ELUCIDATING APOSEMATIC PATTERNS IN NORTH AMERICAN HYLIDS

by

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ABSTRACT

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Recognizing form and function of animal defenses is paramount to understanding the ecological and evolutionary forces behind predator and prey dynamics. Color patterns are strongly related to defensive strategies in animals. Some rely on camouflage to avoid detection, while others are brightly colored and conspicuously signal their noxiousness to potential predators. Still others combine cryptic dorsal coloration with colorful patches that are concealed in resting position but are facultatively unveiled by special behavior or simply during activity. Such hidden conspicuous color patches may be an intermediate stage in the evolution from camouflage to aposematism. We investigated whether conspicuously colored thighs serve as an aposematic signal in members of the North American Hylid treefrog family. Many Hylids are generally considered to be cryptic, yet show colorful patches on their flanks or thighs and have defensive skin secretions that may have unpalatable properties. First we conducted a palatability study to test whether Gray Treefrog (*Hyla versicolor*) skin secretions are unpalatable to model predators, and a clay model field study to test whether the conspicuous black and yellow thigh pattern is an aposematic warning coloration that reduces predation. Frog secretions were discriminated against during palatability assays, and clay models painted with a spotted black and yellow pattern suffered lower predation rates. Second we investigated whether thigh color was indicative of palatability in nine species of Hylid. We found species with thigh colors to be significantly more unpalatable than members without. Our findings support the hypothesis that hidden color patches act as aposematic signals in Nearctic Treefrogs and suggests that more species benefit from aposematic coloration than currently appreciated.

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Chapter one: Hidden black and yellow thigh color acts as an aposematic signal in the Eastern Gray Treefrog (*Hyla versicolor)*

INTRODUCTION

Organisms that deter predatory attacks and reduce predation pressure are at a selective advantage (Cott, 1940; Ruxton et al., 2019). Animals use a variety of protective mechanisms, including the presence and display of morphological weaponry such as spines, claws, sharp teeth, shells, and scales or chemical defenses (Bücherl, 1971; Edmunds, 1974; Utkin, 2015). Chemical defense is exhibited in two major ways: First some animals are equipped with injectable chemicals that harm predators or are used to immobilize and consume prey (Bücherl, 1971). Second, some animals secrete chemicals that render the assailed animal noxious and unpalatable to potential predators (Cott, 1940).

Many animals with chemical defenses display bright coloration and conspicuous patterns to warn potential predators of negative effects should they consume the animal. The advertisement of this unpalatability and chemical defense is known as aposematism. Color combinations common among aposematic taxa include: red, black and white; yellow, black, and white; red and black; orange and black and white and black (Prudic et al., 2007). The colors of black and yellow are widespread among aposematic taxa, and occur in species as diverse as salamanders, snakes, wasps, frogs and mammals (Brodie, 1993; Wells, 2007; Nekaris et al., 2019). It is the combination of these bright colors arranged in distinctive patterns such as spots, stripes and mottling that increases the conspicuousness of aposematic animals to potential predators (Ruxton et al., 2019). Furthermore, aposematic animals are often diurnal and occupy open habitats where they would be highly visible to predators (Dugas et al., 2015).

In addition to strikingly conspicuous species, there are those that have a cryptic dorsum but conspicuous colorful patches on body surfaces that are normally hidden but become exposed when signaling to conspecifics, fleeing, or as part of a defensive posture (Hödl, & Amézquita, 2001; Starnberger et al., 2014). These species may gain the combined advantages of both crypsis when at rest, and aposematism during movement, when crypsis is less effective. Such hidden color signals are taxonomically widespread and may represent a transitionary state in the evolution towards greater conspicuousness (Loeffler-Henry et al. 2019; 2023).

Amphibians are an excellent group to study chemical defense and aposematism. Almost all species have granular poison glands that secrete defensive skin secretions (Toledo & Jared, 1995; Wells, 2007; Dodd, 2013). These may be synthesized or sequestered and represent a range of chemicals including alkaloids, bufadienolides, tetrodotoxins and peptides that when secreted are unpalatable to predators (Wells, 2007; Bolton et al., 2017). Amphibians also have a range of different color patters. Highly conspicuous species such as Dart-Poison Frogs, Harlequin Toads and Fire Salamanders, or those with brightly colored ventral surfaces like Fire-belly toads or Pacific Newts, have been shown to be aposematic (Saporito et al., 2007; Wells, 2007). But many species, especially treefrogs of the family Hylidae, also show colorful patches on their flanks or thighs that are concealed when the animal is in a resting position. These species have received considerably less attention, partly because they are nocturnal and a key component of aposematism is considered to be the ability for a predator to see the warning colors of aposematic species (Cott, 1940), and partly because their defensive skin secretions consist mainly of peptides, whose unpalatable properties have not been appreciated until relatively recently (Clark, 2019).

The Eastern Gray Treefrog (*Hyla versicolor*) is a widespread Nearctic Hylid. The species displays exceptional color changing ability (Dodd 2013; Kapfer, 2022), and when at rest likely relies on crypsis as an anti-predator mechanism. Hidden body surfaces, however, are brightly colored: yellow flanks and groin, and conspicuously spotted black and yellow thighs (Dodd, 2013; Kapfer, 2022). These colorful areas become exposed and visible when the frogs assume an elevated posture, float in water, or are walking or jumping (Cannizzaro pers.obs). Moreover, handling these frogs can cause sneezing, skin rashes, and a burning sensation when making contact with a researcher's eye.

Here we test the hypothesis that the spotted black and yellow thigh coloration of *Hyla versicolor* is an aposematic signal (Fig.1). This hypothesis makes two predictions: (1) Frogs should be chemically defended and distasteful to potential predators, and (2) the spotted black and yellow thigh color should deter predation attempts. To test whether skin secretion of *H. versicolor* are distasteful, we used palatability assays with *Drosophila melanogaster* fruit flies. *Drosophila* are used as a model organism to study taste preferences (Meunier et al., 2003; Sellier et al., 2011**)** and have been used to study unpalatability of skin secretions in other frog species (Clark, 2019). To test whether the thigh coloration deters predation, we conducted a clay model study in which we placed plastalina models with putative aposematic (spotted black and yellow) and cryptic (plain black) painted thighs into the natural habitat of the frogs and scored for differences in predation rates (Madsen, 1987; Brodie, 1993).

