

Coloration, toxicity, and vertical distribution in larval phyllomedusine frogs: is there an anti-predator syndrome?

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Abstract

Visually aposematic prey warns their potential predators about their noxious condition by exhibiting conspicuous coloration. The larvae of some phyllomedusine frogs bear bright patches on the body or tail that suggest an anti-predator role, yet the experimental evidence of aposematism in anuran larvae is notoriously scarce. Here, we assessed and compared the conspicuousness and skin toxicity of three species of phyllomedusine larvae which differ in coloration: *Phyllomedusa vaillanti*, *P. bicolor*, and *Callimedusa tomopterna*. The conspicuous colorations found on the dorsal area of the larvae could be directed to aerial predators, therefore we evaluated the distribution of the larvae in the depth of the water column. We also experimentally assessed their palatability to dragonfly naiads as model predators. Additionally, we observed that all these traits that make up the antipredatory syndrome are modified along the ontogeny of the larvae. For this reason, we assessed body size as another trait that conforms to the syndrome. Our results support an antipredatory syndrome in larvae of *P. vaillanti* and to a lesser degree in *P. bicolor*. The most conspicuous larvae were indeed the most toxic. Moreover, these larvae were attacked less often by predators which, in turn, died within a few hours. In both species, the larvae remained close to the water column surface longer to advertise their noxious condition towards predators. After we included body size in the anti-predator syndrome, we observed that only the *P. vaillanti* larger larvae were more conspicuous. Toxicity and palatability were not related to body size. Nevertheless, vertical distribution varied ontogenetically in *P. bicolor*. The largest larvae remained close to the water surface most of the time, while the small ones were distributed evenly throughout the water column.

Introduction

Conspicuous coloration is a very effective signal to warn and deter predators (Wallace 1876; Endler 1990), particularly when combined with prey unpalatability and predator learning (Shettleworth 1972; Turner 1977; Siddiqui et al. 2004). The evolution of aposematic coloration has been thoroughly studied in adult anurans (Summers and Clough 2001; Darst et al. 2006; Mann and Cummings 2012; Barnett et al. 2018), despite the intense predation pressure experienced by anuran larvae; they are attacked by insects (Caldwell et al. 1980), fish (Smith et al. 2008), snakes (Warkentin 1995), spiders (Luiz et al. 2013), crabs (Gray and Christy 2000), and birds (Gontijo 2018). Unsurprisingly, tadpoles of many species evolved traits that increase the probability of survival, by avoiding, confusing, or escaping the potential predators. Tadpoles may also be toxic or unpalatable (Brodie and Formanowicz 1978), and cryptic or conspicuous in coloration (Wassersug 1971; Thibaudeau and Altig 2012). In response to chemical cues, they often adjust their activity (Hews and Blaustein 1985; Petranka et al. 1987) and distribution (Lima and Dill 1990; Spieler and Linsenmair 1999). A single species can exhibit several of these strategies, which combined represent an antipredator syndrome (Cooper and Blumstein 2015).

In some species of anuran larvae, the dark or patterned coloration of the eyes, mouth, or tail may reduce predation risk by concealing their body shape (Blair and Wassersug 2000), or redirecting attacks to other areas of the body (Van Buskirk et al. 2003). However, the role of conspicuous coloration in anuran larvae

is mostly unknown (Thibaudeau and Altig 2012; Gontijo 2018); some hypotheses suggest that conspicuous coloration in some body areas may confound or intimidate predators (Altig 1972); it may also produce a "lure effect" redirecting the attack to less vulnerable areas of the animal such as the tail (Caldwell 1982). Importantly, some of these colorations appear as phenotypic plasticity, after exposing the larvae to various species of predators, which further supports its alleged functional value in reducing predation risk (Van Buskirk et al. 2004; Touchon and Warkentin 2008).

