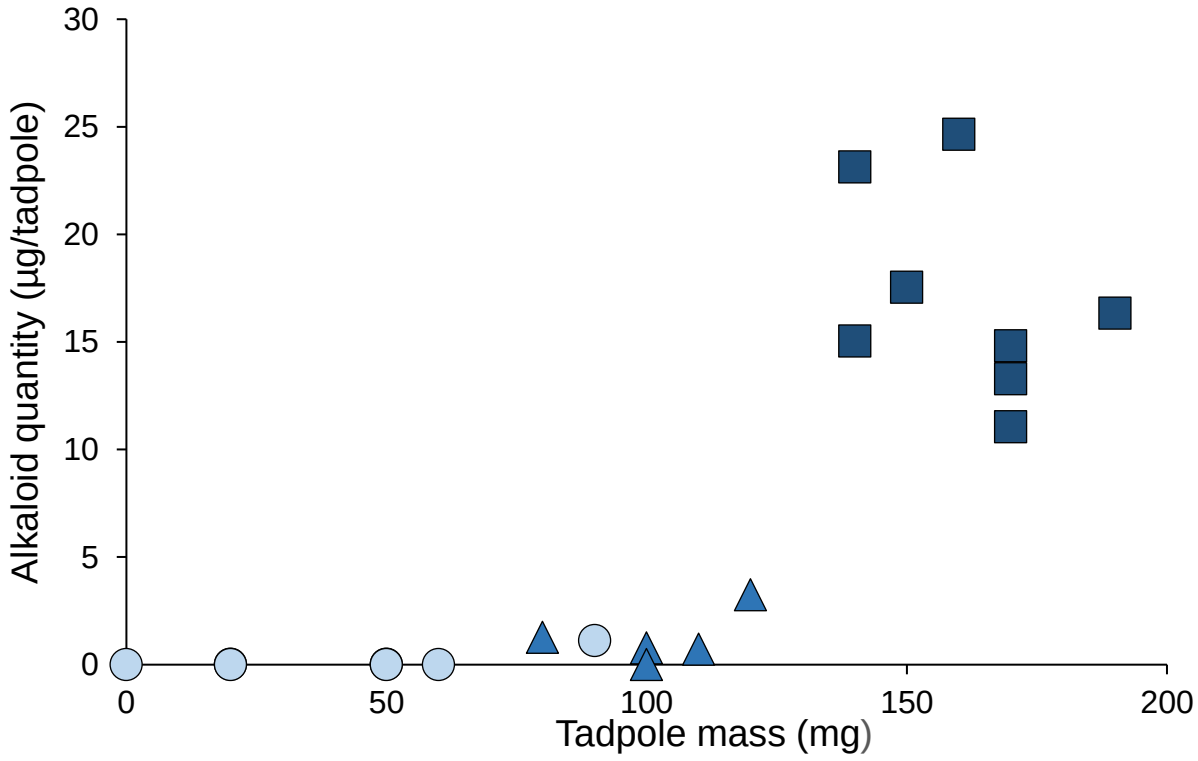
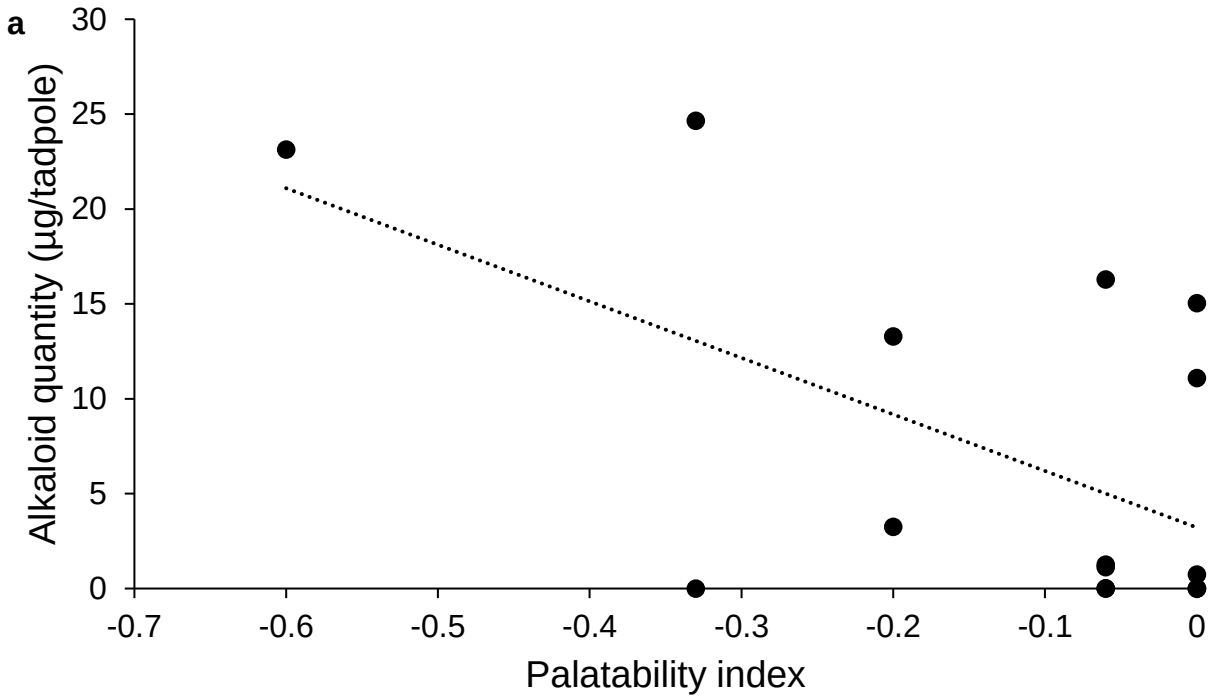
