



NATÁLIA DALLAGNOL VARGAS

**EFEITO DA COLORAÇÃO DO SAPINHO-DE-BARRIGA-
VERMELHA *MELANOPHRYNISCUS CAMBARAENSIS* NA
PERCEPÇÃO DE SUA IMPALATABILIDADE POR PREDADORES**

Dissertação apresentada ao Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências da Universidade Federal do Rio Grande do Sul como requisito parcial à obtenção do título de Mestre em Biologia Animal.

ÁREA DE CONCENTRAÇÃO: BIOLOGIA E COMPORTAMENTO ANIMAL

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UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PORTO ALEGRE

2017

EFEITO DA COLORAÇÃO DO SAPINHO-DE-BARRIGA-VERMELHA
MELANOPHRYNISCUS CAMBARAENSIS NA PERCEPÇÃO DE SUA
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Aprovada em 31 de março de 2017.

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Porto Alegre

2017

*Dedico este trabalho a todos que, de uma maneira ou outra,
ajudaram-me a fazê-lo.*

*“Nunca se deve nem cogitar
que é possível entender uma outra
espécie a julgar por nossos valores.
Penso que seja arrogância humana
achar que compreende como as outras
formas de vida se sentem. [...]*

*Dito isso, mesmo que não
possamos compreendê-las, elas são,
sem sombra de dúvida, vizinhos
que merecem o nosso respeito.”*

(Parasyte - Hitoshi Iwaak)

AGRADECIMENTOS

Aqueles que acompanharam, ao menos em parte, meu mestrado viram que ele foi corrido, cheio de projetos (totalmente diferentes) e tentativas. Eu o descreveria como uma montanha-russa. Então, conseguir fazê-lo e seguir em frente após cada projeto que não deu certo só foi possível por causa de várias pessoas. A todas essas sou eternamente grata de coração.

Começo agradecendo a minha família, meu suporte para tudo. Em especial, meus pais e meu irmão - as pessoas que eu mais amo -, por todo o apoio, amor, companhia, amizade, carinho e, “simplesmente”, por existirem, pois eu não seria nada sem vocês. Obrigada por serem as pessoas mais especiais da minha vida e sempre acreditarem em mim. Também às nossas anjinhas Athena (*in memoriam*) e Mel, por sempre nos trazerem tanta alegria.

Agradeço ao meu orientador, o professor Márcio, por toda a dedicação, orientação (desde o meu segundo semestre na graduação!), ensinamentos e amizade. Por sempre acreditar na minha capacidade e me receber sorrindo (independentemente do momento). Por representar mais do que apenas um orientador, mas também um grande amigo e um segundo pai para mim.

Agradeço ao Murilo por ter aceitado me co-orientar (antes até de me conhecer). Agradeço pela amizade, por tudo o que me ensinou (e continua ensinando) e por mostrar que o R não é tão monstruoso assim. Por todas as palavras de motivação e por sempre acreditar em mim.

Agradeço à equipe das massinha, Debs e Valen, por sempre me ajudarem e tornarem esse projeto possível. Gurias, vocês foram extremamente importantes em todo o trabalho. Obrigada por toda a amizade, carinho, risadas, conversas e por estarem dispostas a me ajudar em todos os momentos. E por sempre comemorarem junto comigo quando conseguíamos dar mais um passo.

Agradeço ao Lui, grande amigo e colega da Herpeto desde que eu entrei (há quase seis anos atrás), por toda a amizade e ajuda. Por todas as discussões e ideias para os meus projetos, mesmo estando longe, e por sempre me dar apoio em tudo.

Agradeço ao Matheus, colega e irmão que a Herpeto me deu, por toda a amizade, dedicação, esforço, apoio e ajuda. Mesmo não participando tão ativamente desse projeto que deu origem à minha dissertação, sempre esteve comigo e foi essencial, principalmente no meu primeiro projeto. Por sempre ser um ombro amigo e por estar ao

meu lado nos piores campos que já fiz (e que não sinto saudade alguma) e em alguns dos meus piores momentos. Agradeço muito também a toda a família do Matheus (isso inclui a Dandara, claro) por toda a hospitalidade e carinho.

Agradeço a todos meus indispensáveis ajudantes de laboratório e/ou de campo (Amu, Barbara, Debs, Deivid, Goku, Irina, Leo, Lilith, Mariano, Matheus, Pâm, Patri, Paulo Barradas, Pedro Luz, Rógger, Stephanie, Thore, Valen, Vini Ferri, Vini Santos e Yuri) por toda a ajuda, risadas, conversas, ideias e experiências. Ao Deivid e ao Patrick novamente, por coletarem os indivíduos de *Ischnocnema henselii* para mim.

Agradeço a todos os meus queridos amigos (colegas da Bio, de laboratório, da escola e da vida) - que caminharam ao meu lado nesta jornada - por toda a amizade, companhia, ajuda e momentos inesquecíveis. Por sempre me apoiarem e me trazerem alegria. Em especial àqueles que me acompanharam mais de perto, meus fiéis stormtroopers (Lucas e Vero), Malu, Mila, Lu e Dani por sempre me escutarem e estarem comigo. A Bruna por, além de me acompanhar, me ajudar a entender o R.

Agradeço aos meus colegas e ex-colegas, todos meus amigos também, do Laboratório de Herpetologia da UFRGS, minha segunda casa, por toda a amizade, disposição, ajuda e aprendizado ao longo desses anos. Muito do que sei aprendi com vocês! Um agradecimento especial a todos que me ajudarem neste trabalho de alguma outra maneira (Carol Z., Diego D., Diego J., Ismael e Mi).

Agradeço à Edenice Brandão Avila de Souza e toda a equipe da FLONA-SFP pela autorização para a realização do trabalho no local (mesmo nesses tempos tão difíceis) e por toda a dedicação e carinho.

Agradeço ao Luiz Gustavo Pereira e a Milena Sulzbach por nos emprestarem o espectrômetro; ao Ronaldo Gemerasca pela confecção do molde que utilizamos para fazer os modelinhos; e ao motorista do PPG Ecologia pela carona em um dos campos.

Agradeço a minha banca de acompanhamento, doutoras Helena Piccoli Romanowski e Laura Verrastro por todas as discussões e sugestões para os meus projetos; e a minha banca examinadora, doutore(a)s Laura Verrastro, Mirco Solé e Patrick Colombo, por aceitarem o convite.

Agradeço ao PPG Biologia Animal por todo o apoio; a CAPES pela bolsa concedida; e ao ICMBio pela licença n° 53177-2.

Agradeço a todos os outros que também me ajudaram de alguma forma em qualquer um dos meus vários projetos de mestrado.

A todos vocês o meu mais sincero MUITO OBRIGADA!!

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RESUMO

Há duas estratégias principais entre as espécies de presas para evitarem a predação: o criptismo e o aposematismo. Através do criptismo, animais previnem sua detecção por combinarem com o ambiente, e, por meio do aposematismo, as espécies de presas advertem sua falta de rentabilidade através de colorações conspícuas. Entre os anfíbios, tanto o criptismo quanto o aposematismo são bem conhecidos para várias espécies. A maioria das espécies dos sapinhos-de-barriga-vermelha *Melanophryniscus* apresenta cores dorsais aposemáticas, além da coloração ventral conspícua e do comportamento conhecido como reflexo “unken”. *Melanophryniscus cambaraensis*, contudo, possui coloração dorsal verde, que é usualmente associada à camuflagem. Como essa espécie é diurna e apresenta toxinas, mas a presença da coloração ventral vermelha associada ao reflexo “unken” não parece impedir o ataque de predadores, este estudo objetiva avaliar experimentalmente se a coloração dorsal verde de *M. cambaraensis* funciona como um sinal aposemático para predadores visualmente orientados. Para testar essa hipótese, foram conduzidos experimentos de predação em campo utilizando massa de modelar, representando a espécie (modelos verdes) e uma rã críptica (modelos marrons), e a predação em presas artificiais foi comparada entre as duas cores. A taxa de predação por aves foi a mesma entre os modelos verdes e marrons de massa de modelar, sugerindo que a coloração dorsal verde da espécie não funciona como um sinal aposemático, mas como uma coloração críptica. Além do que, houve significativamente mais ataques na parte anterior das réplicas, indicando que os modelos provavelmente foram interpretados como presas. Como a maioria das espécies de *Melanophryniscus* apresenta cores dorsais aposemáticas, a coloração de *M. cambaraensis* provavelmente evoluiu secundariamente. Isso possivelmente ocorreu devida à pressão seletiva imposta pela ausência de predadores visualmente orientados especializados em predação de anfíbios no local de estudo, a qual conduziu a uma redução de conspicuidade do sinal aposemático. Esses resultados reforçam que o aposematismo nem sempre é a melhor estratégia para presas impalatáveis.