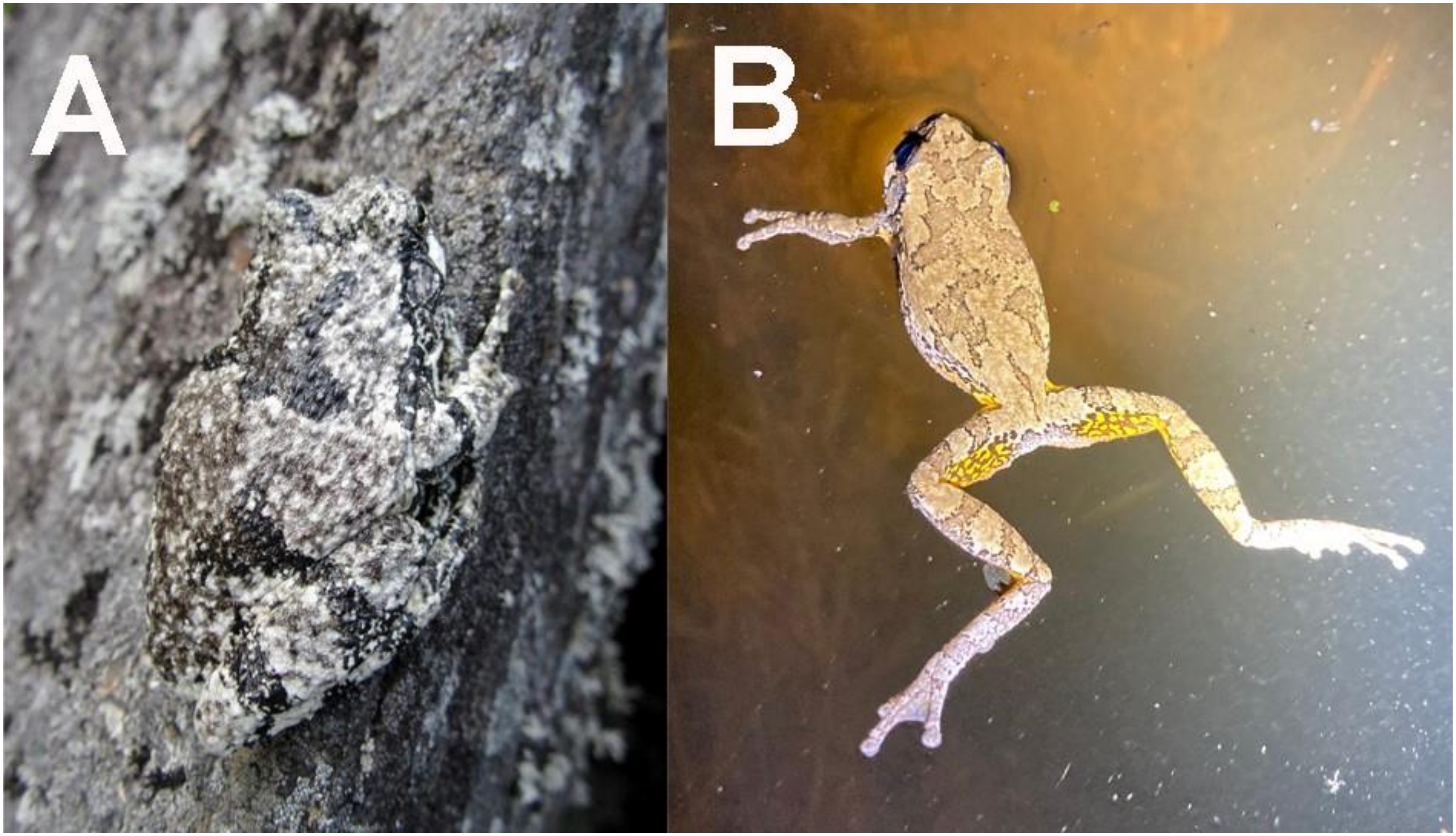

Figure 1. (A) Dorsal crypsis and (B) spotted black and yellow thigh coloration of *H. versicolor*.

METHODS

Study species and study site

We conducted the study at the University of Wisconsin-Milwaukee Field Station (UWM-FS) (43°23'12.72"N, 88° 1'48.50"W) in June 2022 and 2023. To obtain skin secretions for the palatability assay, we collected 10 adult male *H. versicolor* (3 in 2022 and 7 in 2023) and transported them to the lab for sampling. The clay model predation study took place adjacent to known *H. versicolor* breeding habitat at (UWM-FS).

Palatability assay

We collected skin secretions using a Transcutaneous Amphibian Stimulator (TAS, Grant & Land 2002; Bolton et al., 2017). The TAS was applied to the dorsum and ventral side of each frog for 30 seconds. We used a sterile six-inch cotton tipped applicator (Fisher brand REF 22363162, Lot 20211028) to collect the secretion, following the methods of Bolton et al., (2017). After collection, each applicator was broken off at the tip and placed into a four ml glass vial (Quorpak Clear Borosilicate glass vials with Teflon lined cap). Each vial contained two milliliters (ml) of 100% ethanol and the sample. Each frog was sampled once and released to the site of capture.

We conducted a palatability assay in which *Drosophila* flies were offered a sugar solution containing *H. versicolor* skin secretions (dyed blue using food coloring) and a control sugar solution without frog secretions (dyed red using food coloring). For the red control, we made a stock solution that contained 20 ml of 20% sucrose solution in ultrapure water (RX biosciences), 50% ethanol and 100 μl of red food coloring. For the blue treatment solution we also used a 20% sucrose solution in ultrapure water, 50% ethanol containing skin secretions, but since we only mixed 1ml aliquots, we added only 5µl of blue food coloring (McCormick & Company Inc., Hunt Valley, MD). Previous studies had established that fruit flies do not have preferences for certain dye colors (Meunier et al. 2003; Sellier et al. 2010; Bolton et al., 2017). Nevertheless, we ran five control trials to test whether flies used in this study showed a bias for red or blue food coloring; they did not (t = 1.64, df = 4, P = 0.18). In order to examine if palatability is perceived by flies in a dose-dependent manner, we prepared and tested three concentrations per frog (50%, 25% and 12.5%) (Bolton et al., 2017; Clark 2019).

Flies used in the palatability trials were 2–17 days old and were grown on standard fruit fly media (Josh's frog standard media mix®). For the palatability trials, circa 20 individual *Drosophila melanogaster* (wingless, wild type, MKE Rain Frogs®) were starved for 24 h. Starved flies were placed in a 9 cm petri dish (Fisher brand, 100 mm × 15 mm, sterile, Polystyrene), lined with filter paper (Fisher brand P8, 09-795C) dampened with deionized water (to provide moisture for the fruit flies). For the trial, 10 µl of each solution (Red control solution, Blue *H. versicolor* treatment solution) was pipetted onto double welled microscope cavity slides (1X3 inch, 1.0-1.2mm thick, Eisco®). Similar to the methods of previous studies (Sellier et al., 2011; Devambez et al., 2013; Bolton et al., 2017), flies were allowed to feed on the solutions for 2-4 h in complete darkness (inside a close cabinet), and then euthanized by freezing for circa 40-60 minutes in a -20 C freezer. We ran 5 palatability assays at each of three concentrations (50%. 25%, 12.5%) for 10 individual frogs (= 5 assays x 3 concentrations x 10 frogs = 150 total assays) (Clark, 2019).

We examined feeding preferences of individual *D. melanogaster* by putting them under a dissecting microscope to examine the abdomen for the colors blue, red or purple (both blue and red solutions consumed), as *D. melanogaster* have clear abdomens (Bolton et al., 2017). From the counts of red, blue and purple flies, we calculated a palatability index. The index was derived using the following formula: ((# of blue fruit flies - # of red fruit flies - 0.5 * # of purple fruit flies) / (# of blue fruit flies + # of red fruit flies + # of purple fruit flies) (Bolton et al., 2017).The palatability index is a value that ranges from −1 to +1, where positive and negative numbers represent a palatable and unpalatable solution respectively (Bolton et al., 2017; Clark 2019).