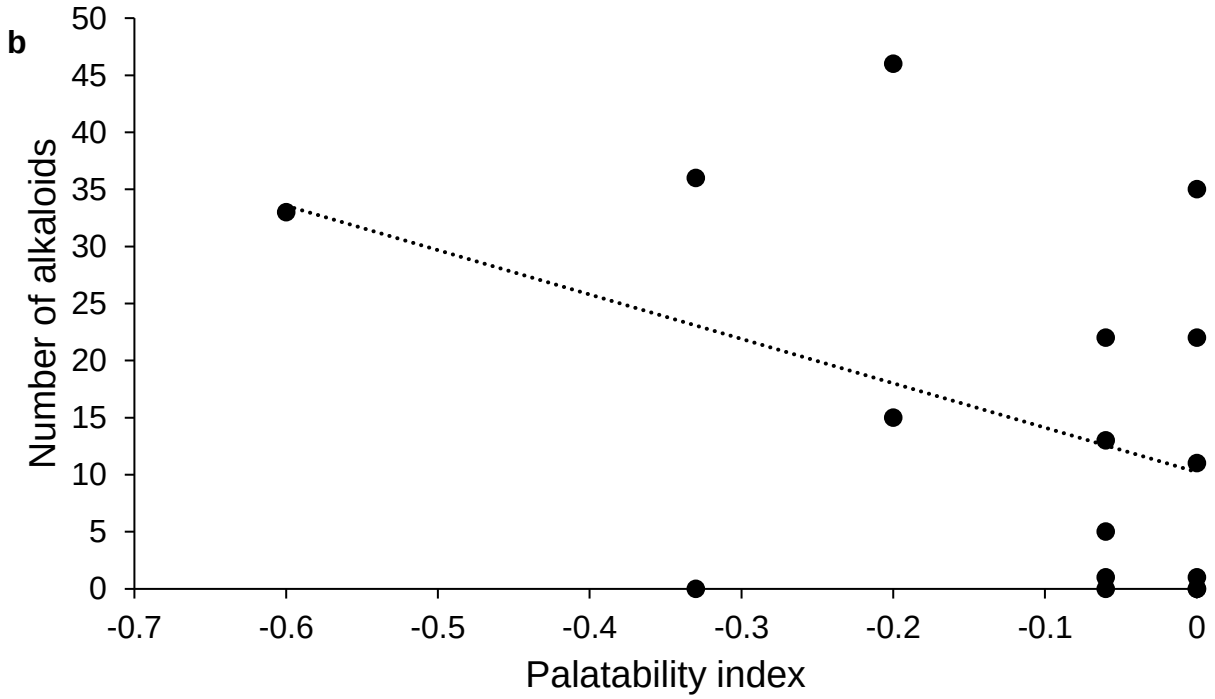