Palavras-chave: predação; camuflagem; pressão seletiva; evolução; predadores visualmente orientados; aves; modelos de massa de modelar; *Melanophryniscus cambaraensis*.

CAPÍTULO I

Introdução geral

Introdução geral

Há duas estratégias principais entre as espécies de presas para evitarem a predação: o criptismo e o aposematismo (Aronsson & Gamberale-Stille, 2008). Através do criptismo (Fig. 1a), os animais possuem coloração parecida com a do ambiente, prevenindo sua detecção por predadores (Stevens *et al.*, 2006). Já por meio do aposematismo (Fig. 1b), ao invés de se esconderem, as presas anunciam sua impalatabilidade e periculosidade por colorações e/ou padrões conspícuos (Poulton, 1890; Ruxton, Sherratt & Speed, 2004; Mappes, Marples & Endler, 2005; Speed & Ruxton, 2007). Essa estratégia evolutiva é utilizada por vários animais, como gastrópodes, milípedes, insetos, anfíbios, peixes, serpentes e aves (Ruxton *et al.*, 2004). Enquanto os animais de coloração críptica confiam na capacidade de passarem despercebidos no seu habitat, a eficácia do sinal aposemático depende da capacidade dos predadores de o associarem à impalatabilidade da presa (Mappes *et al.*, 2005). Os predadores podem aprender a evitar organismos aposemáticos a partir de experiências anteriores, ou sua resposta pode ser inata (Ruxton *et al.*, 2004). Estudos já demonstraram que galinhas domésticas aprenderam a associar a coloração aposemática de anfíbios da família Dendrobatidae com o sabor que elas apresentavam, evitando até mesmo o ataque a espécies de dendrobatídeos mímicas e menos tóxicas (Darst & Cummings, 2006; Darst, Cummings & Cannatella, 2006).



Figura 1 Exemplo de animal: (a) críptico (a rã *Physalaemus lisei*) e (b) aposemático (a cobra-coral ou coral verdadeira *Micrurus altirostris*). Fotos: Natália D. Vargas.

Espécies aposemáticas terrestres geralmente utilizam vermelho, amarelo e laranja, combinados, muitas vezes, com preto, para advertirem sua toxicidade e perigo a predadores visualmente orientados, como as aves (Endler & Mappes, 2004). Colorações

conspícuas como essas favorecem um rápido reconhecimento de que a presa não é palatável (e.g. Gittleman & Harvey, 1980). Além disso, sinais mais chamativos facilitam um aprendizado mais rápido comparado a sinais menos visíveis, e estão associados a um maior tempo de retenção na memória dos predadores (Roper & Redston, 1987). Apesar das vantagens de colorações bastante conspícuas, há várias espécies impalatáveis que são consideradas quase crípticas (Endler & Mappes, 2004). O limiar que divide o aposematismo da camuflagem (termo que inclui todas as estratégias de ocultação de um organismo, e.g. criptismo) (Stevens & Merilaita, 2009) apresenta critérios subjetivos, e um organismo aposemático pode parecer críptico em certos ambientes, como, por exemplo, a espécie de rã *Dendrobates auratus* (Girard, 1855) (Wollenberg & Measey, 2009). Inclusive, segundo várias teorias, os sinais aposemáticos parecem ter evoluído inicialmente em populações crípticas (e.g. Harvey *et al.*, 1982; Leimar, Enquist & Sillen-Tullberg, 1986). O mais importante neste processo é que o sinal emitido seja discriminável e compreendido pelos seus predadores. Sendo assim, a coloração pode até mesmo ser relativamente críptica, desde que os predadores associem o padrão com a impalatabilidade ou risco (Sherratt & Beatty, 2003).

Entre os anfíbios, apesar de a camuflagem e o criptismo serem estratégias bem comuns para evitar predadores (Wells, 2007), o aposematismo também é bem conhecido para várias espécies, principalmente das Ordens Anura (sapos, rãs e pererecas) e Caudata (salamandras e tritões). Em anuros, essa adaptação evoluiu múltiplas vezes em diferentes grupos (e.g. Summers & Clough, 2001). O exemplo mais estudado de aposematismo são as famosas rãs venenosas da família Dendrobatidae Cope, 1865, que possuem colorações de alerta e sequestram alcaloides impalatáveis de suas presas (e.g. Daly *et al.*, 1994a, 1994b). Em uma experiência utilizando modelos de rãs feitos de massa de modelar simulando a espécie *Oophaga pumilio* (Schmidt, 1857) e outros sem coloração aposemática (marrons) observou-se que as taxas de predação em modelos castanhos eram quase o dobro dos modelos vermelhos (Saporito *et al.*, 2007).

Além da coloração aposemática dorsal, algumas espécies de anfíbios possuem cores ventrais conspícuas e, para mostrá-las aos predadores, arqueiam o corpo, elevando a cabeça e a região posterior (Griffiths, 1995; Hinsche, 1926; Toledo & Haddad, 2009). Esse comportamento, conhecido como reflexo “unken”, é compartilhado por famílias filogeograficamente distintas, tanto de anuros, quanto de salamandras, e parece funcionar como um sinal aposemático (Hinsche, 1926; Johnson & Brodie, 1975; Brodie, 1977). Em um recente trabalho, Bordignon (2016) testou essa hipótese na espécie

Melanophryniscus cambaraensis Braun & Braun, 1979 contra predadores visualmente orientados, e verificou que a posição em “unken” pode não ser o suficiente para repelir eventuais predadores. Para este estudo, foram utilizados três tipos de modelos de sapo de massa de modelar, simulando indivíduos da espécie na posição: (a) normal, com corpo e extremidades verdes, (b) em reflexo “unken”, com corpo e extremidades verdes, e (c) em reflexo “unken”, com corpo verde e extremidades vermelhas. Não houve diferença no número de modelos atacados nas três posições, indicando que a presença da coloração vermelha associada à posição estática de reflexo “unken” parece não ser o suficiente para evitar o ataque de potenciais predadores (Bordignon, 2016). Uma possibilidade é que a coloração dorsal verde da espécie já possa funcionar como um sinal aposemático, apesar desta cor estar associada normalmente à camuflagem (Wells, 2007).

O gênero *Melanophryniscus* (Anura: Bufonidae) inclui 29 espécies válidas (Frost, 2016), sendo considerado o táxon irmão de todos os demais gêneros da família Bufonidae (Pramuk *et al.*, 2008). Distribui-se na América do Sul, e a maioria das espécies apresenta naturalmente distribuições pequenas e está categorizada em algum grau de ameaça (Zank *et al.*, 2014). São conhecidos popularmente como sapinhos-de-barriga-vermelha por apresentarem coloração ventral aposemática vermelha ou laranja, que pode ser exibida através do reflexo “unken” (Fig. 2) (Santos & Grant, 2011). Além disso, a coloração dorsal também é conspícua em várias espécies do gênero. Secretam alcaloides (Daly *et al.*, 2008; Jeckel, Grant & Saporito, 2015) e apresentam hábito diurno (e.g. Langone, 1994; Kwet & Di-Bernardo, 1999), apesar de algumas espécies já terem sido registradas vocalizando à noite também (Santos & Grant, 2011).

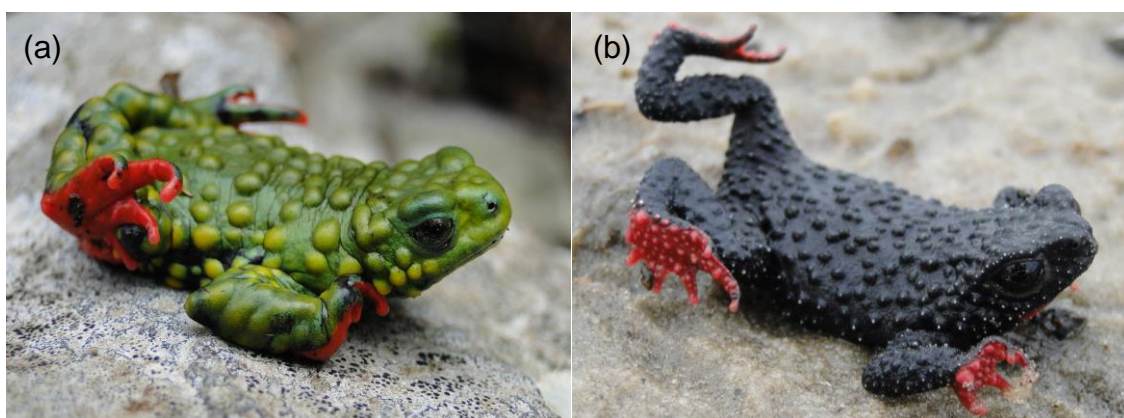


Figura 2 Exemplar de (a) *Melanophryniscus admirabilis* e de (b) *M. dorsalis* exibindo o comportamento de reflexo “unken”. Fotos: Natália D. Vargas.