Clay model predation study

We constructed *Hyla versicolor* models using a silicone rubber mold obtained from an average sized (44.5 mm) preserved male frog following methods described in Yeager et al., (2011). We used gray colored plastalina modeling clay (Van-Aken international silver gray #10115) to construct frog models in a posture displaying thighs. Van-Aken clay is non-toxic and has been demonstrated to preserve predator dentition marks (Yeager et al., 2011). We then used yellow and black Sherwin Williams non-toxic animal safe, water based, acrylic paint (6907 Forsythia, and 6258 Tricorn black) to paint two model types: one with a potentially non-aposematic thigh (black paint), and one with a potentially aposematic thigh (black background with yellow spot pattern, replicating the diagnostic black and yellow spotted thigh pattern typical for *H. versicolor*). Prior to paint selection, we used an Ocean Optics spectrometer to sample a large number of color swatches from a Sherwin Williams Paint booklet until we found paint colors that matched the frog colors: Yellow (#131-C4) and Black (#237-C7). Twelve hours before deploying the models into the study site, we scented all models by spraying them with water in which *H. versicolor* had been sitting for 24 hrs (Bocz & Purger, 2021).

We started by deploying 320 clay models (160 spotted = black and yellow, 160 plain = black) along 16, 100 m-transects within known *H. versicolor* hardwood forest habitat. Transects were spaced apart by 25 meters. 20 models were placed per transect, with 10 plain and 10 spotted per transect. Model order on the transect was randomized by pulling pieces of paper out of a hat with either spotted or plain written on them. No more than two of the same model pattern were placed next to each other. This was done to reduce potential predator color bias (Paluh et al., 2014). Each model was placed five meters away from the nearest model. Models were left in the field for 72 hours, and scored for predation attacks every 24 hours. All models were scored by a single observer (JSC IV) to avoid disparities in detection and or scoring bias. Each model was scored by picking it up and inspecting it for predator marks. Models were inspected for bird peck marks, mammal dentition, or unknown attacks. (Bateman et al., 2017; Brodie, 1993; Paluh et al., 2014**)**. If predator marks were discovered, the model was collected and placed into a ziploc bag for further identification, and replaced with a new model of the same type. If models could not be located after a search time of 5 minutes they were scored as missing (Paluh et al., 2014). All models were retrieved from the field after 72 hours. Since depredated models were replaced, we ended up deploying a total of 365 clay models (174 spotted = black and yellow, 191 plain = black).

To survey potential predators visiting the study area, we placed six wildlife trail cameras (Vikeri model 1) attached to trees facing models along three transects, with two cameras per transect, spaced at least 20 meters from each other. An equal number of model types were placed in front of trail cameras.

Statistical analysis

Palatability Assay: We used one-tailed independent T-tests to see if *H. versicolor* secretion was unpalatable to fruit flies for each individual frog (i.e., average palatability indices for all individual frogs were collated to a hypothesized mean of zero; (Bolton et al., 2017; Dyer et al., 2003). To test whether the unpalatability score was dose-dependent, we used a Least Squares Mean model (implementing REML) with palatability score as the test variable, replicate (5 per frog/concentration) and concentration (50%, 25%, 12.5%) as test variables, and frog ID as a random term. For the test of dose-dependence, the concentration term is the one of interest. All tests were performed in JMP Pro version 13.1.0 (JMP® SAS Institute Inc. 2015. Cary, NC, 1989-2019).

Clay Model Predation Study: We used a GLMM with binomial distribution, with model type as the fixed effect and predation (yes/no) as the test factor (implemented in JMP) to test whether a particular model type (plain black or spotted black and yellow) was attacked significantly more frequently. We used an online Chi-square test calculator (https://www.socscistatistics.com/tests/chisquare2/default2.aspx) to test whether a particular model type (plain or spotted) was attacked more frequently by a specific predator type (mammal, bird, other).

Ethical note

All procedures were approved by the Animal Care and Use Committee of the University of Wisconsin Milwaukee (Protocol Number: 21–22#46). All frogs were released unharmed at site of capture.

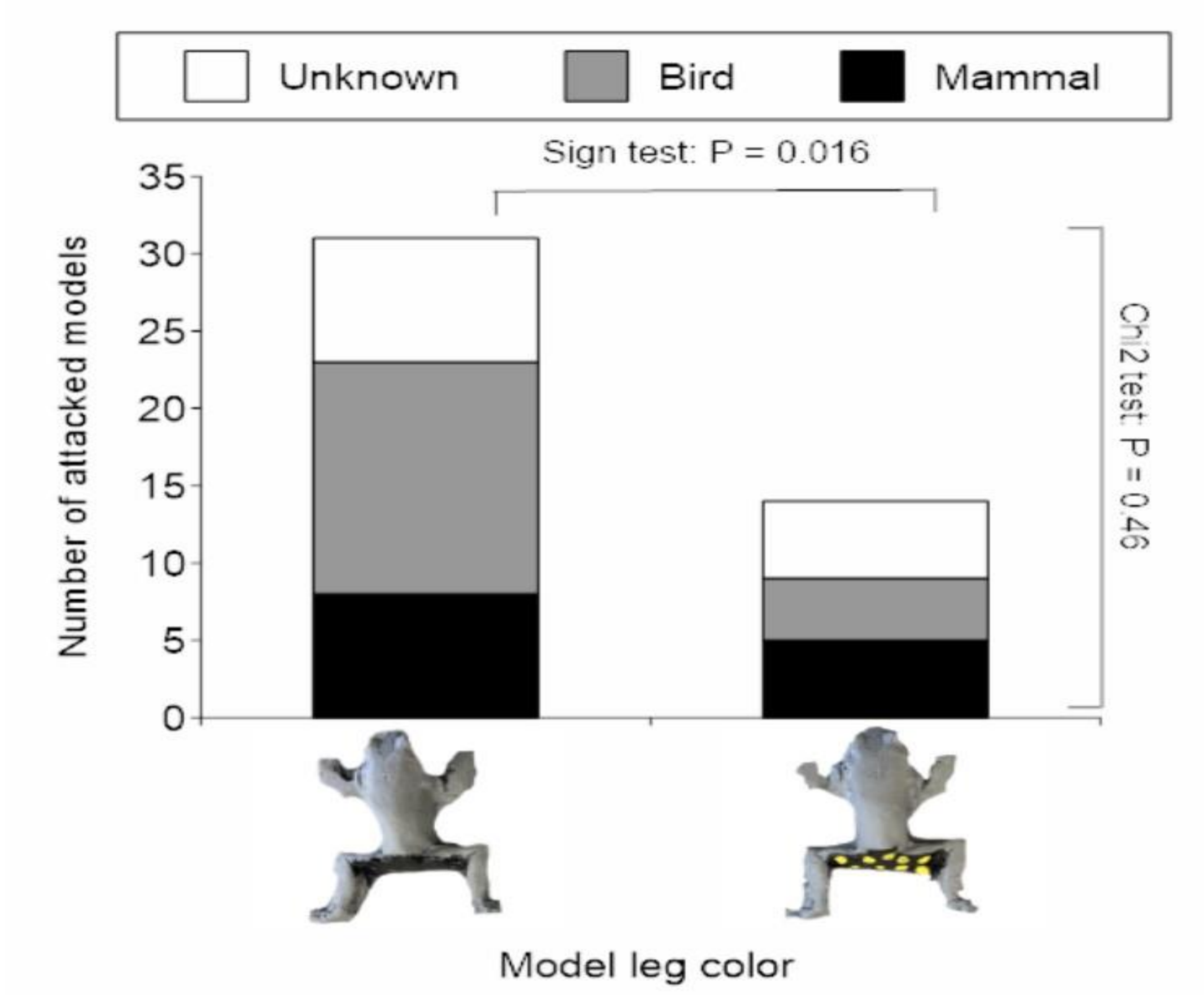

Figure 2. Number of attacked clay models by model color and predator type.

