Color polymorphism and variation in microhabitat selection: a defensive strategy in males of *Boana pulchella* (Anura: Hylidae)?

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ABSTRACT. Anurans vary in body coloration and frequently exhibit color polymorphism, with selection by predation pressure favoring more cryptic animals. Spatial differentiation may also favor color polymorphism in some species, since cryptic morphotypes can use substrates that better match their coloration. *Boana pulchella* is a polymorphic species with green and brown morphotypes, but its polymorphism remains poorly understood. The aim of the present study was to determine the functionality of color polymorphism in *B. pulchella* as a defense strategy. Artificial green and brown clay models were placed in environments with green and brown background to determine if the two morphotypes of *B. pulchella* experience different frequencies of attacks. We expected that more conspicuous morphs are attacked more frequently. The attack rate, which suggests predation intent, on the models was 9.57%. The marks observed in the artificial models were left by both mammals and birds. The number of damaged models did not significantly differ between the different background environments. The results indicate that *B. pulchella* morphotypes are equally cryptic to predators in the studied environments. In conclusion, probably color polymorphism in *B. pulchella* is not operating as a defensive strategy, and other selective forces may be acting in the maintenance of color polymorphism in this species.

Keywords: Cryptic coloration; artificial models; heterogeneous habitat; amphibians.

Received on April 21, 2021.
Accepted on March 29, 2022.

Introduction

Anurans exhibit a remarkable variety of body coloration patterns. Camouflage as a defense strategy is widespread in the group (Rojas, 2017) because of high predation rates by several vertebrate and invertebrate animals (Toledo, Ribeiro, & Haddad, 2007). Predation favors colorations with protective roles for the individual, such as cryptic colorations that hinder detection via background color-matching (Stevens & Merilaita, 2011). Cryptic coloration can be effective alone or combined with some behavioral strategy (Rojas, 2017). For example, there are indication that individuals select substrates with color similar to their body color (Morey, 1990; Wente & Phillips, 2005). Additionally, some species are able to change their body color to correspond to the substrate (Polo-Cavia, Oliveira, Villa, & Márquez, 2016). Most notably, in polymorphic species with distinct morphs, individuals may select background substrates matching their color morph, thus making their detection harder (Rojas, 2017; Toledo & Haddad, 2009).

Color polymorphism is abundant among anurans and occur in a great number of species. It can be manifested in several ways, from variation in the whole-body color to variation in particular patterns (Hoffman & Blouin, 2000). The occurrence of polymorphism can vary between populations or occur in a generalized manner across populations and different ontogenetic stages or between the sexes (Toledo & Haddad, 2009). Although there are aposematic polymorphic anuran species (Hegna, Saporito, Gerow, & Donnelly, 2011), cryptic polymorphic species are more widespread (Rojas, 2017). The maintenance of polymorphism can probably be related to selective pressures by predation (Morey, 1990; Rojas, 2017), but most evidence for this remains correlational (Rojas, 2017). Furthermore, ecological drivers of polymorphism in anuran species remain poorly understood (Hoffman & Blouin, 2000).

Spatial differentiation may influence the evolution of coloration in polymorphic species, once cryptic polymorphic animals can use substrates that match their coloration, thus making them more cryptic to predators (Rojas, 2017; Toledo & Haddad, 2009). For example, differential selection of substrates by different
morphotypes (green and brown) of *Pseudacris regilla* was found to be an adaptive response to visually oriented predators (Morey, 1990). Moreover, substrates used by anurans vary in the amount of vegetation, from exposed soil to densely vegetated areas, and can even include the surface of water bodies (Navas, 1996). These background environments frequently vary widely in color or pattern, both spatially and temporally, and some species are known to exhibit seasonal variation in the frequency of color morphs that seems to match changes in backgrounds (Rojas, 2017). Nonetheless, studies evaluating the importance of color polymorphism as a defense strategy in anurans, especially using artificial models, have been strongly tied to aposome variants (Hegna, Saporito, & Donnelly, 2013; Hegna et al., 2011; Saporito, Zuercher, Roberts, Gerow, & Donnelly, 2007), and have neglected cryptic polymorphisms.