The skin of adult phyllomedusine treefrogs secrete peptides such as bradykinin and dermorphin peptides, which are known to be highly unpleasant to predators and cause highly noxious effects (Sazima 1974; Lacombe et al. 2000; Birth 2013). The tadpoles of *Phyllomedusa bicolor* and *P. vaillanti* display metallic colorations in various regions of the body (Branch 1983; Thibaudeau and Altig 2012), which are clearly visible and contrasting to the human naked eye. At their breeding pond, they coexist with predator insect larvae and even with some fish, which are known as voracious predators that exclude anuran larvae from many water bodies (Magnusson and Hero 1991). Also, these phyllomedusinae tadpoles appear to be gregarious, a behavioral trait alleged to increase the survival of aposematic animals (Bertram 1978). Tested predators learn faster and retain the information longer when exposed to gregarious prey that exhibits contrasting coloration (Gamberale and Tullberg 1998). Altogether, the conspicuous metallic coloration, the co-existence with fish, and the gregarious behavior strongly suggest aposematism as an antipredator strategy in these anuran larvae.

In this study, we aimed at collecting evidence to test the hypothesis that the larvae of *P. bicolor* and *P. vaillanti* exhibits an antipredator syndrome consisting of conspicuous coloration, non-palatability or toxicity, and body size. Because metallic coloration occurs on the head and would be visible particularly to aerial predators, we further tested whether these tadpoles differentially use the water surface in the water column. Because coloration appears to change throughout ontogenetic stages, we further tested the effect of this variation on most measured traits. Lastly, to strengthen the interpretation of our results, we conducted the same measurements on *Callymedusa tomopterna*, a closely related species (Duellman et al. 2016), that lacks both the metallic coloration and the gregarious behavior.

Material and methods

Study system

The monkey frogs or leaf frogs (family Phyllomedusidae) currently comprise eight genera, including *Phyllomedusa* (15 species) and *Callimedusa* (six species) (Duellman et al. 2016; Frost 2017). The larvae of *Phyllomedusa vaillanti* and *P. bicolor* exhibit a greenish coloration on most body surface, which becomes clearer towards the tail. The loreal zone is yellow-orange and presents yellow iridescent reflections towards the sides. In addition, *P. vaillanti* has a yellow or green metallic interocular patch that differentiates it from *P. bicolor* (Caramaschi and Jim 1983, Lynch and Suárez-Mayorga 2011). *C. tomopterna* instead has a uniform translucent grey or dark orange coloration and black fins, with a dark spot at the rear end of the tail (Figure 1-A). The tadpoles of the three species present a pear-shaped body, with a relatively narrow tail and a downward curving tail tip; the eyes are big and are located dorsolaterally. Species are widely spread in the Amazon and live in both primary or secondary forests (Duellman 1974).

Our research was conducted at the Reserva Natural Tanimboca (4 ° 07'10.98 " S, 69 ° 57'04.57 " O), 11 kilometers north to the city of Leticia, in the Colombia's Amazon region. Average annual rainfall is 3432 mm, with maximum values between January–April, and minimum between July–September. The average annual temperature is 25.7 ° C and relative humidity is around 87-90% throughout the year (Higuera 1996; Mueses-Cisneros 2007). We conducted five field trips in 2014 and 2015, two during rainy seasons (December–January) and three during dry seasons (April–June).

Vertical distribution

To corroborate whether our study tadpoles dwell mostly on the pond surface, we tested for differential use of the water column. We used 12 transparent plastic columns of 70 cm high and 50 cm diameter, marked every

5 cm depth. The columns were filled with water from the same pond in which the tadpoles were captured. During a single assay, seven tadpoles were added to each of the 12 columns. For the acclimation period, we placed the columns in a quiet place all night. The next day we noted the position of tadpoles along the water column. These observations were repeated every 2 h, between 06:00 – 18:00 h. For *P. bicolor*, we used three size groups of tadpoles: small (stage 25-27), medium (stage 28-32), and large (stage 33-39) (Gosner 1960). Due to constraints in the availability of tadpoles, we used only large tadpoles of *P. vaillanti* and could not conduct a similar experiment with *C. tomopterna*.