RESULTS

Palatability Assay

Skin secretions were significantly unpalatable to flies at every concentration tested (50%: t=-6.23, df=49, *p*<.0001; 25%: t=-4.34, df=49, *p*<.0001; 12.5%: t=-5.12, df=49, *p*<.0001). Mean ± SD unpalatability scores per concentration were: -0.35±0.40 at 50%, -0.30±0.45 at 25%, and -0.26±0.35 at 12.5%. Although the mean palatability score decreased with increasing dilution, the palatability of skin secretions was not significantly dose dependent (r^2=0.27; Dilution: $F_{1,135}$=1.78, *p*=.18; Replicate: $F_{4,135}$=0.44, *p*=.78; Frog ID [random] 95CI: -0.005% – 0.08%).

Clay Model Predation Study

Of the 365 clay models used in this study, 45 were attacked by predators (i.e., 12.3 % of all models). Of these, 31 (68%) were plain black and 14 (32%) were spotted. Spotted models received significantly fewer attacks than plain models (GLMM: $F_{1,363}$=5.41, *p*=.02; Fig. 2). Putative predators of attacked models were birds, mammals and unknown predators (Fig. 3). Different colored models were not depredated preferentially by specific predator types (chi-square=1.55, df =1, *p*=.46).

Fifteen models were melted by the sun and were unidentifiable as to whether they were depredated (6 spotted black and yellow, 9 plain black). A total of 65 models were missing (not including the 15 melted ones). Of the missing models 30 were spotted black and yellow and 35 were plain black. So as to not inflate our predation rate, we did not score missing models as "presumed depredated". However, if missing models are "presumed depredated" and included in the study, the above finding that spotted models received significantly fewer attacks than plain black models is still marginally significant (GLMM: $F_{1,363}$=3.67, *p*=.056).

Cameras did not capture predation events but confirmed the presence of a variety of potential predators, including wild turkeys *(Meleagris gallopavo*), Northern raccoons (*Procyon lotor*), and White-tailed deer (*Odocoileus virginianus*).

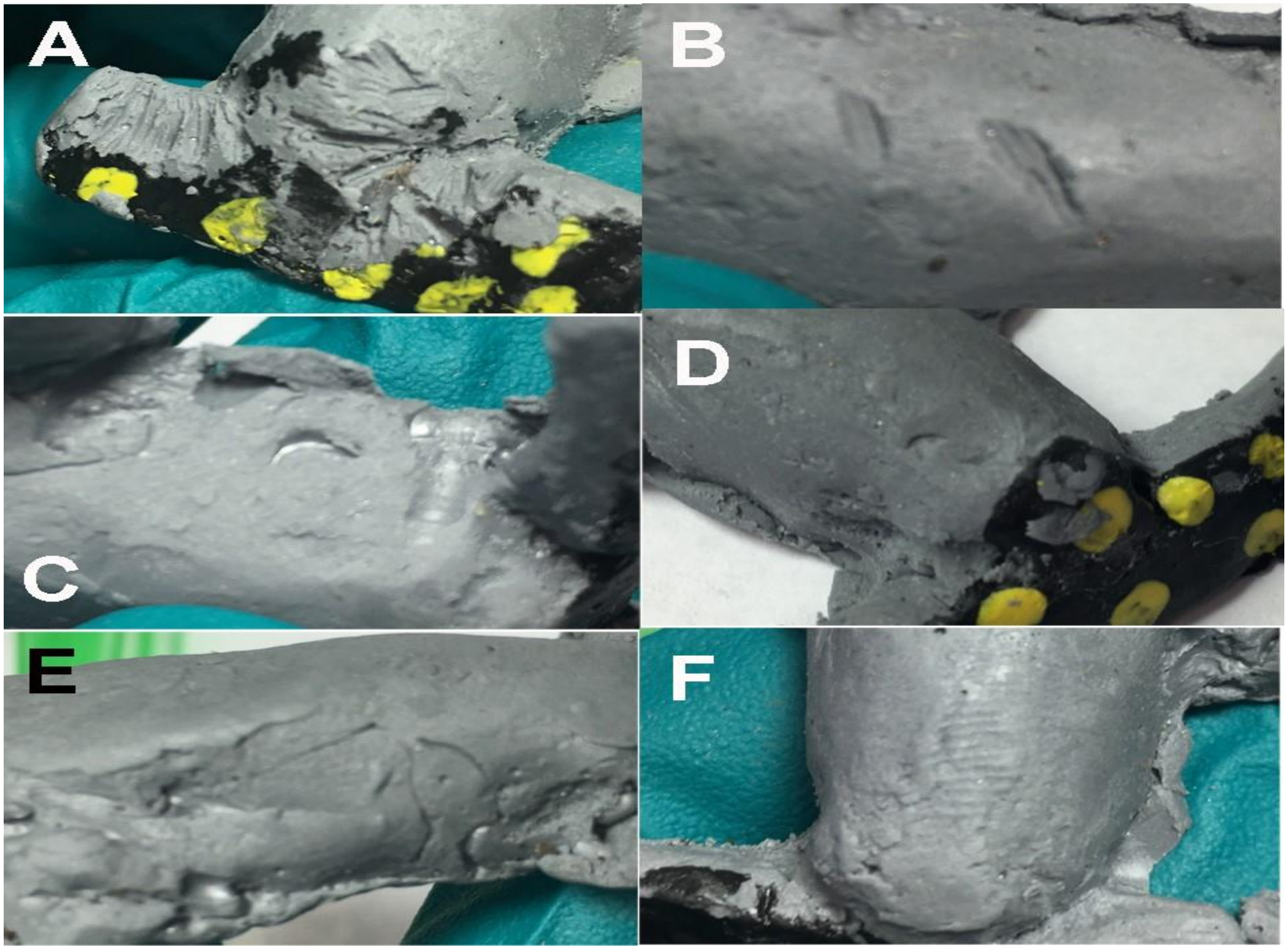

Figure 3. Examples of clay model attacks. (A,B) Dentition imprints suggesting mammal predator; (C,D) Peck marks suggesting avian predator; (E,F) clay models with imprints that suggest predation but could not be classified as either mammal dentition nor bird peck marks.