*B. pulchella* (Duméril & Bibron, 1841) is a cryptic polymorphic tree frog of the family Hylidae, with bright green and light brown morphotypes (Borges-Martins, Colombo, Zank, Becker, & Melo, 2007; Kwet, Lingnau, & Di-Bernardo, 2010). Individuals of this species are known to call from both exposed soil and low vegetation (Kwet et al., 2010) in grassland ecosystems and meadows in the Atlantic Forest (Santos, Colombo, Avila, Oliveira, & Tozetti, 2016). There are reports of individuals preyed upon by a variety of vertebrates, such as birds and rodents, among others (Boelter, Kaefer, Both, & Cecchin, 2012; Ducommun, Quiroga, Beltzer, & Schnack, 2009; Oliveira, 2008; Soibelzon, Daniele, Negrete, Carlini, & Plischuk, 2007). Furthermore, *B. pulchella* is known to exhibit some degree of habitat selection for calling sites during reproduction events, which is considered a defense strategy (Santos et al., 2016). Even though species polymorphism occurs within populations (Kwet et al., 2010), little is known about selective pressures and genetic basis, and whether it is related to phenotypic plasticity.

A better understanding of the importance of color polymorphism as a defense strategy in *B. pulchella* can help explain the emergence of morphotypes and their function, and contribute to a better understanding of polymorphism in general. Our aim, therefore, was to test the functionality of color polymorphism in *B. pulchella* as a defense strategy. We hypothesized that the chances of an individual being preyed upon are directly related to its conspicuousness to the background environment. We used artificial clay models, representing the green and brown morphs of *B. pulchella*, in a natural setting to evaluate the attack rate for each morph in relation to the background substrate. We expected lower predation rates for individuals of the more cryptic morphotype in the respective environment (green individuals in green vegetation background and brown individuals in brown soil backgrounds) based on results of previous studies on anurans (Morey, 1990; Wente & Phillips, 2005).

**Material and methods**

**Study area**

The present study was conducted between December 2015 and May 2016 in the state of Rio Grande do Sul, Brazil. Study sites were selected based on the previously confirmed presence of *Boana pulchella*, according to the literature: three water bodies in Centro de Pesquisas e Conservação da Natureza Pró-Mata (CPCN Pró-Mata); three water bodies in Floresta Nacional (FLONA) de São Francisco de Paula; and a 1-km transect along the banks of a lentic water body in Refúgio de Vida Silvestre Banhado dos Pachecos (RVSBP).

CPCN Pró-Mata is located in the municipality of São Francisco de Paula (29°27’–29°35’S, 50°08’–50°15’W), a region with a humid climate, mean annual rainfall of 2,252 mm and a mean annual temperature of 14.5°C. The vegetation comprises a mosaic of grassland and forests (humid Araucaria forest and Atlantic Forest) (Ferreira & Eggers, 2008). Three water bodies were set as study sites: Pond 1 (Figure 1A; 29°28’49.7’S, 50°10’22.8’’W) with an estimated diameter of 100 meters and a margin with predominance of grasses (around 70%) and emergent vegetation (around 30%); Pond 2 (Figure 1B; 29°28’54.0’S, 50°10’36.3’’W) with an estimated diameter of 25 meters and a margin with shrub and emergent vegetation in equivalent proportions; and Pond 3 (Figure 1C; 29°28’30.1’S, 50°09’51.9’’W) with an estimated diameter of 500 meters and a margin with grasses and emergent vegetation in equivalent proportions.

FLONA de São Francisco de Paula is also located in the municipality of São Francisco de Paula (29°23’–29°27’S, 50°23’–50°25’W). It is located in one of the most humid regions of the state, with mean annual rainfall of 2,200 mm and a mean annual temperature of 14.5°C. The vegetation is steppe formation (Campos de Cima da Serra), along with humid Araucaria forests (Backes, Prates, & Viola, 2005) and areas of Pinus plantations. Three water bodies were set as study sites: Pond 4 (29°29’13.2’S, 50°13’12.0’’W) with an estimated diameter of 50 meters and a margin with grasses and emergent vegetation in equivalent proportions.
proportions; Pond 5 (29°29′13.2″S, 50°13′12.0″W) with an estimated diameter of 300 meters and a margin with predominance of *Pinus* plantations (around 90%) and a very low proportion of emergent vegetation (around 10%); and Pond 6 (Figure 1D; 29°25′34.2″S, 50°25′19.0″W) with an estimated diameter of 100 meters and margin mostly with grasses.

![Figure 1](image)

**Figure 1.** Example of water bodies sampled in the study areas: Pond 1 (A), Pond 2 (B) and Pond 5 (C) located in CPCN Pró-Mata, São Francisco de Paula, Rio Grande do Sul; and Pond 6 (D) located in FLONA de São Francisco de Paula, Rio Grande do Sul.

RVSBP is located in the municipality of Viamão, in the metropolitan region of Porto Alegre (30°05′S, 50°50′W). The area has ecosystems related to Pampas, with the presence of wetlands. The mean annual rainfall of the region is 1,291 mm (Bueno, Cademartori, Forneck, & Cabral, 2013). A 1-km transect along a lentic water body (30°5′55.0″S, 50°51′6.0″W) of variable width (2 – 3 meters) was set as study site. The margin of the water body comprised shrubs and emergent vegetation in equivalent proportions, while there was a great amount of vegetation within the water.

