Adriana Moriguchi Jeckel

Defesa química em *Melanophryniscus moreirae*: a diversidade de alcaloides aumenta à medida que os indivíduos envelhecem?

Chemical defense in *Melanophryniscus moreirae*: does alkaloid diversity increase as individuals grow older?

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Comissão Julgadora:

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof. Dr. Taran Grant
Orientador

Dedico esta dissertação aos meus pais,
Emilio e Cristina, meus maiores exemplos de
seres humanos, profissionais e cidadãos.

“Science is always wrong. It never solves a problem without creating 10 more.”

George Bernard Shaw

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Sumário

Apresentação.....	1
Referências.....	8
Capítulo 1 - Sequestered And Synthesized Chemical Defenses In The Poison Frog <i>Melanophryniscus moreirae</i>.....	16
Capítulo 2 - Age Structure And Life-History Traits In The Brazilian Red Bellied Toad <i>Melanophryniscus moreirae</i>	34
Capítulo 3 - Variation In Sequestered And Synthesized Chemical Defenses In The Brazilian Poison Frog: Age Explains Richness, Size Explains Quantity, Sex Explains Nothing.....	54
Conclusão	66
Resumo	68
Abstract	69
Anexos.....	70
Capítulo 1 – Supplementary Figures	71
Capítulo 1 – Supplementary Tables	73
Capítulo 3 – Supplemental Material	76
Biografia	77

Apresentação

O tegumento dos anfíbios é totalmente desprovido de escamas ou pelos, tornando-os mais susceptíveis às ações do ambiente (dissecação, patógenos, predadores, entre outros). Por isso, esses organismos possuem uma grande diversidade de compostos químicos que mantêm a homeostase da pele para processos fisiológicos e outros que compõem a sua defesa química, contra predadores e patógenos (Wells, 2007). Esses compostos são produzidos e armazenados nas glândulas granulosas, as quais estão localizadas na derme e geralmente estão distribuídas por todo o corpo (Toledo & Jared, 1995). Glândulas granulosas podem se acumular em regiões específicas do corpo, formando macroglândulas como as paratoides em *Rhinella* (Jared *et al.*, 2009) e a tumefação frontal em algumas espécies de *Melanophryniscus* (Naya *et al.*, 2004). Os compostos químicos geralmente são sintetizados pelo próprio animal, como as aminas, proteínas, peptídeos e esteroides, mas também podem ser sequestrados da dieta (Erspamer, 1971, 1984; Daly *et al.*, 1994; Saporito *et al.*, 2009). Algumas linhagens de anfíbios também possuem o alcaloide hidrofílico tetrodotoxina, porém sua origem ainda é desconhecida (Daly *et al.*, 1994; Pires *et al.*, 2002; Brodie Jr *et al.*, 2005; Hanifin, 2010).

Os compostos biossintetizados podem ser produtos do metabolismo secundário de compostos comuns no organismo como serotonina, histidina, entre outros, os quais são precursores das aminas biogênicas (Erspamer, 1971). Algumas das aminas conhecidas são as bufoteninas e seus derivados, encontrados especialmente em espécies de Bufonidae (Ceï *et al.*, 1968) e as leptodactilinas, comuns em membros da família Leptodactylidae (Ceï *et al.*, 1967). Alguns esteróis, também provenientes de metabolismo secundário, como as bufodienolidas, são encontrados em várias espécies de bufonídeos (Daly *et al.*, 2008). Outros tipos de compostos biossintetizados são as proteínas e peptídeos, que estão codificados no material genético do indivíduo. Além de estimularem respostas fisiológicas em vários grupos de vertebrados (Erspamer, 1971), algumas proteínas e peptídeos possuem propriedades antimicrobianas (ver König *et al.*, 2015). Aminas, proteínas e peptídeos já foram encontradas em várias linhagens de anuros e são frequentemente usadas como caracteres para taxonomia e para inferir relações filogenéticas dos grupos (Ceï & Erspamer, 1966; Ceï *et al.*, 1967, 1968; Roseghini *et al.*, 1986; Maciel *et al.*, 2006; Conlon, 2011). A presença de bufotenina em *Melanophryniscus moreirae*, por exemplo, contribuiu para a confirmação da relação do gênero com o restante da família Bufonidae, proposta a partir de outras caracteres morfológicos (Ceï & Erspamer, 1966).

Alguns compostos presentes na pele de anuros são sequestrados da alimentação, como terpenos, encontrados em espécie de *Litoria* da Austrália (Smith *et al.*, 2004), e cantaridina, sequestrado por *Lithobates pipiens* de besouros da família Meloidae (Eisner *et al.*, 1990). Entretanto, a presença desses dois compostos não confere uma defesa contra predadores nos testes realizados. Já os alcaloides lipofílicos, presentes em grandes quantidades em membros de alguns gêneros das famílias Dendrobatidae (Daly *et al.*, 1965), Bufonidae, Mantellidae, Myobatrachidae (Daly *et al.*, 1984) e Eleutherodactylidae (Rodríguez *et al.*, 2011), confere proteção por provocar intoxicação e repelir os predadores (Daly *et al.*, 1967; Saporito *et al.*, 2011). A fonte das centenas de alcaloides lipofílicos encontrados em anuros é a dieta à base de artrópodes que contêm alcaloides, como ácaros, formigas, besouros e milípedes (Daly *et al.*, 2002; Saporito *et al.*, 2007; Saporito *et al.*, 2009).