DISCUSSION

We provide two strong lines of evidence that support the hypothesis that *H. versicolor* is aposematic. First, the palatability assay showed that *H. versicolor* is equipped with skin secretions that renders the frog chemically defended. Second, the clay model study showed that models whose thighs had been painted with contrasting spotted pattern incurred fewer predation attacks than those with plain black thighs. Together, this suggests that *H. versicolor* is aposematic and uses the conspicuous spotted black and yellow thigh color to advertise a noxious taste.

Other studies investigating palatability of potentially aposematic frogs using comparable methods document between-species differences in noxiousness. Clark, (2019) found higher unpalatability scores for the Neotropical Red-eyed Tree frog (*A. callidryas*) (50% Dilution: -0.75 vs -0.35 in *H. versicolor*), another species whose chemical defense is likely derived from peptide-based secretions. Studies with dendrobatid poison dart frogs show very high unpalatability scores at much higher dilutions, demonstrating the potency of their alkaloid skin secretions (Bolton et al., 2017).

Although the palatability scores of *H. versicolor* skin secretions decreased slightly with increasing dilution, analysis did not return a significant dose dependence. On average, frogs were significantly unpalatable even at the lowest concentration tested. A lack of dose-dependence suggests that the predator experiences the full defensive effect of the skin secretion at first contact, and longer handling times do not significantly increase it. By contrast, other studies have document dose-dependence (Bolton et al., 2017; Clark, 2019), but in each case the effect size was of small magnitude ($r^2 = 0.21$ and $r^2 = 0.13$, respectively)

We show that the spotted black and yellow thigh pattern of *H. versicolor* acts as an aposematic warning color that decreases predatory attacks. The majority of predation attempts were a result of avian attacks followed by mammalian attacks. This pattern of primarily avian attack is largely consistent throughout clay model studies (Saporito et al., 2007; Bateman et al., 2017; Nordberg et al., 2018;), and may be due to birds being primarily visual predators, whereas other predators rely on chemoreception (Fernandez-Juricic et al., 2004). Quantitative studies on the predators of *H. versicolor* is lacking, though a variety of birds, meso-mammals, snakes, and arthropods have been observed to prey on adult *H. versicolor* (Carpenter, 1952; Dodd, 2013).

Our camera traps captured a variety of potential predators, the most prevalent being the White-tailed deer (*O. virginianus)*. White tailed deer are primarily generalist herbivores, but they and other cervids have been documented to feed on live birds, bird eggs, nestling birds, fish and even carcasses of conspecifics (Burgess, 1924; Furness et al., 1988;Ellis-Felege et al., 2008). Furthermore, across the United States, including our study site, white tailed deer overpopulation has become a large issue for wildlife managers, leading to overgrazing of vegetation, shortages in food, and increased transmission of disease (Decalesta & Stout, 1997; McShea, 2012). For these reasons, we consider white-tailed deer a potential predator. Additionally, we posit that the missing models in our study could have potentially been depredated and carried away by deer, as they are the largest species known to inhabit our site. Additional predators documented by the cameras include the Wild turkey *(M. gallopavo*) and the Northern Raccoon (*P. lotor*) Both species have been reported to consume amphibians and the Northern Raccoon in particular has been documented to consume *H. versicolor* and is a voracious amphibian predator **(**Hamilton, 1951; Schoonover & Marshall, 1951).

Over 30 studies have used artificial replica to study predation/aposematism in amphibians (Salvidio et al., 2019; Caspers et al., 2020). Not surprisingly, most focus on diurnal, conspicuously colored dart poison frogs (reviewed in Salvidio et al., 2019). Ours is the first study focusing on nocturnal treefrogs. With 12.3 % of model attacked, we saw a similar rate of predation relative to other clay model studies (mean 13%; reviewed Salvidio et al., 2019), documenting that the method is valid for nocturnal species and as well as for species that are not permanently conspicuous, but carry conspicuous patches in normally hidden body locations. A recent study by Loeffler-Henry et al., (2023) suggests that “the evolutionary transition from camouflage to aposematism is rarely direct but tends to involve an intermediary stage where cryptic species evolve conspicuous coloration in hidden body areas that are only exposed to would-be predators on encounter“. Concealed areas of bright coloration on ventral shanks or hindlimbs are frequently found in the family Hylidae, and our study is in line with the hypothesis that many species commonly considered cryptic, like treefrogs, might actually be chemically defended and employ the joint strategy of crypsis when at rest and aposematism when active.

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Chapter two: Hidden thigh color is indicative of unpalatability in North American Hylid treefrogs.

INTRODUCTION:

Colored appendages serve diverse functions across nature. Many animals utilize colored appendages for the luring and capture of prey (Glorioso et al., 2023), sexual attraction of mates, conspecific signaling (Maan and Cummings, 2009; Höbel et al., 2022) and thermoregulation (Hoppe, 1979). Animals have also evolved diverse colors to avoid, discourage and stop the sequence of predation (Bateman et al., 2014; Ruxton et al., 2019). A variety of taxa discourage predatory attacks by displaying conspicuous aposematic colors (Cott, 1940). Honest aposematic colors are hypothesized to advertise a secondary chemical or morphological defense and intimidate potential predators (Wallace, 1867; Edmunds, 1974).

Explaining the evolutionary origin of aposematism has posed a major problem (Harvey et al., 1982; Speed and Ruxton, 2005; Loeffler-henry et al., 2023). If cryptic organisms evolved conspicuous coloration with secondary defenses, then initially, mutants would not be recognized as aposematic by naïve predators, would be detected with greater frequency versus cryptic morphs, and thus would be selected against (Speed and Ruxton, 2014; Caro and Ruxton, 2019; Loeffler-henry et al., 2023). A variety of solutions to this problem have been proposed with limited empirical support. These include heightened predator memory or cognition (Yachi and Higashi, 1998), predator wariness for novel dietary items (Coppinger, 1970, Thomas et al., 2003) and previously evolved conspicuous behavior (Merilaita and Tullberg, 2005). In each of the above solutions, authors propose a direct transition from a totally cryptic organism to one that is fully conspicuous and defended.

Recent work from Loeffler-henry et al., 2023, proposes that the evolutionary transition from camouflage to aposematism is not direct but involves an intermediary stage where cryptic species evolve conspicuous coloration in hidden body areas that are only exposed to would-be predators on encounter. Under this hypothesis, taxa with hidden warning colors are detected by predators at the same frequency as cryptic taxa, due to only unsheathing aposematic colors upon predatory attack. Therefore, these taxa would not be selected against, and would deploy chemical defense following the display of color, thus immediately associating color with defense to naïve predators (Loeffler-henry et al., 2023).

Working with the Eastern Gray Tree Frog (*Hyla versicolor*), Cannizzaro and Höbel (2023) recently demonstrated that a species with a cryptic dorsum and conspicuous thighs is unpalatable, and the hidden conspicuous pattern lowers predation rate. Still, experimental evidence of this evolutionary “steppingstone” is scant and additional studies investigating the phylogenetic relationship and presence of chemical defense in other species with conspicuous coloration in hidden body areas are needed (Loeffler-henry et al., 2023).

