RESEARCH ARTICLE

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

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Abstract

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. Many species, especially treefrogs of the family Hylidae, 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. We investigated whether the conspicuous black and yellow spotted pattern on the thighs of Eastern Gray Treefrogs (*Hyla versicolor*) serve as an aposematic signal. We conducted a palatability study to test whether Gray Treefrog skin secretions are unpalatable, 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. Our findings support the hypothesis that hidden color patches act as aposematic signals in Gray Treefrogs and suggest that more species benefit from aposematic coloration than currently appreciated.

KEYWORDS
clay model study, Hylidae, palatability, peptide, predator defense, skin secretion

1 | 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 III, 1993; Nekaris et al., 2019; Wells, 2007). 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 toward 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 (Dodd, 2013; Toledo & Jared, 1995; Wells, 2007). 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 (Bolton et al., 2017; Wells, 2007). Amphibians also have a range of different color patterns. 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 & Brown, 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 & Brown, 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 *H. versicolor* is an aposematic signal (Figure 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 (Brodie III, 1993; Madsen, 1987).
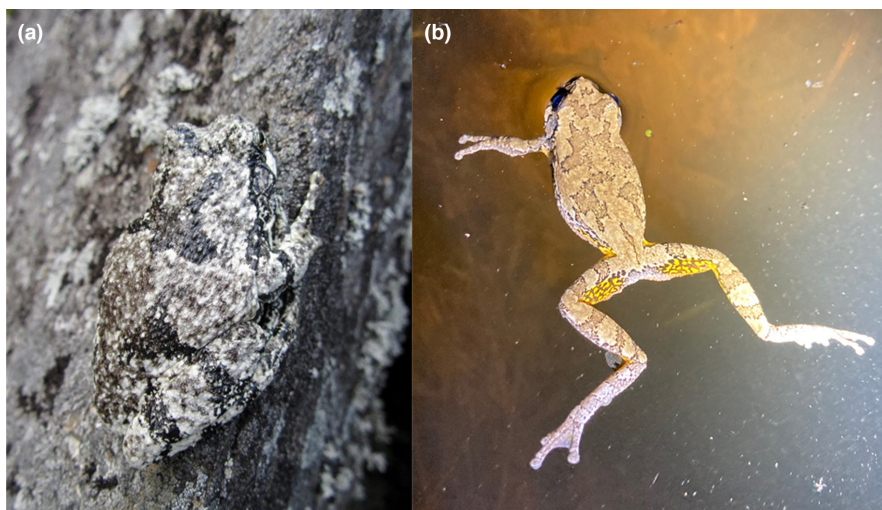

FIGURE 1 (a) Dorsal crypsis and (b) spotted black and yellow thigh coloration of *Hyla versicolor*.

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2 | METHODS

2.1 | 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 laboratory for sampling. The clay model predation study took place adjacent to known *H. versicolor* breeding habitat at (UWM-FS).

2.2 | Palatability assay

We collected skin secretions using a Transcutaneous Amphibian Stimulator (TAS, Bolton et al., 2017; Grant & Land, 2002). The TAS was applied to the dorsum and ventral side of each frog for 30 s. 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 4-mL glass vial (Quorpak Clear Borosilicate glass vials with Teflon lined cap). Each vial contained 2 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 1 mL aliquots, we added only 5 μL of blue food coloring (McCormick & Company Inc.). Previous studies had established that fruit flies do not have preferences for certain dye colors (Bolton et al., 2017; Meunier et al., 2003; Sellier et al., 2011). 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 $= 4$, $p = .18$). 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 *D. 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 (1 × 3 inch, 1.0–1.2 mm thick, Eisco®). Similar to the methods of previous studies (Bolton et al., 2017; Devambez et al., 2013; Sellier et al., 2011), 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 min in a −20 C freezer. We ran five palatability assays at each of three concentrations (50%, 25%, 12.5%) for 10 individual frogs (=5 assays × 3 concentrations × 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).

2.3 | Clay model predation study

We constructed *H. 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 h (Bocz et al., 2022).

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 m. Twenty 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 5 m away from the nearest model. Models were left in the field for 72 h, and scored for predation attacks every 24 h. 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 III, 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 min they were scored as missing (Paluh et al., 2014). All models were retrieved from the field after 72 h. 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 m from each other. An equal number of model types were placed in front of trail cameras.

2.4 | Statistical analysis

2.4.1 | 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).

2.4.2 | 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-squared 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).

2.5 | 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.

3 | RESULTS

3.1 | 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] 95% CI: −0.005 to 0.08).

3.2 | 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: $F_{1,363} = 5.41$, $p = .02$; Figure 2). Putative predators of attacked models were birds, mammals, and unknown predators (Figure 3). Different colored models were not depredated preferentially by specific predator types (chi-squared = 1.55, df = 1, $p = .46$).

