# A Histological Analysis of Coloration and Toxin Sequestration in the Peruvian Mimic Poison Frog (*Ranitomeya imitator*)

Kommentiert [SM1]: Not so much on sequestration in your manuscript!

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## Abstract

Aposematism continues to be a phenomenon of central interest in evolutionary biology. The life history of the mimic poison frog, *Ranitomeya imitator*, relies heavily on aposematism. In order for aposematic signals to be effective, predators must be able to learn to avoid the aposematic phenotype. In *R. imitator*, aposematism is associated with four different color morphs that mimic a complex of congeneric species occurring across the mimic frog's geographic range. Investigations of the underlying mechanics of color production in these frogs can provide insights into how and why these different morphs evolved. We used histological samples to examine divergence in the color production and toxin sequestration mechanisms used by *R. imitator* to produce effective aposematic signals across its geographic range. We measured the abundance of melanophores, xanthophores, and poison glands in each color morph. We find

that morphs that produce orange skin exhibit a higher abundance of xanthophores and lower abundance of melanophores than those that produce yellow skin. In turn, morphs that produce yellow skin exhibit a higher abundance of xanthophores and lower abundance of melanophores than those that produce green skin. Generally, across the morphs, a high ratio of xanthophores to melanophores is associated with colors of brighter spectral reflectance. Additionally, we find differences in the abundance of poison glands across the four color morphs, with the Varadero (red-headed) morph possessing the greatest abundance of glands. This may represent variation in the morphs' anatomical capacity to sequester the alkaloid toxins that reinforce learned predator avoidance of their aposematic signal. However, measurements of glands from wild specimens, exposed to alkaloid toxins, are needed to confirm ~~-~~this result. Together, our results contribute to the understanding of color production in amphibians and document divergence in the histology of a species that is subject to divergent selection associated with aposematism.

## Introduction

The phenomenon of aposematism (the use of conspicuous coloration by prey items to signal unpalatability to predators) has long held the interest of ecologists and evolutionary biologists (Poulton 1898~~-~~; Ruxton et al. 2019). Because aposematic signals act as a color defense that directly ~~impacts~~affects predation rate, aposematic organisms are under significant evolutionary pressure to develop color production mechanisms suitable to the predators in their ~~local~~ environment (Seymoure et al 2018). Likewise, aposematic organisms must be capable of producing or sequestering toxins to maintain learned predator avoidance of their color pattern within a population level.

The mimic poison frog, *Ranitomeya imitator*, is a small dendrobatid frog endemic to Peru (Schulte 1986). Like many poison frogs, *R. imitator* uses bright aposematic colors to signal its

toxicity to potential predators (Stuckert et al. 2014ab). Additionally, *R. imitator* provides a striking example of color polytypism. Rather than converging on a single species-specific color pattern, *R. imitator* has diverged into four distinct color morphs—banded, striped, spotted, and Varadero—across its- geographic- range (Fig 1).

*Figure 1. The four color morphs of R. imitator: banded (upper left), striped (upper right), spotted (lower left), and Varadero (lower right).*

The evolution and persistence of the four color morphs can be explained by Müllerian mimicry (Symula 2001, Twomey 2013). Each morph benefits from taking on a different color pattern because that color pattern resembles another ~~noxious~~ toxic congener—respectively, *R. summersi*, *R. variabilis* highland, *R. variabilis* lowland, and *R. fantastica*—that share the same geographic space. The shared color pattern contributes to reciprocal learned avoidance in local predators (Stuckert et al. 2014a); a predator who has been exposed to an unpalatable model will, in the future, avoid preying upon the mimic and vice versa.

**Color production**

Most color production mechanisms in vertebrates can be categorized either as structural or pigmentary. Structural mechanisms produce bright colors, often blues and greens, by reflecting light off nanoscale structures found in the integument. By contrast, pigments produced by specialized cells in the dermis absorb light of a specific wavelength, leaving the remaining wavelengths visible to an observer (Mills 2008). ~~Structural mechanisms and pigmentary~~Both mechanisms frequently interact to produce colors of varying hue and brilliance (Segami 2017).