Conspicuousness

As a proxy for larvae conspicuousness to aerial and terrestrial predators, we estimated the contrast between the tadpole body coloration and the water background. We estimated conspicuousness from above because phyllomedusinae larvae are known to congregate at the water surface during the daytime (Branch 1983; Caramaschi and Jim 1983). Our experiments (see the previous section) had confirmed this presumption, at least for large-sized larvae (see Results). We are aware that aquatic predators, such as arthropod and fish, also prey on phyllomedusinae larvae (Magnusson & Hero 1991) yet the visibility of these ponds was below 5 cm, as estimated from the “disappearance” of larvae when they swim deeper from the surface. Moreover, the metallic and putatively conspicuous patch was located on the dorsal surface of the tadpole head and is arguably easier to spot from above the water.

The body-background contrast was measured on lateral and dorsal photographs of 193 larvae of *P. vaillanti*, 113 of *P. bicolor*, and 30 of *C. tomopterna* at variable developmental stages. A Kodak color card was included in each photograph as a control for white balance. We then used the software ImageJ (Schneider et al. 2017) to measure image red, green, and blue (RGB) brightness on six areas of the larvae’s body: the interocular patch, the loreal area, the middle dorsal area, the lateral area, the dorsolateral area, and the ventral zone (Figure 1-B). For background measurements, we took photographs of the water surface under daytime natural light and at the very ponds where we found the tadpoles.

To quantify and compare coloration we used the RGB decomposition method on the digital images (Endler 1990; Stevens et al. 2007; Amézquita et al. 2009). Briefly, we estimated relative brightness on each color channel: red $R^* = r / (r + g + b)$, green $G^* = g / (r + g + b)$, and blue $B^* = b / (r + g + b)$. Then, to map those measurements onto a two-dimensional space, we calculated two scores: $LM = R^* - G^*$, and $B^* = B$ (Endler 1990; Amézquita et al. 2009). This approach provides a reasonable estimate (long-medium and short wavelengths) of the photoreceptor’s response in animals with trichromatic vision (Kebler et al. 2003). Finally, we used the multivariate distances between each tadpole area and the background coloration as our proxy for contrast or conspicuousness (Endler 1990).

Palatability

To know whether tadpoles of the three species were palatable to potential aquatic predators that coexist with them, we conducted a predation experiment. As predators, we used odonate naiads in the families Libellulidae and Aeshnidae obtained from the same ponds as the tadpoles or small ponds nearby; naiads are known to be voracious predators with significant importance for the population dynamics of pond-dwelling organisms (Magnusson & Hero 1991; Cobert 1999). During each assay, a single naiad was kept in a 30 x 40 x 15 cm (W x L x H) plastic container and fasted for 12 h prior to the treatment, which consisted in adding a single tadpole. The survival of both the larvae and the naiad was monitored every hour throughout eight hours. To avoid pseudoreplication (Hurlbert 1984), we used a unique naiad for each tadpole: 18 tadpoles of *P. vaillanti*, 9 of *P. bicolor*, and 11 of *C. tomopterna*. The body size (snout to vent length, SVL) of each tadpole and naiad was measured before initiating each trial.

Toxicity

To test for toxicity of our study tadpoles to potential vertebrate predators, we conducted automated behavior analysis (Amézquita et al. 2017) on mice injected with methanolic extracts of skins (Daly et al. 1997) or with saline solution, as control for the effects of manipulation and injection. Mice are a widely used model

system in studies of pharmacology and toxicology (Shimizu 2004), and also specifically in studies with poison frogs (Darst 2006; Darst and Cummings 2006). We first collected 45 larvae of *P. vaillanti*, 24 of *P. bicolor*, and eight of *C. tomopterna* of variable size. The skins were preserved in 96% methanol until arrival to the laboratory. There, each skin was cut and processed with a PRO® 200 homogenizer during 3 min using methanol as a solvent. The resulting extract was filtered using a vacuum filtration funnel with a glass nail and a small piece of filter paper. Using a syringe to induce vacuum. The methanol was afterward evaporated at a temperature of 38° C in a JOUAN RC®1010 vacuum centrifuge for 5 h. The dry pellet was finally resuspended in 0.2 ml of saline solution.