Devido à relação direta com a dieta, existe uma grande variabilidade na composição de alcaloides lipofílicos encontrados em diferentes espécies, populações e indivíduos em diferentes locais e épocas do ano (*e.g.* Saporito *et al.*, 2006, 2007; Daly *et al.*, 2007). Diferenças entre sexos também já foram reportadas (Saporito *et al.*, 2010). Vários fatores podem contribuir para a variação da diversidade de alcaloides, como diferenças na dieta (Donnelly, 1991; Bonansea & Vaira, 2007; Quiroga *et al.*, 2011), a biomodificação de alcaloides sequestrados (Daly *et al.*, 2003; Smith *et al.*, 2002), a diferença na habilidade de sequestrar certos alcaloides entre espécies ou até mesmo entre indivíduos, e a diferença no comportamento e na preferência de alimentos (Saporito *et al.*, 2009). Ainda, Daly e colaboradores (2002) apontaram que, em juvenis, a quantidade de alcaloides era menor quando comparado aos adultos da mesma população. Além disso, Saporito e colaboradores (2010) demonstraram por meio de análises histológicas que as glândulas granulares, que armazenam o veneno no tegumento, tinham um aumento alométrico conforme o crescimento do indivíduo. Tendo em vista que o alcaloide é sequestrado da dieta, pode-se concluir que a diversidade de alcaloides em um indivíduo representa o balanço entre o tempo de vida, liberação do veneno para proteção e a quantidade de alcaloides sequestrados na dieta. Então, é provável que a variação também seja dependente da idade em que os animais são amostrados (Saporito *et al.*, 2011).

Assim como as substâncias sequestradas da dieta, substâncias biossintetizadas por anfíbios também podem ter variabilidade na quantidade e composição de compostos. Diferenças na quantidade já foram relatadas em populações da mesma espécie de distintas localidades (Ceil *et al.*, 1967), em diferentes estágios de vida (Brodie *et al.*, 1978; Hayes *et al.*, 2009), em diferentes épocas do ano (Bowie & Tyler, 2006) e até mesmo entre distintas

regiões do corpo (Maciel *et al.*, 2003; Sciani *et al.*, 2013). Variações na produção de toxinas endógenas também ocorrem de acordo com a quantidade de alcaloides que são sequestrados da dieta em espécies de *Pseudophryne* (Smith *et al.*, 2002). Sugere-se que essas variações sejam geneticamente controladas, mas pouco se sabe sobre a importância ecológica desta variação. No caso da espécie invasora na Austrália, *Rhinella marina*, diferenças na toxicidade em diferentes fases da vida podem ter consequências ecológicas importantes, pois predadores específicos de cada fase são afetados diferentemente e as propostas de controle da espécie devem ser exclusivos para cada estágio de vida (Hayes *et al.*, 2009). Apesar de diferenças de toxicidade em diferentes estágios da vida de anuros terem sido abrangidas por alguns estudos, até agora nenhum estudo foi feito relacionando a idade dos anuros pós-metamórficos com a quantidade e a diversidade de toxinas na pele.

A determinação da estrutura etária de uma população informa dados importantes da dinâmica populacional, como longevidade, idade de maturação e padrões de crescimento, os quais podem ser relacionadas com vários fatores ecológicos, como a toxicidade, por exemplo. Dois métodos de determinação de idade de anfíbios são considerados os mais confiáveis hoje em dia: o método de marcação e recaptura e a osteocronologia (Halliday & Verrel, 1988). Apesar de o primeiro método oferecer dados mais precisos, a grande taxa de mortalidade dos indivíduos e o tempo despendido para a obtenção de um resultado popularizou o uso do segundo método, que dispensa anos de trabalho e fornece informações acuradas, quando interpretadas com atenção (Halliday & Verrel, 1988; Castanet & Smirina, 1990).

A osteocronologia envolve a contagem das linhas de suspensão de crescimento (LAG, em inglês *Lines of Arrested Growth*; Smirina, 1994), formadas durante a época de hibernação ou estivação, alternadas com bandas de crescimento do osso que resultam do rápido crescimento do animal durante os meses de verão. Estações demarcadas por diferença de temperatura são típicas de regiões temperadas (e.g. Sagor *et al.*, 1998; Marangoni *et al.*, 2012; Patón *et al.*, 1991), mas estas linhas de crescimento estão presentes também em anfíbios das regiões tropicais, apesar da ausência de uma estação fria (e.g. Guarino *et al.*, 1998; Lai *et al.*, 2005; Marangoni *et al.*, 2009). A presença de estações chuvosas e secas são as responsáveis pela alternância de crescimento destes animais, que está relacionada com a oferta de alimentos (Guarino *et al.*, 1998). A osteocronologia, por coletar informações demográficas das populações de modo mais rápido que o de marcação e recaptura (Halliday & Verrell, 1988), é muito usada para estudos sobre a idade de maturação (Kumbar & Pancharatna, 2001), longevidade (Guarino *et al.*, 1998) e relações de idade e tamanho do corpo (Liao & Lu, 2010; Esteban *et al.*, 2004; Khonsue *et al.*, 2000) - informações imprescindíveis para um

plano de manejo eficiente para anfíbios em geral, principalmente daquelas que estão ameaçados de extinção (Yetman *et al.*, 2012).