*Figure 2. Illustration of a typical chromatophore unit with subcellular structures. Superficial xanthophores contain pigment vesicles, carotenoid vesicles and pterinosomes. Iridophores contain reflective guanine platelets. Deep melanophores contain melanosomes and may exhibit fingerlike projections that wrap around other chromatophores.*

Amphibians use specialized cells called chromatophores to produce color. Chromatophores are found layered within the dermal and subdermal tissue. Chromatophore layers are usually found in close contact with each other and are frequently referred to as chromatophore units (Dushane 1935). The most superficial chromatophore, the xanthophore, contains pteridine and/or carotenoid pigments. These pigments absorb violet, blue, and green light to produce yellow, orange, and red coloration (Bagnara 1968, Frost 1984, Twomey 2020). Iridophores may be found below xanthophores. Iridophores contain nanoscale guanine platelets that reflect light; traditionally, they have been associated with the production of bright blue and green coloration, although more recently they have been associated with a broader color spectrum (Bagnara 1968, Frost 1984, Twomey 2020). When found beneath xanthophores, iridophores may also increase the brightness of superficial colors (Shawkey 2005, Shawkey 2017, Twomey 2020). The deepest chromatophore, the melanophore, contains eumelanin or pheomelanin pigments. These pigments absorb most of the light in the visible spectrum and produce dark brown or black coloration (Bagnara, 1968, Dushane 1935, Frost 1984). When found below iridophores, melanophores may enhance blue or green colors by absorbing stray light scattered incoherently by the guanine platelets (Shawkey 2017).

**Kommentiert [SM2]:** Not referenced in the text!

**Kommentiert [SM3]:** Represented by what in your figure?

**Kommentiert [SM4]:** same

**Kommentiert [SM5]:** same

**Kommentiert [SM6]:** I like the general outlay and idea of the illustration, however I think it is too simplistic and partly misleading. Particularly the round edged indentation is used to indicating individual cells (and I assume the pink blobs are nuclei?) as well as the entirety of the dermis.

**Toxin sequestration**

While dendrobatid frogs produce most of their colors endogenously, they obtain alkaloid toxins exogenously, by consuming ants and mites (Daly 1994, Saporito et al. 2009). Once ingested, alkaloid toxins are sequestered in specialized granular glands in the frogs' dermal tissue (Saporito 2010). Granular glands are derived from ectodermal precursor cells during embryonic development. However, the glands do not become fully functional until the frogs reach their ~~-~~metamorphic climax, shortly before they begin feeding on ~~ingesting~~ alkaloid ~~toxins from~~containing prey ~~items~~ (Stynoski 2016). As frogs grow from juveniles to adults, the glands enlarge and ~~-~~migrate from the epidermis to the dermis (Saporito 2010, Stynoski 2016). The type and quantity of toxins sequestered in granular glands ~~-~~may vary, depending on the prey items available to dendrobatid frogs (Saporito 2006). In some rare cases, dendrobatid frogs may metabolically alter alkaloids to ~~intensify~~ increase their toxicity (Daly 2003, Saporito 2006).

**Kommentiert [SM7]:** More information would be helpful. This is not enough to prepare me for the relevance of your findings later!

**Kommentiert [SM8]:** Stynoski and Cornell 2017? Stynoski 2016 is not in your references!

**Kommentiert [SM9]:** same

**Study objectives**

Our study aims to address two main questions: 1) does chromatophore abundance vary across color morphs of *R. imitator* and 2) does poison gland abundance vary between color morphs of *R. imitator?* We used histological samples to measure the abundance of melanophores, xanthophores, and poison glands within and between color morphs of *R. imitator* to answer these questions. Answering these questions may contribute to the understanding of an organism's cellular response to an ecological phenomenon, like aposematism, which exerts divergent selection on a species across its geographic range.

## Materials & Methods

**Collection of Specimens**

Individuals were collected from each mimetic morph of *Ranitomeya imitator* from locations near the city of Tarapoto, in San Martin Province, Peru. Six adults from each color morph (24 in total) were sacrificed within 24 hours of collection using 20% benzocaine gel applied to the venter~~, according to the AUP D303)~~. Immediately after the individuals were sacrificed, their dorsal skins were removed and placed in 10% neutral buffered formalin. All animal ~~use~~ handling procedures followed the respective protocols and ~~necessary for this study~~ were approved by Servicio Nacional Forestal y de Fauna Silvestre (SERFOR permiso CITES N°17 PE001718) in Peru and the Institutional Animal Care and Use Committee at East Carolina University (AUP D303).