We used 40 x 20 x 30 cm (W x D x H) glass arenas to film the behavior of each experimental or control mouse; they were adult male *Mus musculus* of the Carworth Farms Swiss Webster (CFW) strain. Four arenas were arranged to be adjacent and filmed from about 80 cm above with a single video camera. Filming was thus conducted in blocks where three arenas contained mice injected with skin extracts and one contained a mouse injected with saline solution. We impeded visual detection among mice by adhering opaque cardboard to the external glass surfaces of the arenas. And we ruled out a potential effect of past chemical cues on mice behavior by thoroughly washing the internal glass surfaces between two consecutive experiments. Mice were given 15 minutes acclimation period prior to the trial start.

To estimate baseline levels of mice behavior, we first filmed them during 5 min in the experimental arenas. Each mouse was then injected with either 0.2 mL of one tadpole's skin extract resuspended in saline solution (experimental treatment) or 0.2 mL of only saline solution (control treatment). The mouse was immediately returned to the arena and filming continued for up to 30 min. At the end, mice were taken to their original cages and provided with food and water for recovery.

To quantify changes in mice behavior and to estimate a toxicity score we analyzed the video recordings on the software Ethovision XT8 (Noldus 2001) following the procedures described elsewhere (Amézquita et al. 2017). Briefly, the automated analysis extracted 68 behavioral variables describing mice mobility (three variables), directionality (three variables), body stretching (two variables), and space use (seven variables). In addition, we directly observed the videos to register the occurrence and frequency of thoracic or limb spasms (four variables). To reduce redundancy among the extracted variables, we conducted a principal component analysis (PCA), which rendered 19 orthogonal PCs that worked as our descriptors of behavior.

Statistical analysis

To compare the palatability of the species, we used a logistic regression model to know whether the tadpoles were depredated for the naiads and we used each species of tadpole as a dependent binomial variable. We also used the glm function with the "logit" link function in ggplot2 package (Friendly and Meyer 2015).

Differences in the conspicuousness of the three species were analyzed with an analysis of variance (ANOVA). To estimate the additional effect of body size, we used an analysis of covariance (ANCOVA). In this case, we used only the conspicuousness values of the dorsal area and the interocular patch of the larvae, which represented the highest contribution percentage in the variance.

To estimate a toxicity score, we conducted principal component analysis (PCA), using 68 variables Ethovision software quantified. We chose variables that were better represented in the PCA and with these, we conducted a discriminant function analyses (DFAs) using as a group discriminating the injection type the mice received: mice injected with skin extracts versus mice injected with saline solution. The values of the first DFA were used as the toxicity score of each tadpole.

Finally, to evaluate the relationship between conspicuity and toxicity score, we used ANCOVA with three species of tadpoles as the covariate. The conspicuousness values were obtained through a PCA. The first PCA was represented by all dorsal colorations of the larvae and the second PCA by the coloration of the interocular patch, which was the most conspicuous and provided the largest contribution in the explained variance.

Results

As expected, most individuals of *P. vaillanti* and *P. bicolor* remained within 5 cm of the water column the majority of the time (Figure 2). Interestingly, the smaller individuals of *P. bicolor* were more evenly distributed along the water column, yet we were unable to obtain data from medium or small larvae of the former species. Both *Phyllomedusa* species were significantly more conspicuous against the water background, compared *C. tomopterna* (Figure 3). In turn, *P. vaillanti* was more conspicuous than *P. bicolor*, particularly concerning the frontal metallic patch (Figure 3). Conspicuousness was higher in larger larvae of the most conspicuous species, *P. vaillanti* (linear regression: $F=24.69$, $p<0.001$, $R^2=0.13$, $N=193$) but this relationship was absent in the other two species (Figure 3).