A contagem das LAGs é feita por meio de cortes histológicos da diáfise de ossos longos, como as falanges, úmero e fêmur (Smirina, 1994; Rozenblut & Ogielska, 2005). Em estudos populacionais, geralmente, se utilizam as falanges para as análises, dispensando o sacrifício dos indivíduos (Halliday & Verrell, 1988; Sagor *et al.*, 1998), o que pode ser um aspecto importante para estudo com populações raras ou em perigo de extinção (Smirina, 1994). Entretanto, já foram reportados erros de interpretação e contagem das LAGs com trabalhos feitos com falanges de salamandras (Wagner *et al.*, 2011). Isto ocorre, pois nos ossos longos as LAGs se encontram no perióstio, que pode ser reabsorvido e remodelado pelo endóstio, levando a subestimação da idade dos indivíduos (Halliday & Verrel, 1988).

A remodelação do endóstio é um dos problemas que mais afetam as estimativas da longevidade apresentadas por alguns trabalhos (Castanet & Smirina, 1990), pois além de destruir as primeiras LAGs, esta região do osso também forma linhas que podem seguir o mesmo padrão da formação das LAGs do perióstio. Por isso, é importante que haja indivíduos que não tenham sofrido reabsorção. Como em muitos casos a identificação da reabsorção e remodelação pelo endóstio não é trivial, a utilização de juvenis e recém-metamorfoseados é importante para a reconstrução da formação de LAGs em uma determinada população (Hemelaar, 1985; Castanet & Smirina, 1990).

Outro problema que pode afetar a estimativa de idade baseada nas LAGs é a formação de linhas duplas, que resultam de duas paradas de crescimento em um ano só, que podem superestimar a longevidade dos indivíduos em uma dada população (Smirina, 1994; Esteban *et al.*, 1996). A única forma de superar esse problema é a observação atenta dos padrões de crescimento na população como um todo (Castanet & Smirina, 1990). Além disso, as LAGs podem não estar distintas impossibilitando a identificação das mesmas (Castanet & Smirina, 1990). Isso pode ocorrer por diferenças individuais na deposição mineral dos ossos (Esteban *et al.*, 1996) ou também por problemas na metodologia (Rozenblut & Ogielska, 2005). Neste caso, não existem muitas formas de superar o problema da falta de distinção de LAGs e, em muitos trabalhos, alguns indivíduos tiveram que ser excluídos das análises (Hemelaar, 1985; Esteban *et al.*, 1996).

Por fim, o problema mais comum em animais com maior longevidade são as linhas depositadas nos últimos anos de vida, que podem ser difíceis de distinguir por se acumularem na periferia do osso. Isso acontece pois, apesar de anfíbios terem crescimento indeterminado, a taxa de crescimento diminui consideravelmente depois da maturação sexual e as linhas de

interrupção do crescimento vão ficando cada vez mais próximas (Castanet *et al.*, 1988; Esteban *et al.*, 1996; Wagner *et al.*, 2011). Portanto, antes de iniciar a contagem e a interpretação das LAGs, é necessário que se entenda bem a história natural da espécie estudada e as peculiaridades do método de osteocronologia, para que não haja nem superestimação nem subestimação da idade (Castanet & Smirina, 1990).

Para o presente estudo, o gênero *Melanophryniscus* foi escolhido para testar a hipótese de idade. *Melanophryniscus* inclui 26 espécies principalmente diurnas (Santos & Grant, 2011; Peloso *et al.*, 2012), e é o único gênero conhecido entre os bufonídeos que possui alcaloides lipofílicos para defesa química (Daly *et al.*, 1984; Hantak *et al.*, 2013). Aproximadamente 170 alcalóides em 15 classes estruturais já foram detectados em nove espécies de *Melanophryniscus* (Daly *et al.*, 1984; Garraffo *et al.*, 1993; Mebs *et al.*, 2005; Daly *et al.*, 2007; Mebs *et al.*, 2007; Daly *et al.*, 2008; Garraffo *et al.*, 2012; Grant *et al.*, 2012), sendo os mais comuns: indolizidinas 5,8-dissubstituídas, indolizidinas 5,6,8-trissubstituídas, pumiliotoxinas, tricíclicos e decahidroquinolinas (Daly *et al.*, 2005; Saporito *et al.*, 2011). Além disso, as espécies deste gênero também são capazes de produzir aminas biogênicas típicas de bufonídeos, como a bufotenina (Ceí *et al.*, 1968; Maciel *et al.*, 2003) e outras indolealquilaminas, como dehidrobufotenina, hidroximetil-bufotenina, 5-hidroxitriptamina, N-metil-5-hidroxitriptamina (Ceí *et al.*, 1968; Maciel *et al.*, 2003; Mebs *et al.*, 2007), e uma fenilalquilamina (Ceí & Erspamer, 1966). Entretanto, as análises realizadas para detectar peptídeos ativos na pele das espécies do gênero deram resultados negativos (Erspamer *et al.*, 1986). Para *M. moreirae*, em particular, os compostos defensivos foram relatados em diferentes trabalhos. Ceí e colaboradores (1968; ver também Erspamer, 1994) relataram grandes quantidades de bufotenina, uma indolealquilamina e, posteriormente, Daly e colaboradores (1984) relataram a presença PTX **267C** e allopumiliotoxina (aPTX) **323B**, provavelmente derivada de uma dieta de ácaros (Saporito *et al.*, 2007, 2009, 2011). A presença de compostos químicos sintetizados e sequestrados em uma espécie permite o estudo de possíveis relações entre os dois tipos de compostos, além da relação destas com as diferentes idades dos indivíduos de uma população.