Amphibians are an excellent class to study the evolution of aposematism. Many Anurans, Caudates, and Gymnophionans are chemically defended, and a variety of taxa display cryptic or conspicuous colors and unique defense behaviors (Noble, 1931; Wells, 2007). This is especially prevalent within the Frogs (Wells, 2007). In a review of anuran anti-predator mechanisms, Ferreria et al., 2019 noted that of 650 species reviewed, 6% of species showed “exposed” aposematic coloration while 38% showed “hidden” aposematism or a cryptic dorsum with conspicuous thigh. Ancestral state estimates for coloration and chemical defense in frogs and salamanders suggest that cryptic coloration is the likely ancestral color state, but that the evidence of whether the basal state was chemically defended or not was equivocal (Loeffler-henry et al., 2023). It is thus not clear whether chemical defense evolves first and is followed by the evolution of conspicuous aposematic displays, or whether the partially hidden conspicuous color state could evolve in the absence of chemical defense.

In the present study we sampled nine Nearctic Hylid Tree Frog species that share the cryptic dorsum but vary in their thigh color from cryptic to conspicuous (plain yellow or white/yellow and black spotted patterns) (Figure 4). We collected their skin secretions and conducted palatability assays with *Drosophila* fruit flies (Masek and Scott, 2010; Bolton et al, 2017). We hypothesized that hidden thigh colors are aposematic. This hypothesis makes the predictions that species with conspicuous thigh color (spot or yellow) have unpalatable skin secretions to model predators, whereas species lacking conspicuous thigh color (cryptic) are palatable to model predators.

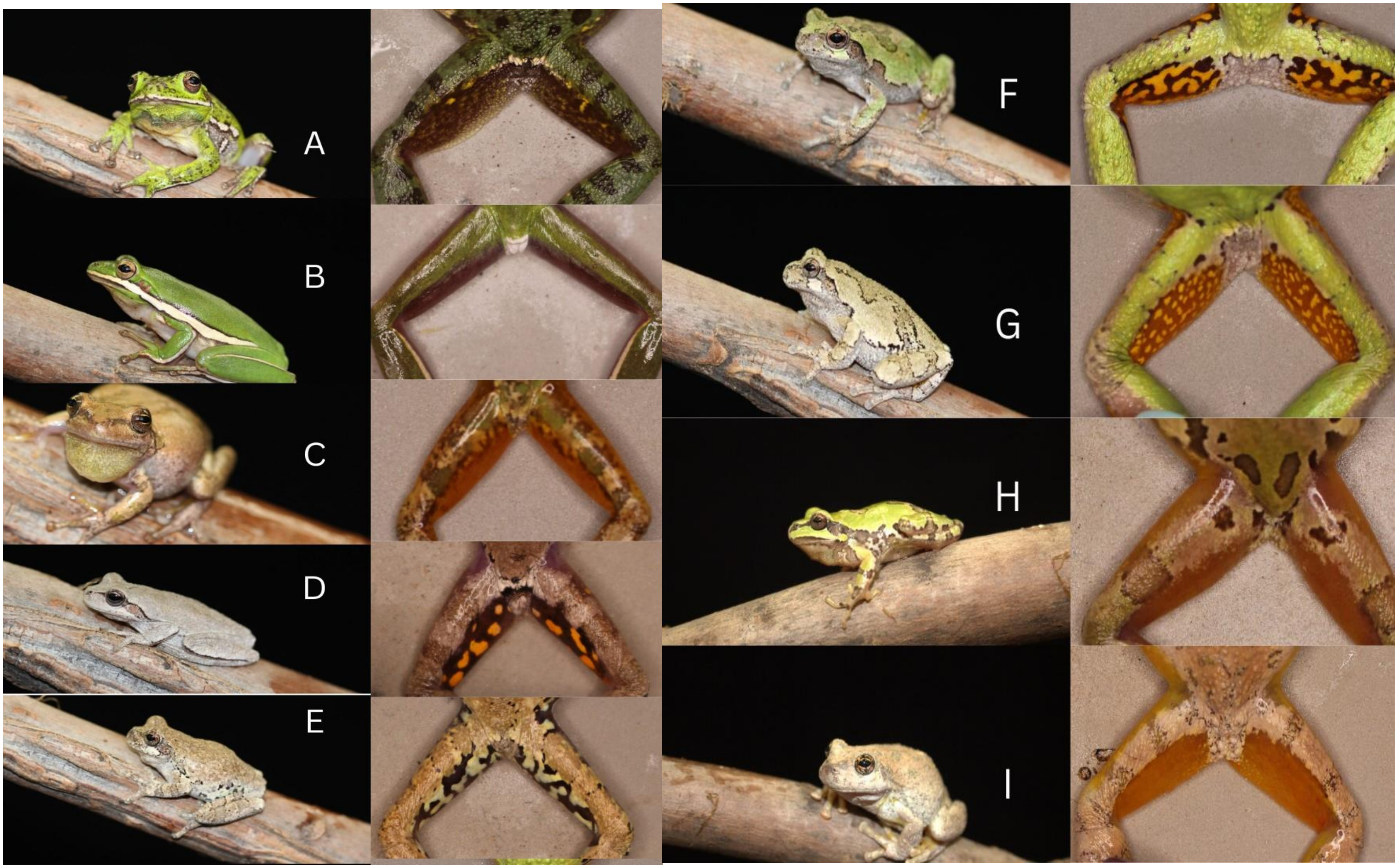

Figure 4. Thigh color of North American Hylid species sampled for the present study. A: *Hyla gratiosa* (Barking Treefrog), B: *H. cinerea* (Green Tree Frog), C: *H. squirella* (Squirrel Treefrog), D: *H. femoralis* (Pine Woods Treefrog), E: *H. avivoca* (Bird-voiced Treefrog), F: *H. versicolor* (Eastern Gray Treefrog),G: *H. chrysoscelis* (Cope’s Gray Treefrog), H: *H. wrightorum* (Wrights Treefrog), I: *H. arenicolor (*Canyon Treefrog*)*.

METHODS

Study species and Frog Collection

Nearctic Hylids are arboreal anurans, that are widely distributed across the North American Continent. They occupy habitats as diverse as desert canyons, cypress swamps and hardwood forests. As well exhibit marked size (21-70 mm) and color variation between species (Dodd, 2023). Ten species are known to inhabit the continental United States, but one is threatened and was thus unattainable for our sample.

We collected three individuals each of nine species of North American *Hyla* treefrogs, for a total of 27 frogs: *Hyla versicolor* (Eastern Gray Treefrog), *H. chrysoscelis* (Cope's Gray Treefrog), *H. cinerea* (Green Treefrog), *H. squirella* (Squirrel Treefrog), *H. femoralis* (Pine Woods Treefrog), *H. avivoca* (Bird-voiced Treefrog), *H. gratiosa* (Barking Treefrog), *H. arenicolor* (Canyon Treefrog) and *H. wrightorum* (Wrights Treefrog) (Table 1). Frogs were transported to our lab at the University of Wisconsin-Milwaukee for body size measurements, extraction of skin secretions and scoring of thigh color pattern.

Thigh coloration

Thigh coloration was scored in three categories: 1=cryptic, 2=conspicuous yellow, and 3= conspicuous spotted (Table 1).