**Slide preparation**

Skins were dissected to separate regions of different colors, which were labeled as black, yellow, orange, or green. Skin sections of approximately 2 x 1 mm were collected from the separated regions. Skin sections were fixed in a 10% phosphate buffered formalin solution for 6-12 hours, then run through several dehydrating and clearing solvents in a tissue processor. Finally, a rotary microtome was used to embed the skins in paraffin wax, section them into sections with a thickness of 5µm, and fix the sections to glass microscope slides. A ~~total of a~~pproximately 1,000 slides were produced, each slide containing 8-10 skin sections.

**Histological staining**

Histological slides were stained according to Newcomer's Schmorl-Melanin protocol, which was designed to identify sites of melanin deposition. ~~-~~Groups of five slides in a slide basket were

**Kommentiert [SM10]:** Pasture, primary forest, urban space? Please specify! From the information given I would not be able to find your frogs!

**Kommentiert [SM11]:** Please give more details on where and under what conditions the specimen were collected (night / day, visual encounter / active search on bromelians or under rocks). Aren't the morphs radiated, could you collect them all from the same area? Can you provide at least some rough coordinates of where the respective morphs were collected? Or at least refer to a publication that provides the information where to find what morph (e.g. Twomey et al 2013 ?)

**Kommentiert [SM12]:** Already mentioned below.

**Kommentiert [SM13]:** That is quite vague, please specify or refer to the used protocol provided in the supplement

**Kommentiert [SM14]:** Didn't knew a microtome can embed tissue! Can you provide the type and manufacturer of the used equipment.

**Kommentiert [SM15]:** One slide per skin sample? I assume so from your statement below… I have to admit it is a bit tedious to gather this information by jumping up and down through your manuscript.

**Kommentiert [SM16]:** Merge with slide preparation?

dipped in a series of reagents, detailed in Supplemental Table 7. Critically, the reagents included a potassium ferricyanide solution to stain sites of melanin deposition black and a Nuclear Fast Red solution to make surrounding cells distinguishable. At the end of the series, coverslips were fixed to slides using a Permount mounting solution. Slidebrite and ethanol solutions were changed after 50 slides, or at the earliest sign of cloudiness or discoloration, whichever came first. Nuclear Fast Red and ferricyanide solutions were changed weekly. A plastic basket was developed so five slides could be submerged in reagent at a time, increasing the efficiency of slide processing.

**Kommentiert [SM17]:** Could be reduced to one sentence in the supplementary file where you specify your staining protocol.

**Microscopy**

Histological slides were examined under a brightfield microscope connected to a computer with a digital display. A magnification strength of 40X was used to collect images. The first, fifth, and last skin section from each slide were selected for imaging, so that sections spanned the length of the original skin sample, except when tissue degradation made a section unusable (gaps in tissue exceeded width of epidermis). When one section was deemed inviable, the nearest viable section was selected for imaging.

**Kommentiert [SM18]:** Resolution of pictures, manufacturer of camera equipment and microscope?

**Kommentiert [SM19]:** 8-10 5µm wide slices = sections per slide?
→ ~ 50 µm of tissue from a 1-2 mm wide skin sample ≠ entire length of skin sample

Did you mount the whole series or 'only' every tenth (or so) section on a slide? Am I missing something?

Also, 3 replicated per slide, from approx. 1000 slides → ~ 3000 pictures to be analyzed 2072 you state …what happened to the remaining 30%, are these the degraded samples you are mentioning?

*Figure 3. Left – degraded tissue section, excluded from the study. Right – intact tissue section, used in the study.*

**Kommentiert [SM20]:** Not sure about the necessity of that figure. Interestingly, there is a tendency to be super detailed on less relevant parts, but unspecific on the actual data. Can you instead provide sections from different skin colors, and try to highlight the anatomical differences, even if they are subtle.

If the figure stays in the manuscript it needs to be referenced and deserves a scale bar and maybe it is better suited for the supplement.

Skin sections on the same slide were assumed to be roughly equivalent, since they represent tissue of the same color taken from the same frog of the same morph at the same depth.

**Kommentiert [SM21]:** Sorry, I am confused now. If you consider all sections on one slide equivalent why do you pooled the first fifth and the last section?

Automatic white balance and exposure times were used to collect images. A total of 2072 images were produced.

**Image analysis**

Images were analyzed using ImageJ Software. A Bamboo stylus was used to outline regions of different cell types, and the area of each region (in pixels) was calculated. In some cases, several discrete regions had to be added together to find the total area of a given cell type.