Melanophryniscus moreirae (Fig. 1) é uma espécie endêmica da Serra da Mantiqueira (1800–2400 m) e pode ser facilmente encontrada se reproduzindo em poças temporárias durante as estações mais quentes no Parque Nacional do Itatiaia, na divisa entre os estados do Rio de Janeiro e de Minas Gerais (Miranda-Ribeiro, 1920; Bokermann, 1967). Essa espécie também já foi reportada nos municípios de Itamonte, Minas Gerais (Guix *et al.*, 1998), Queluz no estado de São Paulo, perto do Pico da Pedra da Mina (Marques *et al.*, 2006), e no

município de Aiuruoca, Minas Gerais (Weber *et al.*, 2007). Devido à elevada altitude, durante os meses de frio, as temperaturas podem chegar a 0 °C ou menos no Parque Nacional do Itatiaia (Plano de Manejo do Parque Nacional do Itatiaia - ICMBio). Sabe-se que *M. moreirae* não está ativo entre maio e setembro (Sluys & Guido-Castro, 2011), e que hibernam durante as estações frias (Fernandez-Carvajalino *et al.*, 2013). A hibernação é extremamente relevante para o presente estudo, uma vez que sugere que os LAGs estarão presentes e que cada linha corresponderá a um ano de vida do animal, fazendo de *M. moreirae* um excelente modelo para este estudo.

O objetivo principal deste estudo é relacionar a diversidade de toxinas defensivas em *M. moreirae* com a idade dos indivíduos. Para isso, outras perguntas tiveram que ser respondidas como: (1) quais toxinas estão presentes na pele dos *M. moreirae*? (2) Existe variação inter-individual na diversidade dessas toxinas? Além disso, para a determinação da idade dos indivíduos, surgiram as seguintes perguntas importantes a serem respondidas: (1) qual o melhor osso longo para a determinação da idade em *M. moreirae* utilizando o método de osteocronologia? (2) Como superar os problemas de reabsorção e remodelação do endóstio para a acurácia dos dados levantados por osteocronologia? Ao responder essas perguntas, foi possível descrever a estrutura etária da população de *M. moreirae* do Parque Nacional do Itatiaia e identificar dimorfismos sexuais em longevidade, idade de maturação e tempo de vida reprodutiva.

Esta dissertação está estruturada em três capítulos em formato de artigos científicos. O primeiro, intitulado "**Sequestered and synthesized chemical defenses in the poison *Melanophryniscus moreirae***", reporta a presença de compostos sintetizados pelos indivíduos e aqueles sequestrados da dieta, além de descrever a variação inter-individual destes compostos. Este capítulo foi publicado na *Journal of Chemical Ecology*, uma revista especializada em ecologia química, que publica frequentemente artigos sobre defesa química em anfíbios. O segundo capítulo, intitulado "**Age structure and life-history traits in the Brazilian Red Bellied Toad *Melanophryniscus moreirae***", descreve a estrutura etária de uma população de *M. moreirae*, relatando características como longevidade, idade de maturação e tempo estimado de vida reprodutiva, relacionando a idade com o dimorfismo sexual encontrado. Além disso, este capítulo testa diferentes ossos longos afim de estabelecer o melhor osso para análises osteocronológicas na espécie. Este artigo será submetido para a *Herpetologica*, que é uma revista especializada em herpetologia com bom alcance para divulgação do trabalho. Por fim, o terceiro capítulo, "**Variation in sequestered and synthesized chemical defenses in the Brazilian poison frog: Age explains richness, size**

explains quantity, sex explains nothing", testa, finalmente, a hipótese de que a diversidade de toxinas encontradas no tegumento pode ter uma relação direta com a idade. Este artigo será submetido para a *Biology Letters*, por sua relevância, que abrange não só a comunidade herpetológica, mas todos os grupos biológicos que possam ter histórias naturais similares, como a existência de um sistema de defesa química, produção e sequestro de compostos químicos e variação etária das mesmas. Cada capítulo seguirá a formatação de referências e cabeçalhos que as revistas mencionadas exigem para a submissão do trabalho. Por questões estéticas, as formatações como margem e espaçamento de linhas seguirá o padrão sugerido pelo modelo de dissertações do Departamento de Zoologia do Instituto de Biociências da Universidade de São Paulo.

A.

B.

Figura 1. *Melanophryniscus moreirae*. (A) Vista dorso-lateral, (B) vista ventral.

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Capítulo 1

SEQUESTERED AND SYNTHESIZED CHEMICAL DEFENSES IN THE POISON FROG

Melanophryniscus moreirae

ADRIANA M. JECKEL¹, TARAN GRANT¹, AND RALPH A. SAPORITO²

¹ Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, 05508-090 São Paulo, São Paulo, Brazil

² Department of Biology, John Carroll University, University Heights, Ohio 44118, USA

Abstract – Bufonid poison frogs of the genus *Melanophryniscus* contain alkaloid-based chemical defenses that are derived from a diet of alkaloid-containing arthropods. In addition to dietary alkaloids, bufadienolide-like compounds and related indolealkylamines have been identified in certain species of *Melanophryniscus*. Our study reports, for the first time, the co-occurrence of large quantities of both alkaloids sequestered from the diet and a biosynthesized indolalkylamine in skin secretions from individual specimens of *Melanophryniscus moreirae* from Brazil. GC-MS analysis of 55 individuals of *M. moreirae* revealed 37 dietary alkaloids and the biosynthesized indolealkylamine bufotenine. On average, pumiliotoxin **267C**, bufotenine, and allopumilitoxin **323B** collectively represent ca. 90% of the defensive chemicals present in an individual. The quantity of defensive chemicals differed between sexes, with males possessing significantly less dietary alkaloid and bufotenine than females. Most of the dietary alkaloids have structures with branched-chains, indicating they are likely derived from oribatid mites. The ratio of bufotenine:alkaloid quantity decreased with increasing quantities of dietary alkaloids, suggesting that *M. moreirae* might regulate bufotenine synthesis in relation to sequestration of dietary alkaloids.