### Sampling design

Predation intensity on the different morphotypes of *B. pulchella* was measured using artificial models made of Acrilex® non-toxic modeling clay in the brand colors green and brown available in the market. Modeling clay was used because it preserves identifiable marks of predator attacks (Hegna et al., 2013; Saporito et al., 2007), and has been successfully used in several other studies on predation (e.g. Dell’Aglio, Toma, Muelbert, Sacco, & Tozetti, 2012; Hegna et al., 2013; Roslin et al., 2017; Saporito et al., 2007). Clay does not reflect UV light. The artificial models were made manually using a plaster mold shaped using a preserved adult male museum specimen. This individual (snout-vent length of 50.48mm) was collected in São Lourenço do Sul, state of Rio Grande do Sul, Brazil, and was deposited in the Scientific Collection of Amphibians and Reptiles of the Science and Technology Museum of Pontifícia Universidade Católica do Rio Grande do Sul (specimen number MCP 11279). The artificial models thusly had the same dimensions and proportions of a real animal; marks were added to the artificial models with a black permanent marker to simulate eyes.

The green (GM) and brown (BM) models were equally distributed between two natural background colors: green (GB) - margin vegetation, and brown (BB) - exposed soil. An experimental block design was adopted.
with four different treatments (different combinations of model color and background color) in each block: GM+GB; GM+BB; BM+GB; and BM+BB. Therefore, predators were exposed to all four treatments at each encounter with artificial models, in which two would be more conspicuous and, according to our hypothesis, more frequently attacked. This methodology follows previous studies (Bordignon et al., 2018; Hegna et al., 2013, 2011; Saporito et al., 2007), in the sense that it uses artificial models in a natural habitat to assess predation, but instead of using fewer large transects with many models, more blocks with one copy of each treatment were used to avoid pseudoreplication. We offered a total of 188 models sorted among 47 sampling points (blocks) near the banks of water bodies. The models were placed no more than one meter away from the banks. The sampling points were separated by at least 100 meters from each other around the water bodies to increase sample independence by preventing the same predator from visiting two or more blocks with artificial models.

The artificial models were exposed to predation for a total of 48 hours with equivalent exposure during the day and night periods. Models were considered predated if they showed some kind of damage caused by a potential predator (nips, bites, etc.). Predated models were photographed and stored for later identification of the found marks. Artificial models were positioned on the ground to avoid possible damage from falling, which could hinder the identification of any predation marks. This practice was not expected to impact the results since although *B. pulchella* males are known to call from perches, they also call from the ground (Kwet et al., 2010).

**Data analysis**

A Generalized Linear Mixed Effect Model (GLMM) was used to test the hypothesis that predation attempts are lower for individuals of the more cryptic morphotype in a given background, with the response variable being whether or not there was a predation attempt in a binomial dataset. Model color, background color and their interaction were included in the fixed portion of the model, while different study sites were included in the random portion of the model to control the influence of different natural areas on the final result. Analyses were performed using the lme4 package (Bates, Mächler, Bolker, & Walker, 2015) of the software R, version 3.4.1 (R Foundation for Statistical Computing, 2008). Attack rate was evaluated by dividing the number of damaged artificial models by the total number of artificial models (predated models/total models).

**Results**

A total of 18 out of the 188 artificial models exhibited damages, and therefore were considered predated, for an attack rate of 9.57%. Of the 18 predated models, eight were identified as attacked by mammals (Figure 2A) and seven, by birds (Figure 2B), with three having unclassifiable marks. The number of attacked models did not significantly differ between treatments (Table 1). The fixed portion of the statistical model (model color, background color and their interaction) only accounted for a small portion of the variance in predation rate ($R^2=0.0294$), while the random portion (study areas) accounted for a much larger portion of the variance ($R^2=0.5302$).

![Figure 2](image_url). Artificial models of *Boana pulchella* with marks (pecks, bites, etc.) made by a potential predator. Arrows indicate marks identified as caused by mammals (A) and birds (B).
Table 1. GLMM results showing no difference in predation between clay models in different background environments.

<table>
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<th>P-value</th>
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<td></td>
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<td>0.5502</td>
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</table>

Discussion

The different colored clay models (green or brown), representing the color polymorphism of Boana pulchella, were equally unlikely to suffer attacks from potential predators, independent of the conspicuousness in relation to the predominant background color (green or brown). This result contradicts other studies on cryptic coloration, which reported decreases in the risk of predation by visually oriented predators on different morphs by microhabitat selection (Morey, 1990; Wente & Phillips, 2005). Furthermore, most of the variance in predation rate in the present study was related to study sites and not to the different experimental treatments. There was no evident difference in predation between different environments in the study sites.