*Figure 4. Regions of epidermis (E), xanthophores (X), melanophores (M), and poison glands (PG) in a section of orange skin tissue.*

~~Per previous research, regions~~ Regions stained dark brown or black pigmentation below the epidermis but above the dermis were considered to be melanophores (Bagnara 1968, Franco-Belussi 2020, Frost 1984). Regions of translucent cellular material with interspersed nuclei between the epidermis and the melanophores were considered to be xanthophores (Bagnara 1968, Frost 1984). Round regions of empty vesicles between the epidermis and the dermis were considered to be poison glands (Stynoski 2016). The pale region with parallel fibers was considered to be dermal tissue, and the pink region with densely crowded nuclei was considered to be epidermal tissue (Bagnara 1968, Carriel 2011, Frost 1984).

**Statistical analysis**

For each image, we used ImageJ Version 1.53k to measure the total area of the skin section, the area of melanophores, the area of xanthophores, and the area of poison glands using. All

**Kommentiert [SM22]:** Merge with microscopy and statistical analysis?

**Kommentiert [SM23]:** Is that relevant? Or is that the tool used in the software, did you use a touch pad?

**Kommentiert [SM24]:** That's why resolution would matter!

**Kommentiert [SM25]:** Automatically by the program?… using what tool?

**Kommentiert [SM26]:** Except for figure 1 none of your figures is referenced in the text!
D for dermis? Maybe you can combine figure 4 and 2 to highlight the characteristics of the different types of chromatophores? Maybe add a detail shot of an poison gland?

**Kommentiert [SM27]:** With that explanation unexperienced researches may even mistake a blood vessel as gland! How did you distinguish poison glands from mucus glands and even (ungranulated) ordinary skin glands.
If you did not differentiate between any of them (particularly due to resolution limitations or alteration during fixation) that has to be discussed elaborately!

**Kommentiert [SM28]:** Stynoski 2016 is not in your references! Did you mean Stynoski and O'Connell 2017? …and if so, this paper addresses only the tadpoles in a different genus!
Maybe a paper with (at least) post-metamorphic stages would be more suitable as reference!

**Kommentiert [SM29]:** Did you actually count the number of poison glands? Did you correct for the number of glands or is it just the relative area they covered? - Particularly you could calculate a relative size per gland (hypertrophy could indicate more active sequestration)

**Kommentiert [SM30]:** And what did you measure with these, the total amount of screened area?

**Kommentiert [SM31]:** Even after reading it several times, I still don't get what data you are actually comparing at the end and how it was standardized. You provide percentages for the different chromatophores in each morph for each skin color type, but why not for the poison glands in the respective skin color section?

measurements were recorded in a Microsoft Excel spreadsheet. Statistical analysis were conducted with SAS Enterprise Guide 7.1 software.

Abundance of each chromatophore type was calculated by dividing area of the chromatophore type by total area of the skin section.

Although the data set generated by the present study was large (2072 images were measured), not all the data points could be considered independent samples (24 adult frogs provided all skin sections imaged). A hierarchical relationship exists between the four color morphs and the six frogs from each color morph that were sampled. In order to manage the hierarchical nature of the data set, measurements taken from each individual frog were averaged and compared to each other in a one-way ANOVA ~~test in SAS Enterprise Guide 7.1~~, since the averages could be treated as independent samples.

The one-way ANOVA tests run ~~within SAS Enterprise Guide 7.1~~ compared the variance observed between six individual frogs of the same color morph (within group variance) to the variance between frogs from different color morphs (between group variance) to test the null hypothesis that color morph has no effect on chromatophore abundance (or that all color morphs belong to the same statistical group). As Post-Hoc tests we conducted pairwise

~~One-way ANOVA tests were followed up by~~ Tukey's ~~Studentized Range (HSD)~~ tests~~, .~~ ~~Whereas the F-values and P-values generated by one-way ANOVA tests merely allowed us to support or reject the null hypothesis that all color morphs belong to the same statistical group, Tukey's Studentized Range test performed pairwise comparisons~~ to ~~quantify the differences~~identify differences in ~~mean between group~~group means ~~s and indicated which groups were statistically different and which were not~~ (Tables S1 – S6).