Dragonfly naiads preyed upon 73% of the tested frog larvae, and we detected no significant differences in the vulnerability of the three species of tadpoles ($X^2=0.102$, $df= 2$, $p= 0.94$ (Figure 4-A) Nevertheless, seven out of the 13 naiads (54%) that preyed upon *P. vaillanti* died within 4 h, whereas none of them died after consuming *P. bicolor* or *C. tomopterna* larvae (Figure 4-B). We also found evidence of larvae toxicity for the tested vertebrate. The tadpole skins of *P. bicolor* and *P. vaillanti* significantly affected mice behavior: and eliciting spasms, lethargy, and decreased movement throughout the arena. In contrast, the mice injected with skin extracts of *C. tomopterna* were indistinguishable in behavior from the mice injected with saline solution (Figure 5). Regarding body size, we found no evidence supporting a relationship between larval size and palatability ($F = 0.60$, $p = 0.54$, $N = 38$), or larval size and toxicity ($F = 1.86$, $p = 0.11$, $N = 76$) in any of the frog species we studied.

At the species level, some relationship between toxicity and conspicuousness was apparent: the non-toxic species (*C. tomopterna*) was the less conspicuous as well. And the two *Phyllomedusa* species differed in conspicuousness but were comparable in toxicity at least for our study system (Figure 5). At the individual level, however, only the tadpoles of *P. vaillanti* showed a positive relationship between toxicity and the conspicuous of the front patch (Figure 6).

Discussion

Our results suggest that *P. vaillanti* and *P. bicolor* show conspicuous colorations in some areas of the body are unpalatable, have toxins and prefer to remain on the surface of the water column. These strategies may be evolving in tandem, and represent an adaptive syndrome that reduces the risk of predation (Relyea 2001). Most of the larvae were depredated; the mortality of the naiads that consumed the *P. vaillanti* larvae is an indication of the presence of toxicity from tadpole's skin. However, there is a great variation in the effect that a toxin can cause to different predators and in how a predator can more easily recognize a non-palatable prey than another (Adams et al. 2011). For example, fish and amphibians can recognize and identify a toxic prey faster than aquatic insects (Kats et al. 1988). This is because vertebrates consume whole prey including skin, while insects use buccal parts to suck the prey content (Brodie et al. 1978; Gunzburger and Travis 2005). Therefore, it is necessary to corroborate the lack of palatability using other predators that can coexist with the larvae.

Toxicity assays helped us to reinforce the hypothesis about the presence of alkaloids in the skin extracts of these larvae. The presence of these toxins as an aposematic signal in anuran larvae is a rare characteristic in which few studies have focused (Toledo and Haddad 2009). Some investigations have shown that various larvae of the genus *Atelopus*, *Rhinella* and *Oophaga* are fed with unfertilized eggs the female deposits and they possess chemical defenses for the tadpoles. Also, some eggs of these same species hatch with chemical defenses the female has provided during oviposition as protection against predators (Pavelka et al. 1977; Hayes et al. 2009; Stinosky et al. 2014). In the *Phyllomedusa* larvae, the acquisition of chemical defenses has not been reported; however, we can be sure the toxicity assays are reliable to demonstrate these tadpoles possess toxic substances, although we cannot prove their origin. The presence of glands that secrete opioid peptides such as dermorphins, bradykinin and deltorphine by some adult *Phyllomedusa* are highly unpleasant to predators because they can modify cardiac function, induce regurgitation, or produce catalepsy (Sazima 1974; Negri et al. 1992). These same substances may be appearing along larval growth, as well as serous glands, which could function as regulatory and defense mechanisms in the aquatic environment (Delfino et al. 1998).