Key Words –Alkaloids, Amphibia, Ants, Anura, Bufonidae, Bufotenine, Mites, Oribatids, Pumiliotoxins, Sequestration.

INTRODUCTION

Chemical defenses are widespread among amphibians and represent complex adaptations to protect against predators, microbes, and parasites (Conlon 2011a, b; Mina et al. 2015; Savitzky et al. 2012; Toledo and Jared 1995). Skin secretions of amphibians contain a broad diversity of defensive chemicals that include biogenic amines, peptides, proteins,

bufadienolides, tetrodotoxins, and lipophilic alkaloids (Daly 1998; Daly et al. 2005; Saporito et al. 2012). Amphibians produce most of these defensive chemicals, but multiple lineages of poison frogs, including some bufonids (*Melanophryniscus*), dendrobatids (*Epipedobates*, *Ameerega*, and Dendrobatinae), mantellids (*Mantella*), and myobatrachids (*Pseudophryne*), sequester alkaloid defenses from dietary arthropods (Hantak et al. 2013; Saporito et al. 2009, 2012). It is also likely that the alkaloids present in certain species of Eleutherodactylidae (*Eleutherodactylus limbatus* group) from Cuba result from dietary sequestration (Rodríguez et al. 2010).

Although dietary alkaloids are generally considered the main defensive compounds in poison frogs, the presence of both sequestered and biosynthesized defensive chemicals has been reported in a few species. Myobatrachids (*Pseudophryne* spp.) sequester pumiliotoxin alkaloids (PTXs) from a natural diet of arthropods and also synthesize unique pseudophrynamine alkaloids (Smith et al. 2002). Sequestration of large quantities of PTXs appears to inhibit the synthesis of pseudophrynamines, suggesting that *Pseudophryne* can regulate the production of their manufactured defenses in response to the availability of sequestered defenses (Smith et al. 2002). The biosynthesized indolealkylamine, 5-hydroxytryptamine, has also been reported in the skin of *Pseudophryne* (Erspamer 1994). Similarly, in addition to sequestered alkaloids, small amounts of the biosynthesized peptide carnosine and trace levels of bufadienolide-like compounds have been reported in certain dendrobatids (Daly et al. 1987, Table 1 and references therein). Small quantities of bufadienolide-like compounds have also been identified in skin extracts of certain bufonids in the genus *Melanophryniscus* (Daly et al. 2008; Flier et al. 1980; but see Mebs et al. 2007a), as well as a number of indolealkylamines, including 5-hydroxytryptamine, N-methyl 5-hydroxytryptamine, bufotenine, and dehydrobufotenine (Ceï et al. 1968; Daly et al. 1987; Erspamer 1994; Maciel et al. 2003; Mebs et al. 2007a) and the phenol hydroquinone (Mebs et al. 2005, 2007b).

The Brazilian red belly toad *Melanophryniscus moreirae* has been reported to contain both sequestered alkaloids and the biosynthesized indolealkylamine bufotenine, albeit not in the same studies. Ceï et al. (1968; see also Erspamer 1994) reported large amounts of bufotenine in the skin of 3,190 individuals of *M. moreirae*. Subsequently, Daly et al. (1984) reported PTX **267C** and allopumiliotoxin (aPTX) **323B** in the skin of 52 individuals, which were likely derived from a diet of oribatid mites (Saporito et al. 2007, 2009, 2011). The occurrence of both large quantities of biosynthesized indolealkylamines and dietary derived

alkaloids in *M. moreirae* provides a rare opportunity to explore the relationship between synthesized and sequestered chemical defenses in a poison frog.

The main goals of the present study were to corroborate the co-occurrence of bufotenine and alkaloids in skins of *M. moreirae* from Serra da Mantiqueira, Brazil, and determine if a relationship exists between the quantities of these biosynthesized and sequestered chemical defenses (bufotenine and alkaloids, respectively). Dietary alkaloids are known to vary among individuals, between sexes, and over time (Saporito et al. 2012), so variation among individuals and between sexes in both the quantity of bufotenine and the composition (type, quantity, and number) of alkaloids was examined in *M. moreirae*.

METHODS AND MATERIALS

Sample Collection. A total of 55 adult toads (40 males, 15 females) were collected in Itatiaia National Park (Serra da Mantiqueira, Rio de Janeiro, Brazil, GPS coordinates: 22°23'05.88" S, 44°40'41.83" W) on November 30, 2013. All toads were measured for snout-to-vent length (nearest 0.1 mm), sexed, and weighed (nearest 0.1 mg). The entire skin was removed from each individual toad. Skin samples were stored in individual 4 mL glass vials with Teflon-coated lids, containing 100% methanol (hereafter, referred to as methanol extracts). Specimens are deposited in the amphibian collection of the Museu de Zoologia da Universidade de São Paulo under voucher numbers MZUSP 154089–154091, 154093–154106, 154109–154146.