## Results

**Kommentiert [SM32]:** That would be relative abundance, with abundance I expect a count data!

**Kommentiert [SM33]:** Number of individual skin samples would be more interesting here!

**Kommentiert [SM34]:** Not sure whether hierarchical is the right word: Foremost your samples from one individual are dependent (as stated), maybe stick to that expression.

**Kommentiert [SM35]:** What has been averaged, the replicates of a skin color samples from one individual? Then also the number of repeats (mean and SD) per color and individual are interesting, can't find that information in your manuscript are there any?

**Kommentiert [SM36]:** This reads a bit like you magically eradicated the dependency of the repeats; the average is a new value! I assume you are saying you didn't find differences between the dependent samples and therefore averaged them?

**Kommentiert [SM37]:** In general, it is hard to understand the composition of your data set and I guess a lot of relevant information is hidden in the supplement that deserves being displayed in the main text.

As far as I understood it correctly…
-You obtain a relative value for each of the different chromatophores from one section?!...
-Then you average 3 sections (1st, 5th,10$^{th}$ section) from one slide, which corresponds to a single skin sample of a particular color?!...
But how many repeats of a certain skin color did you sample per morph?

As poison glands can be distributed unevenly throughout the skin, how did you account for that?

Can you provide a table showing number of skin samples per color and individual, total of different colored skin samples per morph and ultimately the number of analyzed pictures per color?

**Kommentiert [SM38R37]:** Just saw the latter (number of pictures) is provided in the supplement tables…

**Black skin**

All morphs of *R. imitator* exhibit a dark black dorsal background upon which different colors are presented. Black skin from all morphs was typified by a thick band of melanophores directly below the epidermis and a complete lack of other types of chromatophores. Finger-like projections of melanophores were observed surrounding poison glands. The results of the ANOVA suggest no statistically significant differences in melanophore abundance in black skin tissue from the striped, spotted, or banded morphs ($F = 2.190$, $P_r > F = 0.1209$, $df = 3$). However, the Tukey's HSD test indicated that the varadero morph was statistically different from the other three morphs, with approximately 2% less melanophore abundance than the other morphs (Table S1).

**Yellow and orange skin**

Of the four morphs of *R. imitator*, three exhibit patches of yellow or orange skin. The striped morph exhibits yellow skin, and the banded and varadero morphs exhibit orange skin. (No yellow or orange skin sections were collected from spotted morphs). Yellow and orange skin tissue sections were typified by a thin layer of xanthophores located directly below the epidermis and directly above the melanophore layer. At the level of magnification used in this project, the boundary between the xanthophore and melanophore layers appeared discrete and was not characterized by projections of melanophores into the xanthophore layer. An iridophore layer could not be identified with confidence using our staining procedure.

The results of the ANOVA suggest a statistically significant difference in the abundance of xanthophores among color morphs ($F = 11.7$, $P_r \geq F = 0.0009$, $df = 3$). The Tukey's HSD test specifies a difference between the banded and striped morphs ($\bar{x}_{banded\text{-}striped} = 5.115$) and the banded

**Kommentiert [SM39]:** Which is also the case in ordinary mucus glands!

**Kommentiert [SM40]:** Font size?

**hat formatiert:** Nicht Hochgestellt/ Tiefgestellt

**Kommentiert [SM41]:** Here and elsewhere, that's very small! Maybe abbreviate and increase font size?

and varadero morphs ($\bar{x}_{banded\text{-}varadero}$ = 4.940). However, there was no significant difference between the varadero and striped morphs (Table S2). The abundance of melanophores in yellow and orange skin sections was also found to be significantly different across the color morphs (F = 10.120, $P_r$ > F = 0.0017, df = 3), with the striped morph exhibiting the greatest abundance of xanthophores, followed by the varadero morph, then the banded morph (Table S3).

**Green skin**

Of the four morphs of *R. imitator*, two exhibit green skin: the striped morph and the spotted morph. Green skin sections were characterized by a thin layer of xanthophores directly below the epidermis and above the melanophore layer. Presumably, iridophores were also present in green skin tissue, but they were not detectable under the staining protocol and magnification level used in this project.

A one-way ANOVA test indicated a significant difference in the abundance of both xanthophores (F = 21.15, $P_r$ > F = > 0.0001, df = 3) and melanophores (F = 21.11, $P_r$ > F = > 0.0001, df = 3) between green skin sections and the previously examined yellow/orange skin sections. Likewise, the Tukey's HSD test found statistically significant differences in the abundance of xanthophores for all but the striped and varadero morphs (Tables S4, S5).