Extraction of skin secretions

We collected skin secretions using a Transcutaneous Amphibian Stimulator (TAS, Bolton et al., 2017). The TAS was applied to the ventral and dorsal side of each frog in a downward sweeping motion for 30 seconds. We collected the secretion using a sterile six-inch cotton tipped applicator (Fisher brand REF 22363162, Lot 20211028). Post collection, each applicator was broken off at the tip and placed into a four ml glass vial (Quorpak Clear Borosilicate glass vials with Teflon lined cap). The vial contained two milliliters (ml) of 100% ethanol and the sample.

Palatability assay

To test the palatability of the different frog species, we conducted two-choice feeding assays. *Drosophila* fruit flies were allowed to feed on two different sugar solutions. One that contained *Hylid* skin secretion and one that did not. Blue food coloring was added to the solution containing frog skin secretions and red food color was added to the plain control sugar solution. For the red control, we made a stock solution that contained 20 ml of 20% sucrose solution in ultrapure water (RX biosciences), 50% ethanol and 100 µl of red food coloring. For the blue treatment solution we also used a 20% sucrose solution in ultrapure water, 50% ethanol containing skin secretions, but since we only mixed 1ml aliquots, we added only 5µl of blue food coloring (McCormick & Company Inc., Hunt Valley, MD) (Bolton et al., 2017; Clark 2019, Cannizzaro and Höbel, 2023). *Drosophila* fly abdomens are transparent, which allowed us to determine which colored solution they fed on during each trial: red control, blue treatment, or a mix of both solutions that resulted in purple abdomen color.

Flies used in the palatability trials were 2–17 days old and were grown on standard fruit fly media (Josh’s frog standard media mix®). For each palatability trial, circa 20 individual Drosophila *melanogaster* (wingless, wild type, MKE Rain Frogs®) were starved for 24 h. Starved flies were placed in a 9 cm petri dish (Fisher brand, 100 mm × 15 mm, sterile, Polystyrene), lined with filter paper (Fisher brand P8, 09-795C) dampened with deionized water (to provide moisture for the fruit flies). For the trial, 10 µl of each solution (Red control solution, Blue *Hylid* treatment solution) was pipetted onto double welled microscope cavity slides (1X3 inch, 1.0-1.2mm thick, Eisco®). Similar to the methods of previous studies (Bolton et al., 2017; Cannizzaro and Höbel, 2023), flies were allowed to feed on the solutions for 2-4 h in the dark, and then euthanized by freezing for circa 40-60 minutes in a -20 C freezer. We ran five trials per individual, for a total of 135 trials (9 species x 3 individuals x 5 trials).

We examined feeding preferences of *D. melanogaster* by putting them under a dissecting microscope to examine the abdomen for the colors blue (treatment), red (control) or purple (both blue and red solutions consumed) (Bolton et al., 2017, Cannizzaro and Höbel, 2023). From the counts of red, blue and purple flies, we calculated a palatability index for each individual. The index was derived using the following formula: ((# of blue fruit flies - # of red fruit flies - 0.5 * # of purple fruit flies) / (# of blue fruit flies + # of red fruit flies + # of purple fruit flies) (Bolton et al., 2017).The palatability index is a value that ranges from −1 to +1, where positive and negative numbers represent a palatable and unpalatable solution, respectively (Bolton et al., 2017; Clark, 2019).

Statistical Analysis

We used one-tailed independent *t*-tests to see if the skin secretions of the different frog species were significantly unpalatable to fruit flies (palatability indices for the 15 fly trials per species were collated to a hypothesized mean of zero) (Bolton et al., 2017; Dyer et al., 2003).

We used Standard Least Squares Models with mean palatability index as the test variable, and color pattern (1=cryptic, 2=yellow and 3=spotted) and species nested in color pattern as the predictor variables to test whether thigh coloration and unpalatability were associated. Then we ran post-hoc analysis (Tukey HSD) to compare whether yellow and spotted pattern were different from each other and from the cryptic color.

The frog species included in the study vary considerably in size and body shape. To account for resulting differences in skin area over which the samples were collected, we took a representative picture of each species (dorsal side up) and measured the surface area (in mm^2) of the torso using ImageJ software (Schneider et al., 2012). We obtained size adjusted palatability scores by dividing the raw scores by the body area.

Table 1: Thigh color description and collection site of *Hyla* treefrogs included in the study. Thigh colors are scored in three categories: 1=crypic, 2-conspicuous yellow, and 3= conspicuous spotted.

Species	**Description of thigh coloration**	**Color score**	**Collection site**
Hyla arenicolor	uniform yellow to orange	2	Gila Co., Arizona
Hyla avivoca	spotted; dark background with large white spots	3	Wheeler Co., Georgia
Hyla chrysoscelis	spotted; dark background with small yellow spots	3	Payne Co., Oklahoma
Hyla cinerea	uniform dark gray	1	Ben Hill Co., Georgia
Hyla femoralis	spotted; dark background with large yellow spots	3	Telfair Co., Georgia
Hyla gratiosa	uniform dark gray	1	Jenkins Co., Georgia
Hyla squirella	uniform yellow	2	Ben Hill Co., Georgia (2) Chatham Co., Georgia (1)
Hyla versicolor	spotted; dark background with yellow spots	3	Ozaukee Co., Wisconsin
Hyla wrightorum	uniform yellow	2	Coconino Co., Arizona