Chemical Analyses. Alkaloids and bufotenine were isolated from individual methanol extracts using an acid-base extraction (following Saporito et al. 2006). In brief, 10 µg of nicotine ((-)-nicotine ≥ 99%, Sigma-Aldrich) in a methanol solution (internal standard) and 50 µL of 1 N HCl were added to 1 mL of the original methanol extract. This combined methanol extract was concentrated with nitrogen gas to 100 µL and then diluted with 200 µL of deionized water. This solution was then extracted four times, each time with 300 µL of hexane. The aqueous layer was then treated with saturated NaHCO₃, followed by extraction 3 times, each time with 300 µL of ethyl acetate. The combined ethyl acetate fractions were dried with anhydrous Na₂SO₄, evaporated to dryness, and then reconstituted with methanol to 100 µL.

Gas chromatography-mass spectrometry (GC-MS) analysis was performed on a Varian Saturn 2100T ion trap MS instrument coupled to a Varian 3900 GC with a 30 m 0.25 mm i.d. Varian Factor Four VF-5ms fused silica column. GC separation was achieved by

using a temperature program from 100 to 280 °C at a rate of 10 °C per minute with helium as the carrier gas (1 mL/min). Alkaloid/bufotenine fractions were analyzed with both electron impact MS (EI-MS) and chemical ionization MS (CI-MS) with methanol as the CI reagent. Vapor phase Fourier-transform infrared spectral data (GC-FTIR) were obtained using a Hewlett-Packard model 5890 gas chromatograph, with an Agilent J&W DB-5 capillary column (30m, 0.25 mm i.d., 0.25 µm), using the same temperature program as above, coupled with a model 5965B (IRD) narrow band (4000-750 cm⁻¹) infrared detector.

Individual alkaloids were identified by comparison of the observed MS properties (and FTIR properties for PTX **251D** and bufotenine) and GC retention times (R_t) with those of previously reported anuran alkaloids (Daly et al. 2005). Most anuran alkaloids have been assigned code names that consist of a bold-faced number corresponding to the nominal mass and a bold-faced letter to distinguish alkaloids of the same nominal mass (Daly et al. 2005). Identification of pumiliotoxin **251D** and bufotenine was based on comparisons to reference standards of each compound (PTX **251D**: Daly et al. 2003; **Bufotenine**: bufotenine solution, B-022, Cerilliant, Sigma-Aldrich). Isomers of previously characterized alkaloids were tentatively identified based on comparisons of EI mass spectral data and GC retention times. Following the methods of Garraffo et al. (2012), alkaloids were considered new isomers if they shared identical EI-MS data to a previously identified alkaloid, but differed in their $R_t \pm 0.15$ min from the R_t previously reported (Daly et al. 2005). The only exception to this was a tentative new isomer of aPTX **305A**, which differed by only 0.07 min from the previously identified aPTX (see *Supplementary Information*).

Individual frog skin extracts were analyzed in three chromatographic replicates and the average quantity of defensive compounds was determined by comparing the observed alkaloid peak areas to the peak area of the nicotine internal standard, using a Varian MS Workstation v.6.9 SPI. It should be noted, however, that our quantification using nicotine should be considered ‘semi-quantitative’. The response of the MS detector is expected to differ for individual alkaloids, and ideally, a unique standard for each alkaloid should be used for absolute quantification; however, standards are not available for most alkaloids, especially new alkaloids, and therefore this was not possible.

Statistical analysis. Non-metric multidimensional scaling (nMDS) was used to visualize and compare alkaloid composition (number, type, and quantity of alkaloids), and a one-way analysis of similarity (ANOSIM) was used to test for statistical differences between males and females. nMDS and ANOSIM were based on Bray-Curtis similarity matrices, and

these analyses were performed using PRIMER-E version 5. Differences in the quantity of sequestered alkaloids and manufactured bufotenine between males and females were examined using independent samples t-tests. The quantity of alkaloids and bufotenine were corrected for wet skin mass, and analyses for both the corrected and uncorrected quantities are reported. Linear regression was used to determine if the (1) quantity of alkaloids is related to the number of alkaloids, (2) quantity of alkaloids is related to the snout–vent length (SVL) and wet skin mass, and (3) number of alkaloids is related to the SVL and mass of males and females. Linear regression was also used to examine differences in the ratio of bufotenine:alkaloid quantity as a function of the total quantity of alkaloid per frog. In order to meet the assumptions of normality and homoscedasticity, these data were log₁₀-transformed. All parametric statistical analyses were performed using the statistical package R-3.0.1 (R Core Team 2013). All error is reported as ± 1 S.E.

RESULTS

GC-MS analysis of 55 individual *Melanophryniscus moreirae* skin extracts resulted in the detection of 37 dietary alkaloids (including isomers), representing seven different structural classes (Table 1). In addition, all of the samples contained the indolealkylamine bufotenine. We did not detect the phenol hydroquinone in any samples. Five of the dietary alkaloids present in our samples have been reported in other species of *Melanophryniscus*, including allopumiliotoxin (aPTX) **323B** and the pumiliotoxins (PTX) **251D**, **265D**, **267C**, and **323A** (Table 1). Eleven alkaloids are new and have not been detected previously in poison frogs, and a number of tentative new isomers of previously characterized alkaloids were identified (Table 1). The mass spectral data and retention times for all 11 new alkaloids are available in Figure 1 of the *Supplemental Information*, and the retention times for all of the new isomers are included in Table 1 of the *Supplementary Information*.