**Kommentiert [SM42]:** ≥ ?, > ?, = ?

**Kommentiert [SM43]:** ≥ ?, > ?, = ?

**Poison glands**

Like other dendrobatid frogs, all morphs of *R. imitator* have specialized glands in the integumentary system in which toxins are sequestered and can be released. In the skin sections collected for this project, the poison glands appeared as round regions of tissue containing empty vesicles and spanning from the dermis to the epidermis. Melanophores often wrapped around the perimeters of the glands.

**Kommentiert [SM44]:** The locality argument to differentiate type of glands should be mentioned in the M&M section!

The results of a one-way ANOVA test indicate a statistically significant difference in the abundance of glands in skin tissue of the four color morphs ($F = 86.81$, $P_r \geq F = > .0001$, $df = 3$) with the varadero morph exhibiting the greatest abundance of glands ($\bar{x}_{varadero} = 10.438$) and the spotted and banded morphs exhibiting no significant differences and tying for least abundance of glands ($\bar{x}_{spotted} = 4.593$, $\bar{x}_{banded} = 4.466$) (Table S6).

**Kommentiert [SM45]:** The morph with the least amount of black skin! Latest now I wonder whether poison glands are more abundant in the brighter skin sections!

**Kommentiert [SM46]:** Value for the striped morph?

**Kommentiert [SM47]:** Can you add a graph for these results? Did you research the differences in number / abundance of glands in respect of skin color in a morph. After all, poison gland densities may vary across the body surface and may coinciding with areas of brighter color, see Prates et al. 2011

## Discussion

Coloration is a key trait in aposematic organisms, although little is known about the mechanisms of color production in polytypic animals. Here we attempt to document phenotypic divergence on a cellular level in *R. imitator*, an aposematic and polytypic frog under divergent selection across its geographic range. In documenting histological differences between the four color morphs of *R. imitator*, the results of this study contributes to the growing body of evidence that the color polytypism observed in *R. imitator* and other dendrobatids may be associated with speciation at an early stage (Gray 2007, Segami 2017, Servedio 2011, Twomey 2014, Yang 2019).

### No variation in melanophore abundance in black tissue

Although some dendrobatids do exhibit dorsal backgrounds of varying darkness (Posso-Terranova. et al. 2017), no difference in background color has previously been described in *R. imitator*. Moreover, the pressure for *R. imitator* morphs to resemble their models (all of which exhibit dark dorsal backgrounds) would likely act to conserve the dark dorsal background trait within the species. Therefore, it is unsurprising that melanophore abundance in black skin tissue does not vary between three of the four color morphs. The outlying Varadero morph exhibits the smallest area of black skin on its dorsum, and one previous study found that Varadero tadpoles have significantly lower expression of the *mitf* gene, which encodes the melanogenesis

associated transcription factor (Stuckert et al. 2021). Downregulation of melanogenesis in Varadero tadpoles would be consistent with a decrease in melanophore abundance in adult Varadero frogs.

**Xanthophores are more abundant and melanophores less abundant in orange tissue than yellow tissue**

A previous comparison of spectral reflectance across the four color morphs of *R. imitator* found that the banded morph exhibits the brightest and most ~~high~~ contrasting colors (Twomey 2016). In the present study, the banded morph was found to contain the greatest number of xanthophores and the ~~fewest~~ lowest number of melanophores in orange skin tissue, which may be consistent with the production of bright colors. The striped morph exhibited the least abundance of xanthophores, which may indicate that fewer carotenoid/pteridine pigments are required to produce yellow colors than orange colors. Another recent study (Twomey et al. 2020) found that the thickness of guanine platelets in the iridophore layer of *R. imitator* skin can also affect hue in the yellow-to-red region of the spectrum, so this may be an additional factor contributing to overall coloration.

**Xanthophores less abundant and melanophores more abundant in green tissue than orange or yellow tissue**

Both morphs capable of producing green coloration exhibit a lower abundance of xanthophores than those exhibiting orange colors, which suggests that the production of green coloration may depend less on contributions from carotenoid and pteridine pigments and more on contributions from iridophores and unquantified subcellular structures. Frogs capable of producing green coloration also exhibit a higher abundance of melanophores than frogs that produce orange

coloration. Previous studies have proposed that melanophore layers may be thickened below iridophores to absorb light that is scattered randomly by the guanine platelets, which may explain the increased melanophore abundance in green skin (Shawkey 2017).