Melanophryniscus cambaraensis (Fig. 3) é uma das três espécies do gênero que possui coloração dorsal verde. Já sua coloração ventral é predominantemente vermelha, podendo haver manchas de coloração verde, cinza ou preta, além de tubérculos brancos (Braun & Braun, 1979). Apresenta reprodução explosiva, reproduzindo-se apenas após fortes chuvas, quando se deslocam para o sítio reprodutivo durante o dia (Santos & Grant, 2011). É uma espécie pequena (cerca de 35 mm de comprimento rostro-cloacal), endêmica do sudeste do Planalto das Araucárias do Rio Grande do Sul (microrregião dos Campos de Cima da Serra) (Kwet & Di-Bernardo, 1999). São conhecidas apenas duas populações, separadas por aproximadamente 50 quilômetros, nos municípios de Cambará do Sul (no qual não se encontra há mais de duas décadas) e São Francisco de Paula (Braun & Braun, 1979; Garcia & Vinciprova, 2003). A espécie consta na categoria “Vulnerável” em níveis regional - pela Lista de Espécies Ameaçadas de Extinção do Rio Grande do Sul (Decreto Estadual Nº 51.797 de 8 de setembro de 2014) - e nacional - pela Lista Nacional Oficial de Espécies da Fauna Ameaçadas de Extinção do Ministério do Meio Ambiente (Portaria MMA nº 444/2014) -, e como “Dados Insuficientes” em nível global, sendo o fogo e a destruição do habitat suas maiores ameaças (Garcia, Kwet & Silvano, 2004).

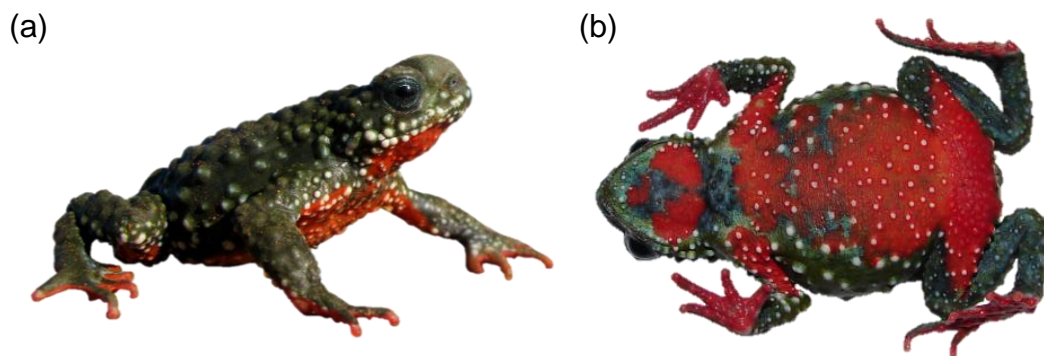


Figura 3 Vista (a) lateral e (b) ventral de exemplares de *Melanophryniscus cambaraensis* (São Francisco de Paula, Rio Grande do Sul, Brasil). Fotos: Valentina Z. Caorsi.

Na literatura não há quase nenhuma informação sobre quais seriam os predadores desse gênero, apenas o relato de que foram encontrados *M. atroluteus* (Miranda-Ribeiro, 1920) em estômagos analisados de serpentes da espécie *Xenodon dorbignyi* (Bibron, 1854) (Miranda, 1966) e um indivíduo de *M. moreirae* (Miranda-Ribeiro, 1920) que foi predado por uma serpente da espécie *Thamnodynastes strigatus*

(Günther, 1858) (Winkler *et al.*, 2011). Apesar disso, sabe-se que as aves podem ser os principais predadores de diversas espécies aposemáticas, inclusive os anuros da família Dendrobatidae (Master, 1998, 1999; Alvarado, Alvarez & Saporito, 2013). As aves dispõem de visão tetracromática, sendo que um de seus cones é sensível à luz ultravioleta, e “cones duplos”, capazes de detectar colorações brilhantes (Bowmaker *et al.*, 1997). Porém, além de existir pouca evidência experimental registrando o ataque de predadores, eventos de predação na natureza sobre as espécies de *Melanophryniscus* também são raramente observados (Bordignon, 2016). Uma solução para realizar estudos de curto prazo com predação é utilizando um método que consiste em obter marcações de ataques em réplicas das espécies de interesse feitas com material macio (como massa de modelar) (e.g. Brodie, 1993). Essa técnica tem sido utilizada para estudar interações de predação no habitat natural das espécies de vários táxons, pois as réplicas das presas são deixadas em campo para os predadores atacá-las (Irschick & Reznick, 2009). Além disso, tal abordagem permite que os predadores sejam identificados posteriormente, por meio das impressões deixadas nas réplicas de massa de modelar (e.g. Brodie, 1993; Saporito *et al.*, 2007; Hegna *et al.*, 2011).

Como são conhecidas apenas duas populações de *M. cambaraensis* e a população do município de Cambará do Sul não é encontrada há mais de duas décadas (Braun & Braun, 1979; Garcia & Vinciprova, 2003), o estudo foi realizado na Floresta Nacional de São Francisco de Paula (FLONA-SFP) (29° 25' 41.3" S, 50° 23' 44.5" W, altitude máxima de 923 m) (Fig. 4), localizada no Município de São Francisco de Paula, Rio Grande do Sul (RS), Brasil. A FLONA-SFP é uma Unidade de Conservação de Uso Sustentável de cobertura florestal composta predominantemente por mata nativa, com a Floresta Ombrófila Mista como formação dominante. O local é constituído por uma área de 1.606 hectares e está inserido no Planalto das Araucárias, microrregião dos Campos de Cima da Serra. O clima é classificado como Temperado Superúmido, com média anual de 14,5 °C e pluviosidade superior a 2.000 mm ao ano (considerado um dos mais úmidos do estado), e com temperaturas variando de -3 a 18 °C nos meses de inverno e de 18,3 a 27 °C nos meses de verão (Maluf, 2000; Backes, Prates & Viola, 2005). A área de amostragem situa-se em um afloramento rochoso (29° 25' 41.3" S, 50° 23' 44.5" W, 866 m acima do nível do mar), em torno de um dos sítios reprodutivos conhecidos para a espécie (Santos *et al.*, 2010). O sítio possui cerca de 20 metros de comprimento e 4 metros de largura e está localizado na borda de uma estreita estrada de terra, na qual, em ambos os lados, há floresta de araucária plantada, arbustos e

ciperáceas (Santos *et al.*, 2010). A vegetação do sítio é parcialmente composta por gravatás (*Eryngium* sp.) e gramíneas (Poaceae) (Santos *et al.*, 2010).



Figura 4 Localização da Floresta Nacional de São Francisco de Paula (FLONA-SFP), São Francisco de Paula, Rio Grande do Sul, Brasil. Fonte: Google.

Ao contrário do esperado, a presença da coloração vermelha associada à posição estática de reflexo “unken” da espécie *M. cambaraensis* parece não ser o suficiente para evitar o ataque de potenciais predadores (Bordignon, 2016). Levando isso em consideração, e o fato de a espécie ser diurna e apresentar toxinas, este estudo pretende testar se a coloração dorsal verde da espécie, por si só, representa um sinal aposemático a predadores visualmente orientados. A hipótese inicial é que os modelos de coloração marrom (crípticos) sejam mais atacados nas duas áreas de amostragem, por simularem uma espécie não tóxica, no caso *Ischnocnema henselii* (Peters, 1870) (Haddad *et al.*, 2013).