Overall, the three most abundant dietary alkaloids were PTX **267C** (average quantity in 55 samples = 279 ± 32 μ g per skin), aPTX **323B** (37 ± 6 μ g per skin), and tricyclic (Tri) **265S** (16 ± 2 μ g per skin) (see Fig. 1 for alkaloid structures), representing 89% of the total dietary alkaloid quantity and 71% of the total mass of chemical defenses (dietary alkaloids + bufotenine) in *M. moreirae*. PTX **267C** was present in all individuals, whereas aPTX **323B** and TRI **265S** were present in all but two individuals. PTX **265D** was present in all but three individuals, and PTX **323A**, aPTX **337D**, and an isomer of PTX **267C** were present in ca. 70% of individuals. aPTX **323B** (isomer), aPTX **305A**, and 5,8-disubstituted indolizidine (5,8-I) **297G** were present in ca. 30% of all individuals examined. The remaining 28 dietary

alkaloids were detected in only a few (1–15) individuals and in variable quantities (0.5–42 µg per skin).

The total number and quantity of dietary alkaloids differ among individual skin extracts. Individuals possessed 5–17 alkaloids (**mean**: 10 ± 1 per skin) and 37–1382 µg of alkaloids (**mean**: 369 ± 35 µg per skin). There is no linear relationship between the total number and quantity of dietary alkaloids among individuals ($F_{1,53} = 0.539$, $p = 0.466$). There is no linear relationship between the number of dietary alkaloids and wet skin mass or SVL (**skin mass**: $F_{1,53} = 0.003$, $p = 0.958$; **SVL**: $F_{1,53} = 0.071$, $p = 0.791$); however, there is a linear relationship between the quantity of dietary alkaloids and wet skin mass ($F_{1,53} = 17.41$, $p < 0.001$) and SVL ($F_{1,53} = 15.19$, $p < 0.001$).

Females are significantly larger than males in SVL ($t_{53} = 10.05$, $p < 0.001$) and wet skin mass ($t_{53} = 8.56$, $p < 0.001$). Dietary alkaloid composition (Global $R = 0.098$, $p = 0.071$; Fig. 2) and the total number of dietary alkaloids did not differ significantly between males and females ($t_{53} = 0.491$, $p = 0.625$; **mean number males**: 10 ± 1 per skin; **mean number females**: 10 ± 1 per skin).. Although females had significantly larger quantities of dietary alkaloids compared to males ($t_{53} = 4.28$, $p < 0.001$; **mean QTY males**: 289 ± 32 µg per skin; **mean QTY females**: 582 ± 72 µg per skin), when corrected for wet skin mass, the total quantity of dietary alkaloids did not differ significantly between males and females ($t_{53} = 1.83$, $p = 0.073$; **mean QTY males**: 2 ± 0.2 µg per skin wet mass; **mean QTY females**: 2 ± 0.3 µg per skin wet mass).

Bufotenine was the second most abundant defensive compound in terms of quantity (average quantity in 55 samples = 103 ± 11 µg per skin), representing on average 22% of the total mass of chemical defenses in *M. moreirae*. Bufotenine was detected in all individuals, albeit in variable quantities (4–395 µg per skin). Although females contained significantly larger quantities of bufotenine compared to males ($t_{53} = 3.45$, $p < 0.001$; **mean QTY male**: 83 ± 8 µg per skin; **mean QTY female**: 157 ± 29 µg per skin), when corrected for wet skin mass, there is no statistical difference in bufotenine quantity between males and females ($t_{53} = 1.30$, $p = 0.200$; **mean QTY male**: 0.5 ± 0.04 µg per skin wet mass; **QTY female**: 0.6 ± 0.1 µg per skin wet mass). There is a positive linear relationship between bufotenine quantity and wet skin mass ($F_{1,53} = 15.98$, $p < 0.001$) and SVL ($F_{1,53} = 14.30$, $p < 0.001$). The ratio of bufotenine:alkaloid quantity decreased significantly with increases in total alkaloid quantity ($F_{1,53} = 44.75$, $p < 0.001$, $r^2 = 0.46$; Fig. 3). When analyzed separately, the ratio of bufotenine:alkaloid quantity decreased significantly with increases in total alkaloid quantity

in males ($F_{1,38} = 49.00$, $p < 0.001$, $r^2 = 0.56$; Fig. 4), but there was no relationship in females ($F_{1,13} = 0.88$, $p = 0.370$; Fig. 3).

The rank order of defensive chemicals by quantity varied extensively among individuals, but the three most abundant chemicals were consistently PTX **267C**, bufotenine, and aPTX **323B** (Table 1 of the *Supplemental Information*). The most abundant chemical was PTX **267C** in 45 individuals (82%), followed by bufotenine in 8 (14%) and aPTX **323B** in 2 (4%). When PTX **267C** was most abundant, it was 1.0–13.6 (3.5 ± 0.4) times more abundant than the next most abundant chemical and comprised 38–79% ($61.2 \pm 0.02\%$) of the total quantity of defensive chemicals; when bufotenine was most abundant, it was 1.1–4.5 (1.9 ± 0.4) times more abundant and comprised 36–71% ($49.2 \pm 0.04\%$) of the total quantity; and when aPTX **323B** was most abundant, it was 1.7–2.1 (1.9 ± 0.2) times more abundant and comprised 34–43% ($38.0 \pm 0.04\%$) of the total quantity. The second most abundant chemical was bufotenine in 39 individuals (71%), followed by aPTX **323B** in 12 (22%), and PTX **267C** in 4 (7%). The third most abundant chemical was TRI **265S** in 22 individuals (40%), followed by aPTX **323B** in 18 (33%), PTX **267C** in 5 (9%), bufotenine in 4 (7%), PTX **265D** in 3 (5%), and PTX **323A**, aPTX **323B**, and aPTX **337D** in a single individual (2%) each.