A previous comparison of spectral reflectance across the four color morphs of *R. imitator* found that the spotted morph exhibits the least bright and lowest contrast colors (Twomey 2020). In the present study, the spotted morph was found to contain the lowest abundance of xanthophores and the greatest abundance of melanophores in colored skin tissue, as opposed to the banded morph, which exhibits the brightest and most ~~high~~ contrasting colors. Together, these results suggest that a high ratio of xanthophores to melanophores may produce bright colors, whereas a low ratio of xanthophores to melanophores may produce dull colors.

*Figure 5. Summary of results from measurements of chromatophores across color morphs. Few significant differences exist in black skin tissue across color morphs. In yellow/orange skin tissue, the striped (yellow) morph exhibits lesser xanthophore abundance and greater melanophore abundance than the banded or varadero (orange) morphs. In green skin tissue, the spotted morph exhibits lesser xanthophore abundance and greater melanophore abundance than the striped morph. The morph with brightest colors (banded morph) has the highest ratio of xanthophores to melanophores, and the morph with the dullest colors (spotted morph) has the lowest ratio of xanthophores to melanophores. Tests of statistical significance can be found in S1 – S5.*

**Poison gland abundance does not predict quantity of toxins sequestered by frogs of different color morphs in a field survey**

Previous studies have suggested that variations in toxicity across color morphs of polymorphic frogs could be attributed either to 1) physiological adaptations allowing some frogs to sequester more toxins than others or 2) differences in the diets available to frogs in dispersed populations (Saporito 2006, Saporito 2010, Stynoski and O'Connell 2017, Saporito 2010). The present study found that the varadero morph may be anatomically equipped to sequester more toxins than other *R. imitator* morphs, having a greater abundance of poison glands than the other morphs. However, a field study found that the varadero morph carries the least amount of alkaloid defenses, compared to other morphs (Stuckert et al. 2014). This discrepancy suggests that the strength of a frogs' defenses may be more closely related to the availability of dietary alkaloids than to anatomical capacity for sequestering toxins.

Additionally, previous studies have demonstrated that the banded morph exhibits the brightest and most ~~high~~ contrasting colors of all the *R. imitator* color morphs, whereas the striped morph exhibits the dullest and least contrasting colors (Twomey 2013). Our results show no significant difference between the abundance of poison glands in the banded and spotted morphs, which suggests that despite its brighter colors, there may not be an anatomical basis for the banded morph to have a higher level of toxicity than the spotted morph. Thus, in the case of *R. imitator*, intraspecific color variation across morphs may not reflect a quantitatively honest aposematic signal (Stuckert et al. 2018).

Importantly, the frogs used in the present study were raised in captivity and were not exposed to prey items carrying alkaloid toxins. The absence of toxins may have affected the development of glands, meaning that poison gland abundance in captive frogs may not accurately represent poison gland abundance in wild frogs. To date, no measurements of poison gland abundance in wild *R. imitator* morphs has been published. The results of this study should

**Kommentiert [SM48]:** ?

**Kommentiert [SM49]:** Abundance or relative area in the section?

**Kommentiert [SM50]:** Interesting, maybe the frog is already investing more into aposematic colors and can economize on venom sequestration? One wonders how that may affect the honesty and evolutionary stability of the mimicry signal along with his mimicking conger…

**Kommentiert [SM51]:** Or that brighter skin section tend to have a higher 'density' of poison glands

**Kommentiert [SM52]:** That is an important finding (if not the most important) and deserves better emphasis!

**Kommentiert [SM53]:** What study are you referring to? Stuckert or or own? Didn't you collect your specimen from the field?
…Your reference used wild animals!

**Kommentiert [SM54]:** I mean this not me misunderstanding the text?!…

This is in clear contrast to your M&M section where you state you collected your specimen in the field and euthanized them within 24 hours after collection!

be considered preliminary until a comparison of poison glands in captive frogs and wild frogs is made.