Clay models have been used to estimate natural predation rates due to their ability to preserve identifiable marks left by an attacker (Hegna et al., 2013; Saporito et al., 2007). Previous research on cryptic coloration with anurans was able to show a decrease in the risk of predation by visually oriented predators (Morey, 1990; Wente & Phillips, 2005). However, contrary to the present study, which were carried out in the natural habitat of the species, these studies involved experiments in the laboratory and real amphibians. It is also important to emphasize that artificial clay models have been employed with significant results with aposematic anurans (Hegna et al., 2013; Saporito et al., 2007) and other groups of animals (e.g. snakes, Dell’Aglio et al., 2012; insects, Roslin et al., 2017; and lizards, Shepard 2007).

The low predation rate observed in the present study is similar to those previously reported by other studies with aposematic organisms (Hegna et al., 2013, 2011; Saporito et al., 2007), even though several studies have suggested that organisms with aposematic color show lower predation rates than non-aposematic organisms (e.g. Brodie III, 1993; Kuchta, 2005; Saporito et al., 2007). However, our predation rate and overall result is similar to that of Bordignon et al. (2018), who assessed the defensive value of the unken reflex behavior in a study with clay models of Melanophryniscus cambaraensis in the same study area. This anuran has a cryptic green dorsal color and a red to orange ventral color (Kwet et al., 2010), which is considered conspicuous (Bordignon et al., 2018). Nevertheless, predation rates did not differ between clay models with and without red marks (Bordignon et al., 2018). Possibly, low predation rate in that study and herein in the present study may be related to low predator density in the study area, which would result in overall low predation, not related to any defense mechanism used by the species. Likewise, predation may also vary in time, although the present study did not account for variation in climate or weather during data collection.

It is known that predators may experience neophobia — fear of a novel item — which may have contributed to the low attack rate in the present study (Hegna et al., 2015; Marples, Roper, & Harper, 1998). This phenomenon, however, may vary widely among individuals in the wild (Marples et al., 1998). Furthermore, Merilaita, Tuomi, and Jormalainen (1999) showed that the optimum defense coloration in environments with heterogeneous habitats could be achieved through a compromise between levels of cryptic coloration in different microhabitats, or by the enhancement of cryptic coloration in a specific microhabitat to the detriment of others. Besides that, it is possible for more than one morphotype to have equally cryptic coloration in a given environment (Merilaita, 2003). Moreover, in visually complex habitats, there is a greater amount of visual stimulus to be processed, which makes detection by predators more difficult. Therefore, camouflage can exploit not only the background coloration, but also the ability of a predator to process information (Merilaita, 2003; Merilaita et al., 1999).

Importantly, individual coloration is not only related to predation pressure, but also to other behavioral and physiological functions (Sowersby, Lehtonen, & Wong, 2015). Other mechanisms that can be responsible for the maintenance of color polymorphism, apart from predatory selection, include: frequency and density dependent selection, when alleles are maintained in stability by selection mechanisms (Endler & Greenwood, 1977).
heterozygote advantage, when an allele persists even though it is deleterious in homozygosis condition (Jones et al., 1977; Roulin, 2004); climate related selection, when coloration causes selective responses to microclimate (Jones et al., 1977); and neutral polymorphism, when both morphotypes have the same fitness and the polymorphic alleles are maintained (Jones et al., 1977; Roulin, 2004).

Finally, other selective forces may be responsible for evolution and maintenance of polymorphism in a same population of *B. pulchella*. For example, there may be a relationship between the microhabitat selected as a vocalization site and the body coloration of a calling male. It is possible that male choice by females (sexual selection) and/or territorial disputes between males (intraspecific competition) may evolve favoring those individuals that are more conspicuous to the background environment, thus increasing visibility to both conspecific receptors. Among anurans, females of the polymorphic *Oophaga pumilio* prefer to mate with males of their own color (Richards-Zawacki, Wang, & Summers, 2012), while females of *Scaphiopus couchii* show a preference for males with bright dorsal coloration, which is an honest signal for better-condition males (Vásquez & Pfennig, 2007). Furthermore, real amphibian may still show behavior and possibly other defensive strategies in addition to morphology. Although the present study represents a starting point for elucidating the function of the cryptic color polymorphism in *B. pulchella*, future studies should investigate other ecological aspects.

### Conclusion

In summary, the color polymorphism in *B. pulchella* may not be related to a defensive strategy against predation, and morphs may be equally cryptic to predators in environments that are heterogeneous in color. However, other selective forces may be acting in the maintenance of color polymorphism in *B. pulchella*.

### References