Fifteen models were melted by the sun and were unidentifiable as to whether they were depredated (six spotted black and yellow, nine plain black). A total of 65 models were missing (not including the 15 melted ones). Of the missing models 30 were spotted black
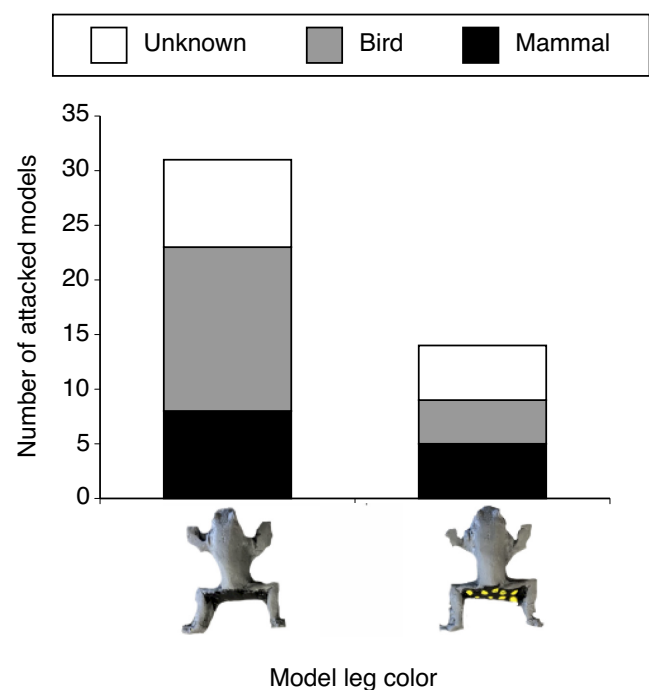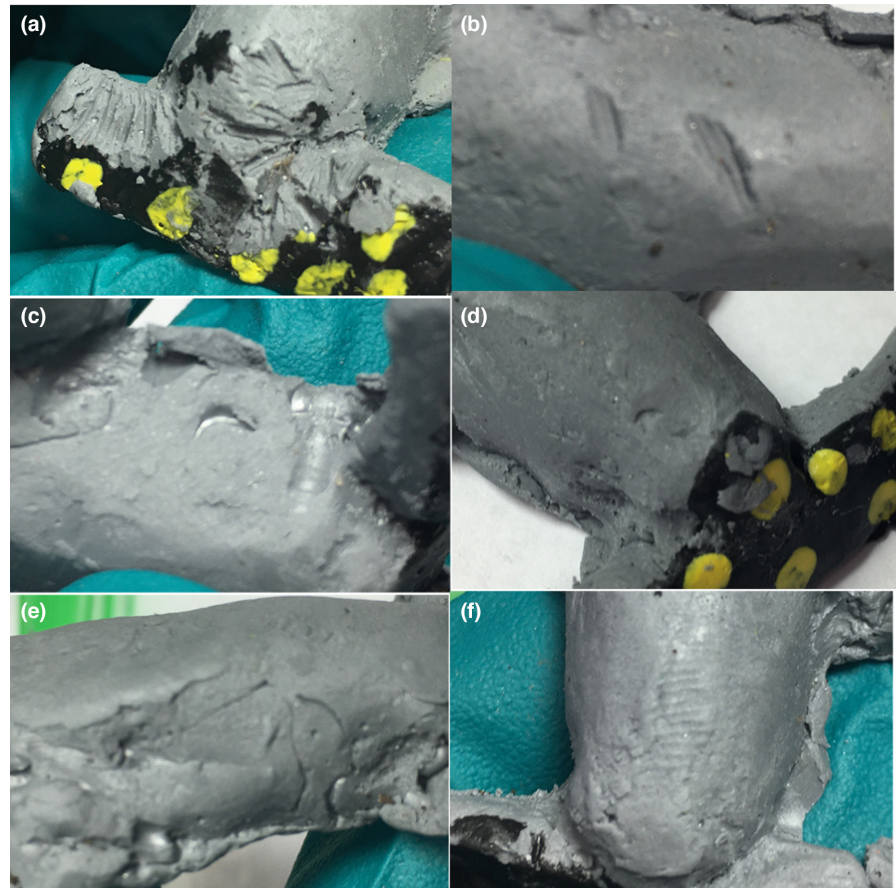

FIGURE 2 Number of attacked clay models by model color and predator type.

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.

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*).

4 | 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. In 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 (Bateman et al., [2017]; Nordberg et al., [2018]; Saporito et al., [2007]), and may be due to birds being primarily visual predators, whereas other predators rely on chemoreception (Fernández-Juricic et al., [2004]). Quantitative studies on the predators of *H. versicolor* are lacking, although 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; Ellis-Felege et al., 2008; Furness, 1988). 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 replicas to study predation/aposematism in amphibians (Caspers et al., 2020; Salvidio et al., 2019). 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.

AUTHOR CONTRIBUTIONS

Joseph S. Cannizzaro: Conceptualization; writing – original draft; writing – review and editing; investigation. **Gerlinde Höbel:** Conceptualization; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data associated with this manuscript are included as supplementary files associated with this manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.