Para testar essa hipótese, portanto, foram conduzidos experimentos em campo utilizando modelos de massa de modelar, representando a espécie venenosa em questão (modelos verdes) e uma espécie de rã não-tóxica (modelos marrons).

Objetivo geral

Este estudo objetivou avaliar experimentalmente, por meio de modelos de massas de modelar, se predadores visualmente orientados associam a coloração dorsal do sapinho-de-barriga-vermelha, *M. cambaraensis*, à toxicidade da espécie, em condições naturais.

Objetivo específico

- a) Analisar se os modelos verdes, aposemáticos, que simulam *M. cambaraensis*, são menos atacados por predadores visualmente orientados do que os modelos marrons, não aposemáticos, semelhantes à espécie não tóxica *I. henselii*.

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CAPÍTULO II

Aposematism or crypsis? Experimental evidence for the green dorsal color function in a poisonous Neotropical red bellied-toad

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(Formatado para ser submetido ao periódico Journal of Zoology)

Aposematism or crypsis? Experimental evidence for the green dorsal color function in a poisonous Neotropical red bellied-toad

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Abstract

There are two main strategies for a prey species to avoid predation: crypsis and aposematism. Through crypsis, animals prevent detection by blending into the background, and through aposematism, prey species signal their unprofitability via conspicuous colorations. Among amphibians, both crypsis and aposematism are well known for many species. Most species of the red-bellied toads *Melanophryniscus* present aposematic dorsal colors, besides their ventral conspicuous coloration and the behavior known as unken reflex. *Melanophryniscus cambaraensis*, however, possess green dorsal coloration, that is usually associated with camouflage. Since this species is diurnal and presents toxins, but the presence of red ventral coloration associated with unken reflex does not seem to prevent the attack of predators, this study aims to evaluate experimentally if the green dorsal color of *M. cambaraensis* works as an aposematic signal for visually oriented predators. To test this hypothesis, field predation experiments employing clay models, representing the species (green models) and a cryptic frog (brown models), were conducted and the predation on artificial prey was compared between the two colors. Avian predation rate was the same on green and brown frog clay models, suggesting that the green dorsal color of the species does not work as an aposematic signal, but functions as a cryptic color. In addition, there were significantly more attacks on the anterior part of the replicas, implying that the models were probably perceived as prey. As the majority of the *Melanophryniscus* species present aposematic dorsal colors, the cryptic dorsal coloration of *M. cambaraensis* has probably evolved secondarily. It possibly occurred due to the selection pressure imposed by the absence of visually oriented predators specialized in preying amphibians

at the local study, which led to a reduced conspicuousness of aposematic signal. These results enhance that aposematism is not always the best strategy for unpalatable preys.

Keywords

predation; camouflage; selection pressure; evolution; visually oriented predators; birds; clay models; *Melanophryniscus cambaraensis*.

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Introduction

In order to avoid predation, there are two main strategies for a prey species: crypsis and aposematism (Aronsson & Gamberale-Stille, 2008). Through crypsis, animals prevent detection by blending into the background (Stevens *et al.*, 2006), while through aposematism, prey species signal unprofitability and unpalatability via conspicuous colorations or patterns (Poulton, 1890; Ruxton *et al.*, 2004; Mappes *et al.*, 2005; Speed & Ruxton, 2007). While the efficacy of crypsis relies on deceiving the detection skills of predators, the efficacy of aposematism depends on increasing their abilities to recognize and avoid unprofitable preys (Mappes *et al.*, 2005). These responses of avoiding aposematic species may be innate or may be learned from previous experiences (Ruxton *et al.*, 2004).

Terrestrial aposematic species commonly use conspicuous colors such as red, yellow, and orange, often combined with black, to favor a rapid recognition by visually oriented predators, like birds, and also enhance predator learning and memory (Gittleman & Harvey, 1980; Roper & Redston, 1987; Endler & Mappes, 2004). Despite the advantages of conspicuous colorations, there are several unpalatable species that are considered almost cryptic (Endler & Mappes, 2004), and only moderately conspicuous

aposematic signals may be advantageous when other biological costs are involved (Stevens & Ruxton, 2012). In fact, the threshold that divides aposematism from camouflage presents subjective criteria, and aposematic organisms may appear cryptic in some backgrounds, such as the frog *Dendrobates auratus* (Wollenberg & Measey, 2009). Actually, aposematic warning displays, according to various theories, must have initially evolved from cryptic prey populations (e.g. Harvey *et al.*, 1982; Leimar, Enquist & Sillen-Tullberg, 1986). The most important for unprofitable prey is that the signal emitted is readily discriminable and understood by predators; therefore, prey coloration can be even cryptic as long as predators discriminate it (Sherratt & Beatty, 2003). Notwithstanding the great theoretical and empirical knowledge acquired in the study of aposematism origins and evolution, the description and understanding of the diversity and efficiency of warning signals in nature is still a major shortfall (Stevens & Ruxton, 2012).

Among amphibians, although camouflage and crypsis are widespread anti-predator strategy (Wells, 2007), aposematism is also well known for many species, mainly in frogs and salamanders. In anurans, at least, aposematism evolved multiple times (e.g. Summers & Clough, 2001). The most studied examples of aposematism in anurans are the poison dart frogs of the family Dendrobatidae, which obtain impalpable alkaloids through sequestration (Daly *et al.*, 1978, 1994). In an experiment with clay frog models, predation rate of cryptic (brown) models was almost two times higher than aposematic (red) models, simulating *Oophaga pumilio* specimen (Saporito *et al.*, 2007). Some studies demonstrated that domestic chickens learned to associate the conspicuous coloration of Dendrobatidae frogs with its taste, avoiding also less toxic mimic species of this same family (Darst & Cummings, 2006; Darst *et al.*, 2006).

Dorsal conspicuous colors are present in most aposematic amphibian species. Additionally, some anurans and salamanders present conspicuous ventral coloration and, in order to display these colors to their predators, most of these amphibians arch their body, raising the head and the posterior region in a behavior known as unken reflex (Hinsche, 1926). It seems to function equally as an aposematic signal (Hinsche, 1926; Johnson & Brodie, 1975; Griffiths, 1995; Toledo & Haddad, 2009). Ventral conspicuous coloration is found in geographically distinct amphibian genera from different families such as *Bombina* (Hinsche, 1926), *Melanophryniscus* (Fernández, 1926), *Taricha* and *Triturus* (Johnson & Brodie, 1975; Brodie, 1977). The nearly 30 species of Neotropical red-bellied toads (*Melanophryniscus* spp.) present ventral and

several times also dorsal aposematic coloration (Baldo *et al.*, 2014). These small toads are diurnal (Santos & Grant, 2011), and secrete bufadienolides and alkaloids (Daly *et al.*, 2008). This genus is the sister taxon of all remaining Bufonidae (Pramuk *et al.*, 2008). Despite *Melanophryniscus* being an appealing model to study many open aspects of aposematic signal evolution, as the mechanistic basis of aposematic markings and the form and diversity of warning signals, almost nothing has been explored. Indeed, the efficiency of aposematic warnings (color and behavior) has been largely neglected in *Melanophryniscus*, which seems to be a general bias in our understanding of aposematism (Stevens & Ruxton, 2012).

Although the unken reflex seems to work as an aposematic signal for visually oriented predators (e.g. Brodie & Howard, 1972; Johnson & Brodie, 1975), its efficiency is essentially ignored and it was not supported by the results of an experimental test with *Melanophryniscus cambaraensis* (Bordignon, 2016). *Melanophryniscus cambaraensis* is a poisonous threatened species, endemic to the Atlantic Forest of southern Brazil (Kwet & Di-Bernardo, 1999). It is one of the three species of the genus with green dorsal coloration (most have black or dark background dorsal coloration). In an experiment, clay models were used to represent the toad in normal position and in unken reflex position (displaying red ventral coloration of hand and foot). There was no preference by visually oriented predators for any model, indicating that the presence of red ventral coloration associated with the static unken reflex position does not seem to be a signal strong enough to prevent the attack of predators (Bordignon, 2016). The lack of preference combined with the low percentage of observed attacks suggested alternatively that the green dorsal coloration could function as camouflage, or an aposematic signal itself. Although green does not seem to be a warning color as common or effective as red (Hegna *et al.*, 2013), and it is usually associated with camouflage (Wells, 2007), there are green aposematic species, like some populations of *Dendrobates granuliferus* (Wang, 2011).