About the presence of conspicuous colorations in our model, we observed that there is a bimodal pattern of coloration. On the dorsal area, the tadpoles have conspicuous colorations, while ventrally the tadpoles can be considered inconspicuous. This would suggest that dorsal coloration would be targeted at aerial or terrestrial predators, whereas the coloration of the ventral area makes them cryptic for predators living underwater (Thibaudeau and Altig 2012). *P. vaillanti* was the most conspicuous species in our experiment. It also had a very significant metalized interocular patch. It is possible this conspicuous patch not only gives it advantages to warning predators, but it can also serve as a disruptive coloration, thus increasing their opportunity to avoid predators (Eterovick et al. 2010). On the other hand, *C. tomopterna*, presents a black spot at the end of the tail, which could be used as a strategy to redirect the predator's attack. Such an attack would be directed toward a less vulnerable area and compensate the absence of some conspicuous coloration (Caldwell 1982).

On the other hand, conspicuousness did not have a correlation with the toxicity in *P. bicolor*. This correlation was only observed in the *P. vaillanti* larvae and only associated to the coloration of the interocular spot, but not all tadpole coloration. The reason can be that the conspicuousness and presence of toxins could operate decoupled. For example, some species of dendrobatids may differ significantly in toxicity, but their coloration is the same. Other individuals may exhibit similar colorations, but their toxicity varies significantly (Darst et al. 2006).

Conspicuous dorsal colorations of these larvae may work together with schools towards the surface of the water. This would suggest a positive effect on predator learning. Other phyllomedusine larvae (*P. gutatta*, *P. jandaia* and *P. vaillanti*), mainly present congregation behaviors supposedly as an antipredatory mechanism. They are predominately grouped on the water surface forming schools with individuals of the same size during the day while at night they are dispersed and located along the water column (Branch 1983; Caramaschi and Jim 1983). This behavior towards the water surface could be strongly related to environmental factors such as light and the different sensory systems of the larvae, which would explain the behavior of *P. vaillanti* and *P. bicolor* (Katz et al. 1981).

On the other hand, it has been evidenced the effectiveness of this conspicuous coloration and the toxicity present in both *P. vaillanti* and *P. bicolor* increases as the individuals' size, which produces greater aversion by predators (Hagman and Forsman 2003). In addition, the palatability experiments support this hypothesis, since it was observed that the larger larvae were not depredated. This has suggested this aposematic signal may evolve favoring larger prey (Lever 2001, Gunzburger and Travis 2005). Depending on the stage of larval development, it is possible for toxins or alkaloids to change their composition and abundance, making the animals less palatable to predators (Brodie and Formanowicz 1987; Peterson and Blaustein 1991; Hayes et al. 2009; Stynoski et al., 2014). For example, there has been an apparent change in the toxicity of *Bufo* during ontogeny. Large mortality of giant water bugs (Belostomatidae) was observed when the insects predated tadpoles in late stages of development (Crossland 1998). In other experiments, were offered to chickens and predatory rejection of larger insects was observed (Gamberale and Tulberg 1998).

In conclusion, *P. vaillanti* and *P. bicolor* larvae may present an antipredatory syndrome using various mechanisms to avoid predators. These combined strategies can reduce the probability of attack, confuse the predator, reinforce the alarm signal, and warn of the presence of toxins in the skin of the tadpoles (Aronsson and Gamberale 2012). Finally, the high cost of having a conspicuous coloration associated with chemical defense and the formation of congregations is a point that merits greater attention in future investigations, specifically at the time the larvae complete their cycle or survival rates associated with the use of these strategies.

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Data Accessibility Statement

The data that support the findings of this study are openly available in Figshare at <https://doi.org/10.6084/m9.figshare.13489260.v1>

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Figure legends

Figure 1. A: Lateral and dorsal view of three larval of phyllomedusinae used as study models and diagrams with reference color codes of the tadpoles; B: dorsal and lateral view of *P. vaillanti* tadpoles indicating areas from which color measurements were obtained in the three species.

Figure 2. Distribution of two species of phyllomedusine frog larvae along the water column under experimental conditions.

Figure 3. Relationship between body length and back conspicuousness (green) and interocular patch (orange) of three species of phyllomedusine frog larvae.