**Limitations**

Two major limitations exist in the present study's methodology: the magnification power used to examine skin sections, and the lack of classification of pigments contained in chromatophores. The brightfield microscope used in this study lacked magnification power to identify one of the major chromatophores, the iridophore, with confidence. Likewise, subcellular structures could not be identified with confidence. Previous studies have demonstrated that the size, distribution, and orientation of subcellular structures, like pigment vesicles and guanine platelets, may have significant influence on coloration, but their influence could not be accounted for in the present study (Frost 1984, Posso-Terranova 2017, Shawkey 2005, Shawkey 2017, Stuckert 2019, Twomey et al. 2020). Each of the chromatophores described in the present study is capable of producing a variety of pigments. Final skin color may vary depending on the pigment the chromatophore is producing (Andrade 2019, Posso-Teranova 2017, Twomey et al. 2020). However, the present study did not classify pigments prior to the processing of skin tissue samples for microscopic analysis and thus cannot account for variation in color due to pigment type.

## Conclusions

*Ranitomeya imitator* provides science with a striking example of color polytypism, produced by the mimic poison frog's need to present aposematic signals familiar to predators across its geographic range. The present study demonstrated that the divergent selection associated with

aposematism has led to divergence in the relative abundance of color-producing chromatophores across the four color morphs. Morphs that produce orange skin exhibit a higher abundance of xanthophores and lower abundance of melanophores than those that produce yellow skin. In turn, morphs that produce yellow skin exhibit a higher abundance of xanthophores and lower abundance of melanophores than those that produce green skin. Generally, across the morphs, a high ratio of xanthophores to melanophores can be associated with colors of brighter spectral reflectance. Additionally, the study documented differences in the relative abundance of poison glands across the four color morphs, with the varadero morph possessing the greatest abundance of glands. This finding may represent variation in the morphs' anatomical capacity to sequester the alkaloid toxins that reinforce learned predator avoidance of their aposematic signal. However, measurements of glands from wild specimens, exposed to alkaloid toxins, are needed to support this result.

**Future directions**

To date, the majority of studies of coloration in dendrobatid frogs have taken advantage of natural color variations to establish correlations between color production mechanisms and observed color patterns. Studies attempting to experimentally manipulate color production mechanisms would be extremely valuable to the field. For example, the present study found that the spotted morph had a much lower abundance of xanthophores in its green tissue than the banded morph had in its orange tissue. Previous studies have identified *pax7* and *xdh* as genes associated with the early development of xanthophores and have found both genes to be differentially expressed across the color morphs of *R. imitator* during development (Stuckert 2020). If the *pax7* and/or *xdh* gene could be overexpressed in spotted *R. imitator* embryos and

the overexpression of those genes led to adult spotted frogs with more abundant xanthophores and a more orange color pattern, there would be direct evidence that increasing xanthophore abundance causes the development of orange skin rather than merely being correlated with it.

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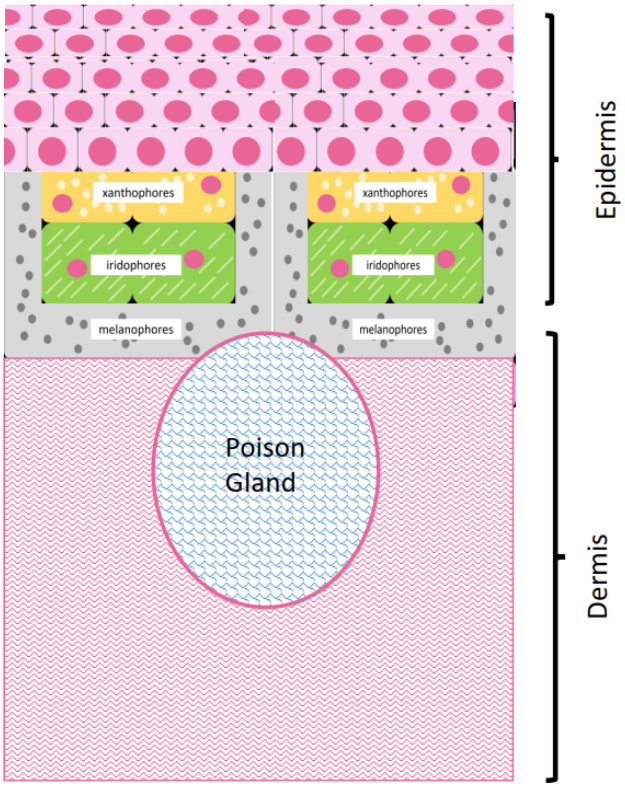


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