Besides the report of predation of *M. moreirae* by a Xenodontinae snake (*Thamnodynastes strigatus*; Winkler *et al.*, 2011), there is no information about the predators of *Melanophryniscus* species. Nevertheless, it is already known that birds may represent the main predators of several aposematic species, including dendrobatid frogs (Master, 1999). Birds are visually oriented predators that possess tetrachromatic vision, are sensitive to ultraviolet (UV) light and are capable of detecting bright colors (Bowmaker *et al.*, 1997). However, it is notably rare to observe predation events in situ.

A common solution to study predation in short periods of time is to use a methodology that consists in obtaining attack marks through soft models that resemble the species of interest (e.g. Brodie, 1993).

As previously mentioned, contrary to expectations, the presence of red ventral coloration associated with the static unken reflex position of *Melanophryniscus cambaraensis* does not seem to be enough to prevent the attack of potential predators, and does not reduce predation attempts when compared to only green models (Bordignon, 2016). Since this species is diurnal and poisonous (presents bufadienolides and alkaloids), this study aims to evaluate experimentally if the green dorsal color of the red-bellied toad *M. cambaraensis* works as an aposematic signal for visually oriented predators. Therefore, to test this hypothesis, we conducted field predation experiments employing clay models, representing the toxic species (green models) and a cryptic edible frog (brown models). If green is actually acting as a warning signal, we expect that green models would experience less bird attacks, because visually oriented predators would discriminate this color and associate it with *M. cambaraensis* unprofitability. If so, green coloration would function as a reliable aposematic signal for this toad species. In this case, an only moderately conspicuous aposematic signal may have evolved, since aposematism is not always the best strategy for unpalatable preys and sometimes there is a selection for reduced conspicuousness (Endler & Mappes, 2004). Alternatively, the green coloration may be a cryptic adaptation for closed evergreen Atlantic rain forest environments.

Materials and methods

The field experiments were conducted at two sites in Floresta Nacional de São Francisco de Paula (FLONA-SFP), southern Brazil: the breeding site of *Melanophryniscus cambaraensis* (Fig. 1a) and its adjacent forest of planted *Araucaria angustifolia* (Fig. 1b). The breeding site (29° 25' 41.3" S, 50° 23' 44.5" W) is approximately 20 m long and 4 m wide and is located on a small, unpaved road on a rocky outcrop (Santos *et al.*, 2010). The experiment occurred between 12 October and 15 October 2016, and was repeated between 13 December and 16 December 2016.



Figure 1 Study sites of aposematism efficiency with clay models at the Floresta Nacional de São Francisco de Paula (FLONA-SFP), southern Brazil: (a) breeding site of *Melanophryniscus cambaraensis* and (b) its adjacent forest.

Clay model design – Frog models were constructed using non-toxic, odorless, pre-colored modeling clays (Corfix® and Acrilex®). Black eyes were drawn on the models with a black permanent marker (Sharpie®). Two model types were constructed: completely green models (representing *M. cambaraensis*; Fig. 2a) (Fig. 2c) and completely brown models (representing the cryptic species *Ischnocnema henselii*, a very common leaf-litter frog that occurs on the site and has a similar size to *M. cambaraensis*; Fig. 2b) (Fig. 2d). Although the two species own morphological differences, the same rubber mold was used to make the models of both species, in order to avoid possible unwanted variables. The mold was made from a specimen of *M. cambaraensis* deposited in the herpetology collection at Fundação Zoobotânica do Rio Grande do Sul (voucher number: MCN 13459), that presents the average size described for the population of São Francisco de Paula (SVL 32 ± 1.6) (V. Caorsi, unpublished data). To ensure the colors of the models matched the color of the species in their natural environment, a mixture of different clay colors was created. Then a spectrometer (Ocean Optics 2200 SD) was used to match the wavelengths of the colors of *M. cambaraensis* and *I. henselii* with our models. Spectrometer was also used to test for ultraviolet (UV) reflectance. Both the frogs and the models (clay and the permanent marker) did not reflect ultraviolet (UV) light.

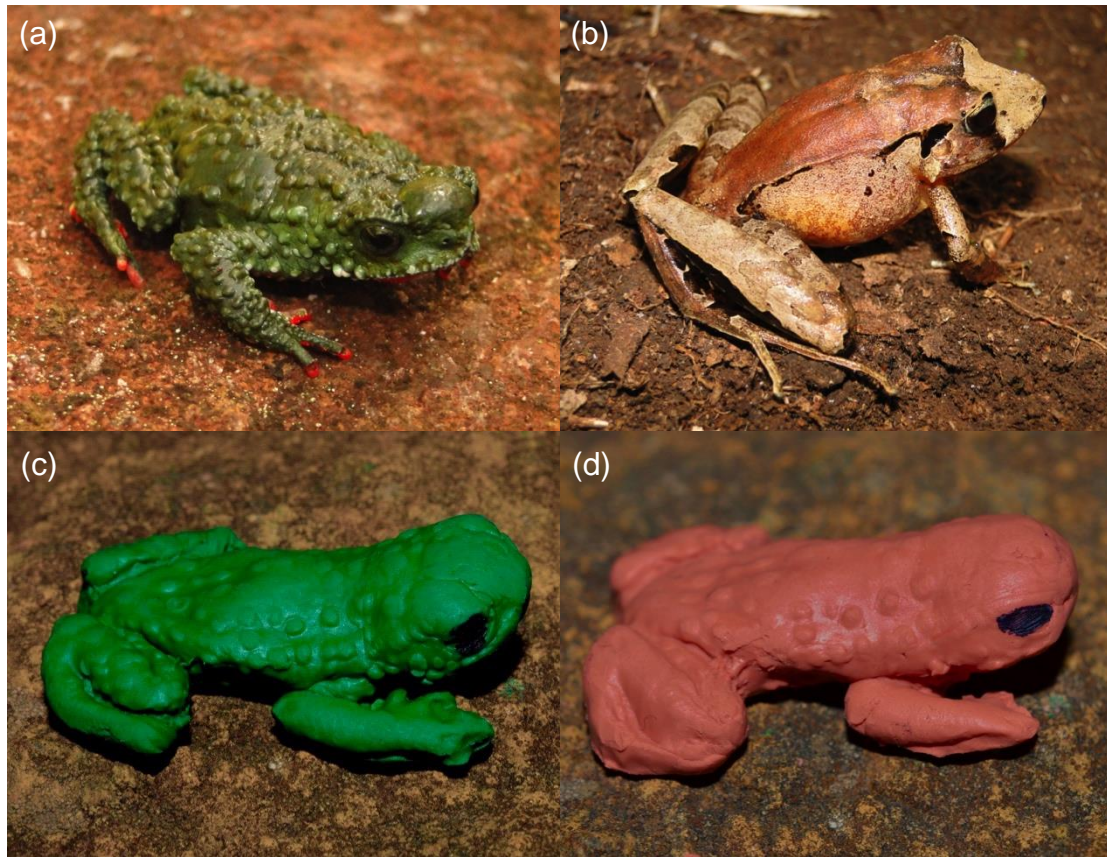


Figure 2 Live specimens and clay model replicas of (a and c) *Melanophryniscus cambaraensis* and (b and d) *Ischnocnema henselii*; used in the predation experiment.

Experimental design – The experimental design followed Bordignon (2016), since the study area and the model species were the same. A total of 600 models was used on each experiment, where 540 models (270 green and 270 brown) were placed in the adjacent forest along 18, 20-m transects (see Supporting Information Figure S1), and 60 frog models (30 green and 30 brown) were placed randomly at the breeding site, since this site is small and a higher density of models could affect the results. Each transect was 90 m in length and had 15 blocks (each block with one green model and one brown model). The blocks were spaced 6 m apart, and the models, within the blocks, were spaced 1 m. The order of the model placement was always alternate. After 72 h, models were collected, photographed and examined for evidence of predation attempts. To avoid disturbance, the transects and the breeding site were not visited between deposition and collection. All models were placed directly on the leaf litter, because in most studies (e.g. Saporito *et al.*, 2007; Hegna *et al.*, 2011), predation attempts by birds are lower in models placed on high contrast backgrounds, such as the white “Rite in the Rain” paper. Furthermore, Bordignon (2016) worked in the same sites

as this study and found no difference in predation by birds between the treatments and, on the white background, the models were less attacked.