Figure 14. The relationship between tadpole alkaloid quantity and tadpole mass (n = 20). Light blue circles = early stage tadpoles (25-29); medium blue triangles = middle stage tadpoles (30-32); dark blue squares = late stage tadpoles (41-44). Categories were selected to represent tadpoles before the development of glands (early stage), tadpoles undergoing the development of glands (middle stage), and tadpoles with more mature glands (late stage).


a
Alkaloid quantity (µg/tadpole)
30
25
20
15
10
5
0
-0.7
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0
Palatability index


b
Number of alkaloids
50
45
40
35
30
25
20
15
10
5
0
-0.7
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0
Palatability index

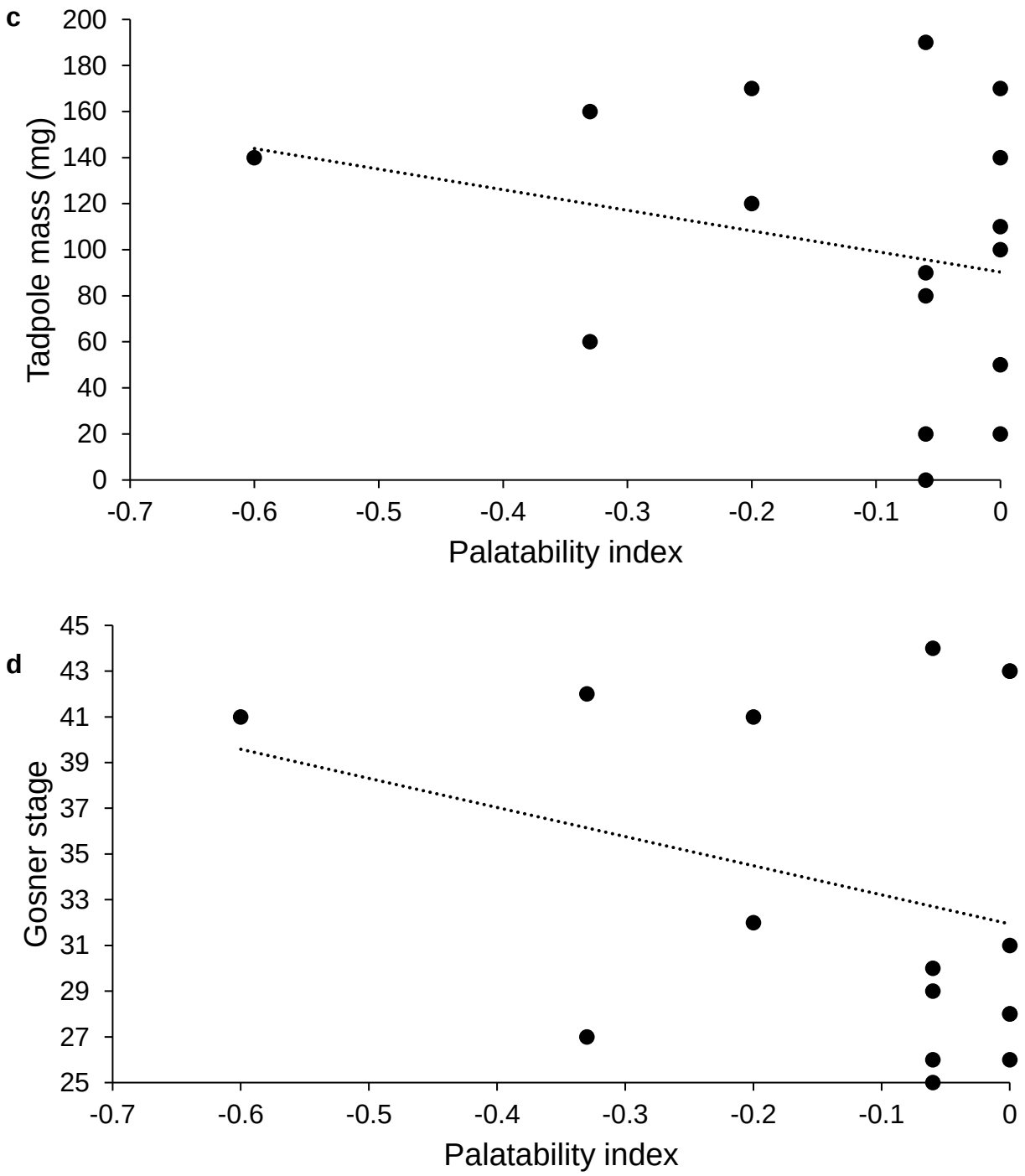


Figure 15. The relationship between *Oophaga pumilio* tadpole (n = 16) (a) alkaloid quantity and palatability, (b) number of alkaloids and palatability, (c) mass and palatability, and (d) developmental stage and palatability