Figure 4. Palatability of three species of phyllomedusine frog larvae, measured as the proportion of larvae that were predated by odonates (N = 18 *P. vaillanti* , N = 9 *P. bicolor* , N = 11 *C. tomopterna*).

Figure 5. Differences in toxicity and conspicuousness in three species of phyllomedusine frog larvae (*P.vaillanti*, *P. bicolor* and *C. tomopterna*). Saline injection was used as a negative toxicity control measure. The black dots indicate the median and the horizontal bars the 95% confidence intervals.

Figure 6. Correlation between toxicity and conspicuousness in three species of phyllomedusine frog larvae. The PC1 represents the conspicuousness of the back of the body and PC2 the conspicuousness of the interocular patch.

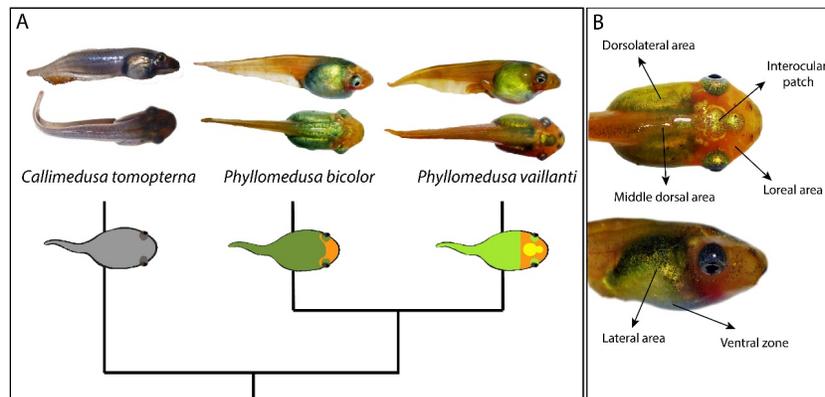


Figure 1

Figure 2

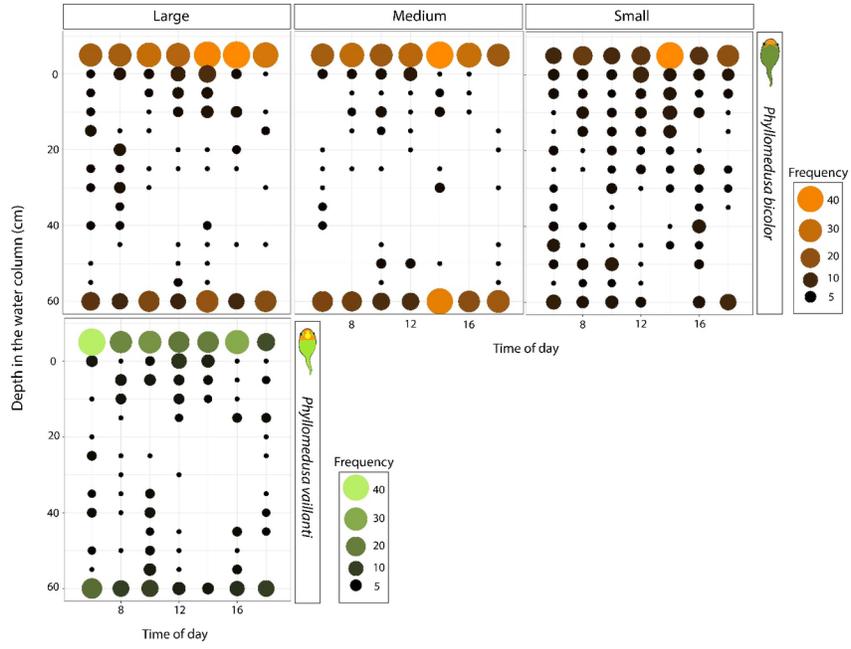


Figure 3

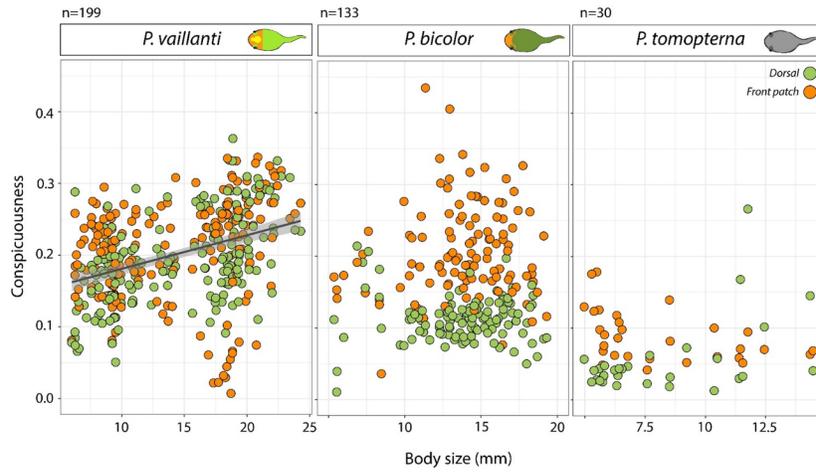


Figure 4

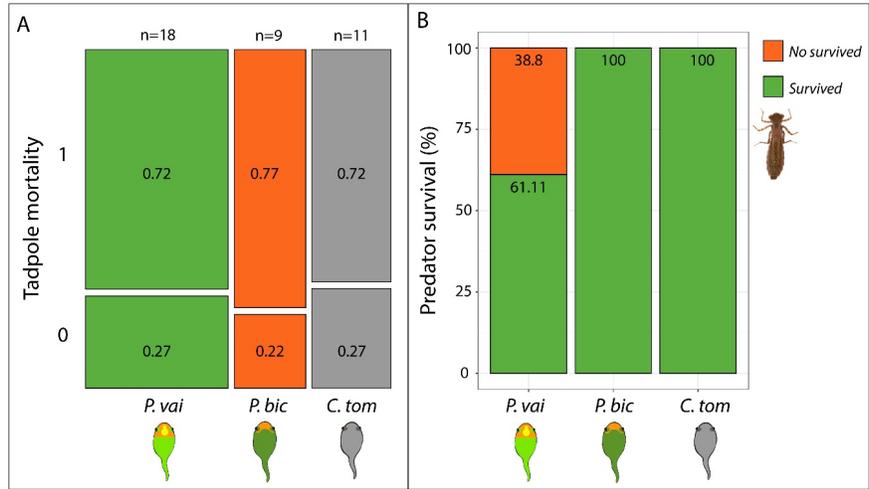


Figure 5

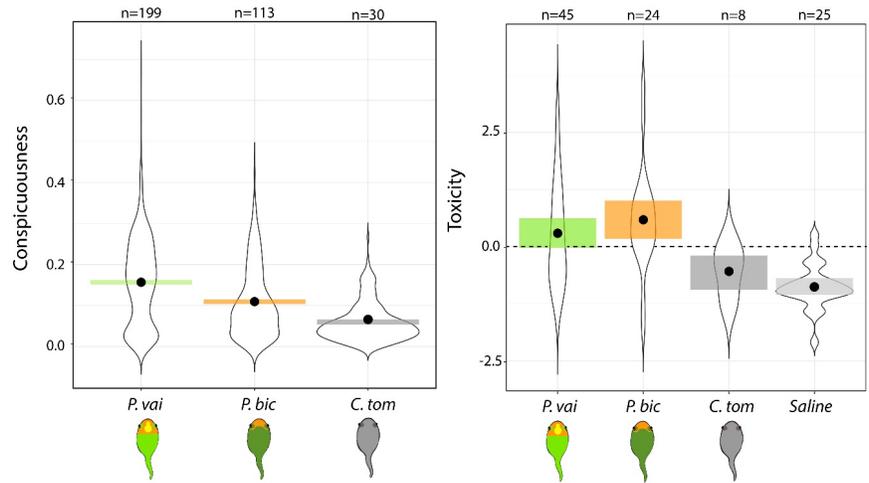


Figure 6