Statistical analysis – Marks on models were classified into four categories: bird, mammal, arthropod and unidentified. Predation attempts left by birds were recognized by U- or V-shaped imprints (Fig. 3a) (e.g. Brodie, 1993). Mammalian predations were identified by incisor marks (Fig. 3b), and arthropod predations were recognized by small notches and mandible impressions (Fig. 3c) (Brodie, 1993; Paluh, Hantak & Saporito, 2014). Marks made on the model surface that did not fit into any of these categories were classified as unidentified (Fig. 3d). Since mammals and arthropods rely primarily on non-visual signals to locate their prey, they can be attracted to frog models regardless of their coloration, so only the avian predation were considered as attempts of interest (Bell & Cardé, 1984; Vander Wall, 1998; Paluh, Kenison & Saporito, 2015). Moreover, multiple attack marks on a single model were considered as a single predation attempt (Saporito et al., 2007). Models that could not be found after 72 h or melted were excluded from the final analysis. The two experiments were carried out with exactly the same methodology and both were performed in the spring. Because we did not find any difference between the two experiments, we present a joint analysis. We account for a potential habitat effect because we performed the experiments in the breeding site and in the adjacent forest, so the analyses were made separately. A general linear mixed model (GLMM) with a binomial error distribution was used to determine if frog model color (green or brown) was a significant predictor of attack rates in the adjacent forest (blocks and transects treated as random effects), and, in the analyses of the breeding site, we used a general linear model (GLM) with a binomial error distribution. All possible interactions between the predictors were accomplished in different models. Finally, we used another Binomial GLM to determine if bird attacks were non-randomly directed at the anterior or posterior body region; the models attacked both in anterior and posterior body regions were excluded from the analyses. All statistical analyses were performed in R version 3.3.1 (R Core Team, 2015). We selected and ranked models using AIC (a measure that compares the quality of statistical models to each other) values.

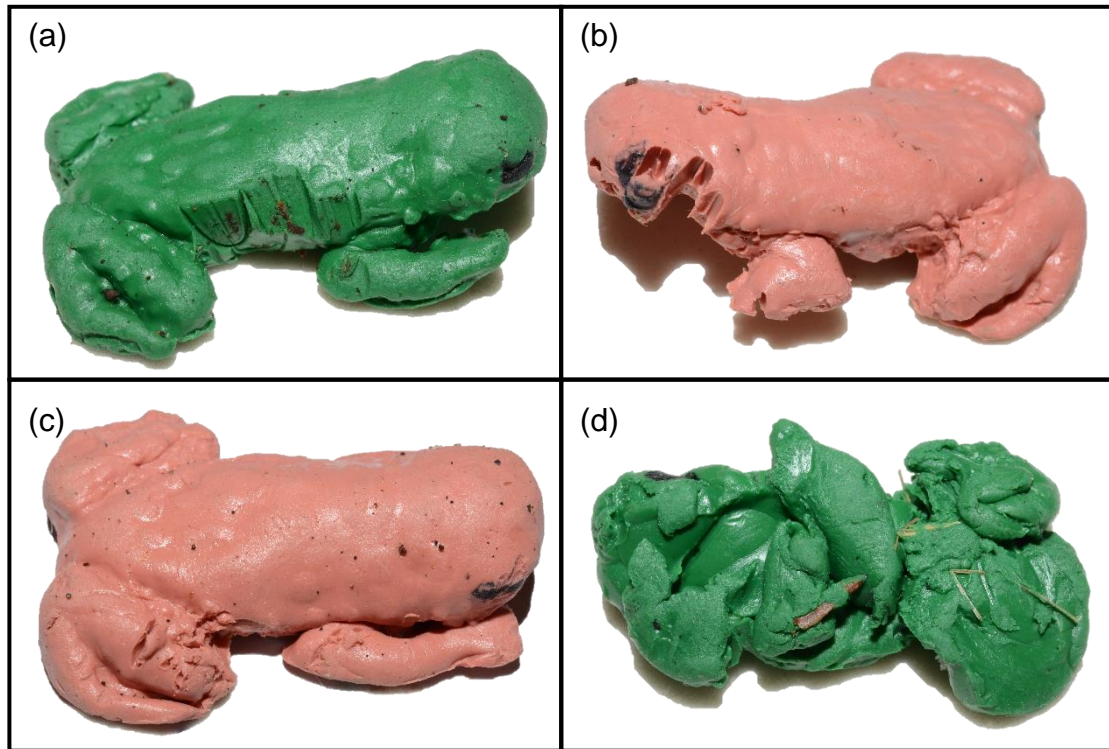


Figure 3 Clay models with (a) avian peck marks, (b) rodent incisor marks, (c) small notches and mandible impressions made by arthropods, and (d) heavily damages caused by an unknown cause.

Results

From the 1200 replicas (600 in each experiment) placed in the field, 20 (1.7%) were discarded from the analyses, due to loss, melting or human treading (seven green models and one brown model in the first experiment; nine green models and three brown models in the second one). Among the remaining 1180, 564 models (47.8%) were attacked (202 in the first experiment and 362 in the second one): 38 (3.2%) attacks were attributed to birds, 76 (6.4%; 35 green + 41 brown) to mammals, 295 (25%; 138 green + 157 brown) to arthropods, and 168 (14.2%; 81 green + 87 brown) were unidentified.

Among the 38 replicas (14 in the first experiment and 24 in the second one) attacked by birds, 30 (16 green models and 14 brown models) were from the adjacent forest and 8 (five green models and three brown models) from the breeding site (all from the second experiment). For predicting avian attack rates in the adjacent forest, the best model, with lower AIC value, was the null model with transects as random factors (Table 1). When this model was compared to the second best ranked model (frog model

color as the predictor variable with transects as random factors), no significant difference was found (likelihood ratio test: $\chi^2 = 0.172$, $P = 0.678$). At the breeding site, the null model had the best AIC value and was not significantly different from the statistic model with treatment ($P = 0.3541$).

Table 1 Model results for avian attacks on clay models during the experiment in the adjacent forest to the breeding site of *Melanophryniscus cambaraensis*. null = null model; treatment = frog model color as the predictor variable; tran = transect; between parenthesis the variables treated as random effects.

Model	Model df	AIC	χ^2	P
Null + (tran)	2	265.14	31.541	<0.001*
Treatment + (tran)	3	266.97	30.841	<0.001*
Null + (block) + (tran)	3	267.10	0.0000	10.000
Null + (block)	2	268.30	92.091	<0.001*
Treatment + (block) + (tran)	4	268.93	0.1736	0.6769
Treatment + (block)	3	270.05	0.0000	10.000
Null	1	275.66	-	-
Treatment	2	277.50	0.1572	0.6918

For each model is reported the degrees of freedom (Model df), the AIC (Akaike's information criterion) values, the chi-square values (χ^2) and the P -values ($\alpha < 0.05$).

Frog color was not associated with avian predator attack rate. 'Transect', however, was significant as a random effect to predict avian attacks in the forest. Although, just counting the replicas placed in the forest, in the first experiment more brown models (five green models and nine brown models) were attacked, and in the second experiment more green models (11 green models and five brown models) were attacked, 'frog model color' continued to not predict avian predator attack rate when the experiments were analyzed separately.

There were significantly more attacks on the anterior part of the replicas (90.5%; Table 2). Seventeen replicas were excluded from the analyses, because they were attacked both in anterior and posterior body regions. Of the 21 remaining, 19 replicas were attacked on the anterior body region and two were attacked on the posterior body region (both brown replicas).

Table 2 Model results for avian attacks on anterior and posterior body regions of clay models. null = null model; body region = anterior or posterior body region as a predictor variable.

Model	Model df	AIC	Resid. Dev.	P
Body region	2	11.38717	0.000	<0.001*
Null	1	25.41216	16.025	-

For each model is reported the degrees of freedom (Model df), the AIC (Akaike's information criterion) values, the residual deviance (Resid. Dev.) and the *P*-values ($\alpha < 0.05$).

Discussion

We found no evidence of differences in bird predation rates on green and brown frog clay models. Our results suggest that the green dorsal color of the red-bellied-toad *M. cambaraensis* does not work as an aposematic color or, at least, is not a signal strong enough to prevent the attack of visually oriented predators. Alternatively, we regard *M. cambaraensis*' green dorsum as a camouflage strategy, what corroborates that green is usually associated with crypsis, as well as other earthy colors such as brown and grey (Wells, 2007). While this result may seem unsurprising, considering the usual association of green with camouflage, it still demands an appealing explanation for the loss of aposematism. This is noteworthy, since aposematism seems to be an ancestral and preponderant characteristic in the "older" South American bufonids of the genus *Melanophryniscus* and *Atelopus* (Pramuk *et al.*, 2008).