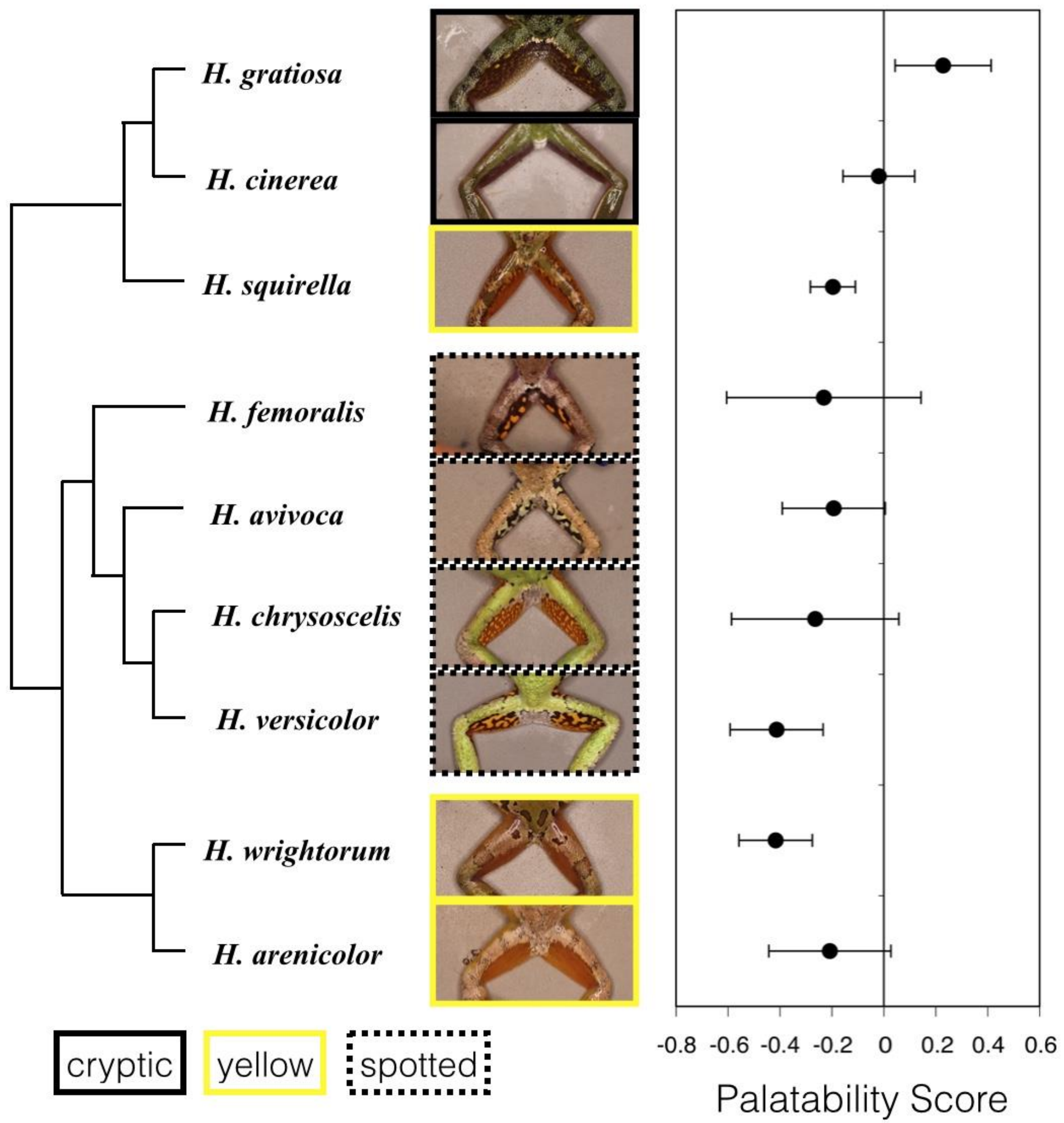

Figure 5. Thigh color and unpalatability scores for the nine tested species. Phylogeny from Wiens et al 2010.

RESULTS Palatability ranged from neutral (*H. gratiosa*, *H. cinerea*) to highly unpalatable (*H. versicolor, H. wrightorum*) (Figure 5; Table 2). Unpalatability was associated with thigh color: Species with yellow and spotted thigh were mostly unpalatable, but those with cryptic thighs were not (Fig. 2; Table 3).

DISCUSSION

Elucidating the evolutionary origin of aposematism has posed a major challenge for researchers (Speed and Ruxton, 2005). How would this tactic evolve if initial mutants are not recognized and thus selected against? Herein we provide robust support for a solution to this problem**.** The expression of hidden aposematic colors likely acts as an evolutionary "stepping stone" between completely conspicuous defended and cryptic undefended taxa (Loeffler-henry et al., 2023). The ability to alternate between a cryptic and aposematic state is beneficial, as taxa with hidden warning colors are detected by predators at the same frequency as cryptic taxa, due to only unsheathing aposematic colors upon attack. Our hypothesis that hidden aposematic frogs are unpalatable to predators was supported. Thigh color was supported as a predictor of palatability. Six out of seven species with thigh color (2,3) were significantly unpalatable. Whereas the cryptic species scored neutral or palatable on the palatability index.

Table 2: Results of one-tailed independent *t*-tests examining whether skin secretions are significantly unpalatable. Significant tests are set in bold. DF = 15 for all tests.

	t	P
Hyla arenicolor	-1.81	**0.046**
Hyla avivoca	-2.24	**0.021**
Hyla chrysoscelis	-2.71	**0.009**
Hyla cinerea	-0.34	0.37
Hyla femoralis	-1.95	**0.035**
Hyla gratiosa	1.34	0.9
Hyla squirella	-1.55	0.07
Hyla wrightorum	-6.97	**<0.0001**
Hyla versicolor	-6.16	**<0.0001**

Table 3: Results of standard least squares models testing the association of thigh coloration with unpalatability

	Raw palatability score				Size-adjusted palatability score		
Source	df	F	P		df	F	P
Pattern	2	8.56	**0.002**		2	4.14	**0.033**
Species [Pattern]	6	1.14	0.38		6	0.63	0.70
Tukey HSD	Cryptic A LSM = 0.104 Yellow B LSM = -0.274 Spotted B LSM = -0.275				Cryptic A LSM = 0.00006 Yellow A B LSM = -0.00055 Spotted B LSM = -0.00062		

Loeffler-henry et al., 2023, found evidence for the ancestral state for salamanders and frogs as being cryptic. Though whether this initial state was chemically defended was not clear. So it is not known whether color first evolved and defense second or vice versa. In the present study, one species with yellow thigh color, the Squirrel tree frog (*H. squirella*) was not significantly unpalatable. *H. squirella* is closely related to the two cryptic species and nested within the cryptic clade. We suggest that this species provides evidence that color could have evolved first, and chemical defense second in aposematic amphibians.

Incorporating this evidence, we observed marked variation of palatability between species even with the same thigh pattern. For example, among conspicuous species (2,3), The Gray Tree Frog (*H. versicolor*) and Wright's Treefrog (*H. wrightorum*) were highly unpalatable ($P<0.0001$), whereas the Canyon Treefrog (*H. arenicolor*) was not as potent ($P<0.046$). Undoubtedly, predator presence, density and within population variation further influence the toxicity of each species. Hague et al., 2016 examined Co-evolutionary reciprocal selection between *Taricha* newts and predatory *Thamnophis* garter snakes. They found that *Taricha* that occurred in sympatry with known *Thamnophis* predators exhibited heightened levels of defensive tetrodotoxin (TTX). Conversely, *Thamnophis* exhibited heightened levels of resistance to TTX. In regions where *Thamnophis* was absent, *Taricha* exhibited lower levels of TTX (Hague et al. 2016).

Evolution from an undefended cryptic to conspicuously aposematic state exists likely not in a unidirectional, but plastic gradient. This gradient is largely dictated by selective pressure from predators and the environment. For example, in evolutionary arms races, where predators have developed heightened resistance and recognition to prey toxins, it may be selective for conspicuous aposemes to revert back to hidden aposematism, in order to lower detection frequency and eventual resistance of predators. Furthermore, anecdotal evidence has suggested predators can discriminate poisonous body regions on aposematic prey. For example, Laciak, 2022 documented a predation event where over eight individual Fire Salamander (*Salamandra salamandra*) heads were found in an unknown raptor nest. All edible body parts were consumed, with only the poisonous head regions left uneaten (Laciak, 2022). In reverting back to hidden aposematism, aposemes are lowering detection probability and therefore the ability for a predator to learn how to consume undefended regions of the body.

Our results illustrate that North American hylids with hidden aposematic thigh color are unpalatable to predators, and that the transitional state of hidden aposematism is supported as a solution to the problem on the origin of aposematism. Future studies investigating selective pressure, habitat and predator density on the expression of this strategy on a population level are warranted.

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