The overall attack rate in this experiments was high (47.8%, counting also the marks classified as unknown) compared to other studies (e.g. Brodie, 1993; Kuchta, 2005; Noonan & Comeault, 2009). However, just 3.2% of the replicas were attacked by visually oriented predators in this study. It represents a lower avian predation rate compared to several other studies (mean rate = 6.75%) (e.g. Brodie, 1993; Saporito *et al.*, 2007; Hegna *et al.*, 2011). Yet, the present work is comparable with some other studies that found a lower avian predation rate and more predation attempts by non-visually oriented predators, such as mammals (e.g. Noonan & Comeault, 2009; Kikuchi & Pfennig, 2010).

Bordignon (2016), in the same study area with the same model species, found 5.7% of the replicas attacked by birds. This difference in the avian predation rate may be explained by the different seasons in which the experiments were carried out. While Bordignon (2016) performed the experiment in the fall of 2016, we performed the two

experiments in the spring of the same year. The research site (FLONA-SFP) presents a rich avifauna, which includes 235 bird species from 57 different families (Souza & Fialho, 2013). However, only 38 of these species could be considered as potential predators of frogs in forests, based on their diets and habitats. These species are generalist predators or, at least, are not specialized in amphibians. This lack of anuran specialists may explain the low avian predation rate observed. Furthermore, environmental factors such as prey availability, abundance of alternate food types, and cover for prey hiding (Wunderle, 1981) likely affect avian predation on frogs (Poulin *et al.*, 2001). In a tropical forest in Panama, frog intake by birds peaked in the dry season (Poulin *et al.*, 2001). Although the forest in FLONA-SFP is subtropical and rainfall is regularly distributed along the year, the most intense rains occur during spring and summer (Backes *et al.*, 2005). Frog activity also varies temporally in the region and is strongly associated with pronounced seasonal differences in temperature. So, despite the lack of local studies, it is plausible that food availability for birds is different through seasons, and amphibians may represent only a seasonal inconstant resource. Inconstancy of resource availability indeed may be especially relevant when considering the red bellied-toads explosive breeding behavior (Santos *et al.*, 2010).

Since frog color was not associated with avian predator attack rate, it indicates that these predators attacked all models equally, regardless of the frog color, in the forest and in the breeding site. In another study, it was proposed that the equal avian attack rate between brown control models and aposematic morphs could be due to the similarity in shape and color of the brown models to other poison frogs present at the local study (Hegna *et al.*, 2013). We do not believe it is a shortcoming of our experiment. Besides *Ischnocnema henselii* (our model species), there are other common brown species at the study, like *Adenomera araucaria* and *Proceratophrys brauni*, which are not toxic and approximately resemble in size and color the brown models. Alternatively, the cururu toad *Rhinella icterica*, is a large noxious bufonid toad, very common at the study area. However, adults of this species are much larger than *M. cambaraensis*, and juveniles with similar size present dark spots, unlike our homogeneous brown models. So, it is unlikely that our control brown models were confounded with *R. icterica* by birds.

Within aposematic species, honest signaling remains a highly debated topic (Blount *et al.*, 2009). It has been already shown that conspicuous coloration and toxicity are inversely related in some poison frogs species (Darst *et al.*, 2006; Wang, 2011).

Moreover, like dendrobatid frogs, the *Melanophryniscus* genus is one of the lineages of poison anurans that sequester alkaloid defenses from dietary arthropods (Saporito *et al.*, 2009, 2012; Hantak *et al.*, 2013). Because alkaloid composition vary temporally and spatially, and among individuals and populations (Kwet *et al.*, 2005; Daly *et al.*, 2007, 2008; Grant *et al.*, 2012), it is also important to consider temporal resource variation to discuss about honest signaling in poison amphibians (Hegna *et al.*, 2013). Although these differences in alkaloid composition among species, populations and individuals are not well understood, they probably occur due to variation in habitat use and arthropod availability (Grant *et al.*, 2012). Since there are differences in the toxicity among individuals of the same population (Saporito *et al.*, 2006; Grant *et al.*, 2012), it is possible that the same occurs in this *M. cambaraensis* population. Besides, *M. cambaraensis* is endemic to a very restricted area in the subtropical southeastern Araucaria Plateau in the Atlantic Forest (Santos & Grant, 2011) and presents explosive breeding behavior (Santos *et al.*, 2010), what makes its encounter by predators more difficult. Other green anuran species are more common at the study area and do not present toxins, like *Aplastodiscus perviridis* and *Hypsiboas marginatus*. Adding all these factors, *M. cambaraensis* toxicity probably represents a weak aposematic signal to be learned by visually oriented predators.

The non-random distribution of attacks in frog models (90.5% were predated on the anterior region by birds) suggests that the models were perceived as potential preys (Kuchta, 2005), at least for birds. Some predators preferentially attack directly the head of preys (Smith, 1973, 1976), especially the dangerous and noxious ones (Kuchta, 2005). It is suggested that this predator's tendency to attack the prey's head may represent an adaptation to immobilize dangerous animals, such as snakes (Smith, 1973, 1976). *M. cambaraensis*, however, is characterized by the presence of a poison macrogland in the frontal top of its head. Among the 38 replicas attacked by birds, 13 attacks (34.2%) reached this frontal swelling (four green models and nine brown models). This result, yet not particularly pertinent to our main question, is the first empirical evidence of directional attacks by birds in the red belly-toads and may elucidate the selective forces behind the evolution of the frontal macrogland present in the *Melanophryniscus tumifrons* group.

Birds have been considered as potential predators of red bellied-toads, mainly because of the aposematic coloration, as birds are the most common visually oriented predators of anurans. Besides that, birds represent the main predators in several

aposematic species, like Dendrobatidae frogs (Master, 1999). However, it is important to consider that there is no information about predators of *M. cambaraensis*. Actually, the only record of predation on *Melanophryniscus* (*M. moreirae*) is from a dipsadid snake (Winkler *et al.*, 2011). Notwithstanding the lack of empirical evidence, the presence of aposematic colors in all *Melanophryniscus* species (at least on ventral surface) associated with the unken reflex is suggestive of evolutive pressures molded by visually oriented predators. Snakes and spiders, alternatively, are common predators of small frogs (Poulin *et al.*, 2001), even poisonous species (e.g. Myers, Daly & Malkin, 1978; Summers, 1999). However, it is not expected that the main drivers of aposematic coloration in the red bellied-toads are snakes and spiders. Furthermore, personal observations in the field on this and other *Melanophryniscus* species suggest that not all individuals perform the unken reflex in dangerous situations. Since there might be no bird species specialized in preying amphibians at our study site, it is possible that the ventral aposematic coloration and the defensive behavior of *M. cambaraensis* represent merely ancestral traits of the *Melanophryniscus* genus. If so, as the main predators are not visually oriented, it may help explain the evolution of the green cryptic dorsal coloration of the species. In addition, the suitability of warning signals can depend on the predator community structure (Endler & Mappes, 2004; Mappes *et al.*, 2005; Noonan & Comeault, 2009; Mochida, 2011). In this case, probably some predators were able to cope with the secondary defenses of the species, so there was a selection for reduced conspicuousness, because prey aposematic coloration increased predation pressure (Endler & Mappes, 2004).

Overall, this study suggests that the green dorsal coloration present in the poisonous diurnal frog *M. cambaraensis* is not aposematic, and may be regarded as cryptic. Since the majority of the *Melanophryniscus* species present aposematic dorsal colors, the cryptic dorsal coloration of *M. cambaraensis* has probably evolved secondarily, at least one time in the *Melanophryniscus tumifrons* group. It possibly took place because of the selection pressure imposed by the presence of a very diverse and generalist fauna of visually oriented predators, not specialized in preying amphibians. Besides, the conjunction of a chemical defense based mainly on alkaloids (supposedly more variable), a very restricted geographic distribution, and an inconstant availability, probably induces a fuzzy and weak unprofitability signal to be learned by visually oriented predators. We suggest that these physiological, biogeographical and ecological factors benefited camouflage and a reduced conspicuousness of aposematic signals.

Although the crypsis in unpalatable, poisonous species remains a complex topic, our work provides indirect evidences that the selection pressure imposed by predators, under certain circumstances, may favor crypsis rather than aposematism, even for poisonous and diurnal species. The genus *Melanophryniscus* is a promising alternative model to study many untested assumptions about the evolution, diversity and efficiency of secondary defense strategies in poisonous organisms.

Acknowledgements

We are very thankful to Edenice B. Á. de Souza and the entire Floresta Nacional de São Francisco de Paula team for allowing us to carry out the experiments; Luiz G. Pereira and Milena Sulzbach, from Institute of Physics – UFRGS, for lending us the spectrometer; Ronaldo Gemerasca, from Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul, for confectioning the mold we used to make all the clay replicas; Amrita Menon, Barbara Vicentini, Deivid Pereira, Filipe M. Bianchi, Irina Oliveira, Lilith S. Bizarro, Mariano Shigihara, Matheus Kingeski, Pâmela Friedemann, Patrick Colombo, Paulo Barradas, Pedro B. Luz, Rógger Antunes, Stephanie Rosswag, Thore Teufel, Vinícius Ferri, Vinícius Santos and Yuri Borges for helping us in the fields and/or constructing the clay replicas; Deivid Pereira and Patrick Colombo for collecting *Ischnocnema henselii* specimens for our work; Helena P. Romanowski and Laura Verrastro for all the discussions; Luis F. M. da Fonte for always being so helpful; Ismael Franz for helping us with the bird species; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship; ICMBio for license n° 53177-2.

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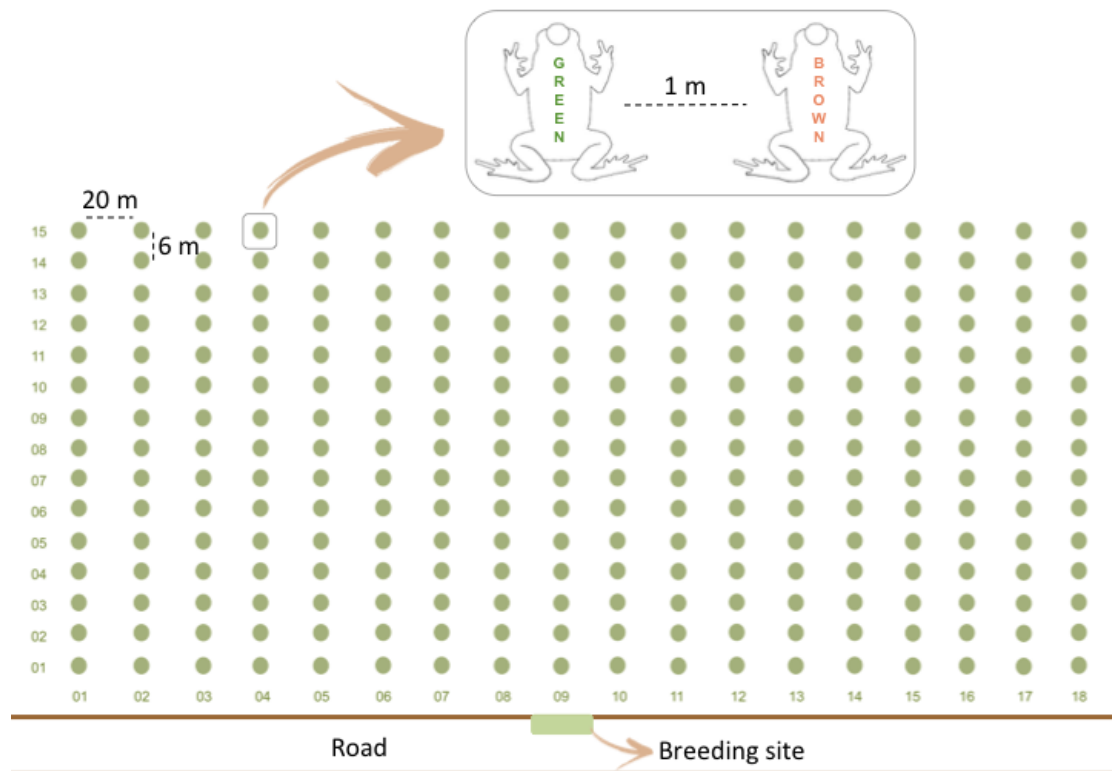
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Supporting Information

Figure S1. Schematic representation of the experimental design utilized in the field experiments of this study.

Supporting Information

Figure S1: Schematic representation of the experimental design utilized in the field experiments of this study. Above the road is represented the adjacent forest: transects are listed on the horizontal, and the blocks of each transect are listed on the vertical. The upper arrow shows in detail how the frog models were distributed in a block. The breeding site of the species is represented by a rectangle on the road.



CAPÍTULO III

Conclusões gerais

Conclusões gerais

Este estudo sugere que a coloração dorsal verde do sapinho-de-barriga-vermelha *Melanophryniscus cambaraensis* não funciona como aposemática, mas como críptica, não corroborando com a hipótese inicial. Isso nos leva a concluir que, aparentemente, assim como a presença da coloração vermelha associada à posição estática de reflexo “unken”, a coloração dorsal não funciona como um sinal eficiente da espécie para alertar predadores visualmente orientados sobre sua toxicidade. Ao contrário, a estratégia utilizado por este anuro tóxico é prevenir sua detecção através da coloração críptica, diferentemente de vários anfíbios tóxicos que possuem coloração aposemática.

A maioria das espécies do gênero *Melanophryniscus*, entretanto, possui tanto coloração aposemática no ventre quanto no dorso, as quais provavelmente representam caracteres ancestrais do gênero, assim como o reflexo “unken”. Então, a coloração dorsal críptica desta espécie possivelmente evoluiu como um caractere secundário. A coloração aposemática dorsal de *M. cambaraensis* devia ser desvantajosa para a espécie em seu habitat. Como cores conspícuas chamam a atenção, um sinal aposemático só é eficiente quando os predadores o associam à impalatabilidade da presa. Já que no local de estudo não há predadores visualmente orientados especialistas em predação anfíbios, a pressão seletiva imposta para estes animais é baixa. Deste modo, além de os predadores se alimentarem apenas ocasionalmente de anuros, eles precisariam aprender a associar a coloração conspícua de *M. cambaraensis* a sua toxicidade. A combinação de uma defesa química baseada principalmente em alcalóides (supostamente mais variável), uma distribuição geográfica muito restrita e uma disponibilidade inconstante nos locais de ocorrência da espécie, provavelmente induziram a um sinal de impalatabilidade fraco e difícil de ser aprendido por predadores orientados visualmente.

Por fim, através dos resultados obtidos neste trabalho, foi possível demonstrar que o aposematismo em espécies tóxicas nem sempre é a estratégia mais vantajosa. A pressão seletiva imposta pelos predadores de um dado ambiente pode favorecer o criptismo, mesmo em espécies venenosas e diurnas. Além disso, o gênero *Melanophryniscus* representa um modelo alternativo promissor para testar várias suposições sobre a evolução, a diversidade e a eficiência de defesas secundárias em organismos venenosos.

ANEXO

Normas para publicação no periódico Journal of Zoology

É estabelecido pelo Programa de Pós-graduação em Biologia Animal que os artigos apresentados na dissertação de Mestrado sejam colocados nas normas editoriais exigidas para publicação direta nos periódicos científicos escolhidos e que as normas sejam incluídas no trabalho. Porém, para facilitar a leitura, algumas normas não foram contempladas na dissertação.

Como há várias diretrizes para os autores seguirem a fim de publicar no periódico Journal of Zoology, neste anexo só foram colocadas as mais importantes para a escrita do artigo. Para ler as normas do periódico na íntegra é necessário acessar o site do periódico: [http://onlinelibrary.wiley.com/journal/10.1111/\(ISSN\)1469-7998](http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1469-7998).

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Papers that report experimental work must comply with the standards and procedures laid down by British national or equivalent legislation and the research

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Examples

Lemelin, P. (1996a). Relationships between hand morphology and feeding strategies in small-bodied prosimians. *Am. J. phys. Anthropol.* (Suppl.) 22, 148.

Lemelin, P. (1996b). The evolution of manual prehensility in primates: a comparative

study of prosimians and didelphid marsupials. PhD thesis, State University of New York at Stony Brook.

Pianka, E. R. (1978). *Evolutionary ecology*. 2nd edn. New York: Harper & Row.

Whitcar, M. (1992). Solitary chemosensory cells. In *Fish chemoreception*: 103-125. Hara, T. J. (Ed.). London: Chapman & Hall.

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