DISCUSSION

Our study reports, for the first time, the co-occurrence of large quantities of both sequestered dietary alkaloids and the biosynthesized indolealkylamine bufotenine in the same skin secretions of *M. moreirae*. The defensive chemicals of nine of the 26 species of *Melanophryniscus* (Frost, 2014) have been examined (including the present study), revealing approximately 200 dietary alkaloids organized into 15 different structural classes (Daly et al. 2007; Garraffo et al. 2012; Grant et al. 2012). As in other poison frogs, the large variety of dietary alkaloids in bufonids appears to reflect a similar diversity of alkaloids in their arthropod prey (Saporito et al. 2009, 2012).

The three most abundant defensive chemicals in the skins of *M. moreirae* were pumiliotoxin (PTX) **267C**, bufotenine, and allopumiliotoxin (aPTX) **323B** (Fig. 1), respectively, which collectively represent almost 90% of the average quantity of defensive chemicals present in an individual toad. Bufotenine alone comprises > 22% of these chemicals, and ranks as one of the three most abundant chemicals in 93% of toads, accounting for up to > 70% of the total quantity of defensive chemicals in individual toads. These findings suggest that bufotenine plays a prominent role in *M. moreirae* chemical defense. Some pumiliotoxins and allopumiliotoxins are known to be quite toxic, with LD50 values of

ca. 2.5 mg/kg mouse (Daly et al. 2005). The specific activity of PTX **267C** is unknown, but it appears to be an effective defense against arthropods (Weldon et al. 2013). aPTX **323B** is a voltage-dependent sodium channel agonist, causing activation of sodium flux (Daly et al. 1990). Bufotenine is a toxic (LD50 = 1.3 mg/kg mouse; Erspamer 1994), hallucinogenic (Ujváry 2014) indolealkylamine with antiviral activity (Vigerelli et al. 2014).

Pumiliotoxin and allopumiliotoxin alkaloids are derived from dietary mites (Saporito et al. 2007, 2009, 2011), and both PTX **267C** and aPTX **323B** are widespread among poison frogs, occurring in bufonids, dendrobatids, mantellids, and myobatrachids (Daly et al. 2005). In contrast, although the serotonin-derived bufotenine is synthesized by frogs and is widespread among amphibians (Daly et al. 1987; Erspamer 1994; Mebs et al. 2007a), it appears to be rare among poison frogs, in which it has been reported exclusively in *M. moreirae* (Cei et al. 1968; Erspamer 1994), *M. cambaraensis* (Maciel et al. 2003), and possibly *M. stelzneri* (small quantity tentatively reported by Cei et al. 1968, but not detected in subsequent studies; e.g., Daly et al. 2007; Hantak et al. 2013).

Individuals of *M. moreirae* that had higher levels of alkaloids also contained more bufotenine, and variation in both bufotenine and the most abundant alkaloids was similar. Interestingly, however, the ratio of bufotenine:alkaloid quantity decreased with increasing quantities of dietary alkaloids (Fig. 3), suggesting that these toads might regulate bufotenine production in relation to the total quantity of sequestered dietary alkaloids. A similar possibility was suggested by Smith et al. (2002), who proposed that high levels of dietary pumiliotoxins in *Pseudophryne* spp. (myobatrachid poison frogs) might turn off biosynthesis of pseudophrynamine alkaloids. It is also possible that bufotenine production is merely correlated with, but is not regulated in response to, alkaloid uptake, and further investigation is required to test the causal relationship between acquired and biosynthesized chemical defenses in poison frogs, including *M. moreirae*.

The quantity of dietary alkaloids and bufotenine increased with skin mass and SVL. The greater quantity of dietary alkaloids in larger individuals is probably related to individual age. Anurans possess indeterminate growth, and among conspecifics, larger frogs are usually older than smaller frogs (e.g., Monnet and Cherry 2002). Dietary alkaloids in poison frogs accumulate over an individual's lifetime, and therefore older individuals usually will have consumed more alkaloids than younger individuals. Although the relationship between age, size, and alkaloid quantity has not been tested among adults, in the dendrobatid *Oophaga pumilio*, larger tadpoles possess more alkaloids than younger, smaller ones and adults possess more alkaloids than juveniles (Stynoski et al. 2014). The increase in amount of bufotenine

with toad size suggests that larger and presumably older individuals either produce or retain more of this indolealkylamine than smaller, younger individuals.

The composition of dietary alkaloids did not differ significantly between sexes. In contrast, the quantity of defensive chemicals did differ between sexes, with males possessing significantly less dietary alkaloid and bufotenine than females; however, females were larger than males, and size-corrected values were not significantly different. Previous studies have reported mixed findings on sex-related differences in dietary alkaloid defenses. The clearest evidence was found in the dendrobatid *Oophaga pumilio*, which exhibits differences in composition, in number, and quantity of alkaloids that are not attributable to differences in size (Saporito et al. 2010). Among species of *Melanophryniscus*, Daly et al. (2007) examined two breeding pairs of *M. stelzneri* and found that nine alkaloids present in trace amounts were restricted to one sex. Garraffo et al. (2012) did not detect sex-related differences in *M. rubriventris*, but the sample included only a single female. Overall alkaloid composition did not differ significantly between males and females of the mantellid poison frog *Mantella bernhardi*, although certain alkaloids were more common in one sex (Daly et al. 2008). Sex-related differences in quantity of bufotenine have not been studied previously.

In addition to PTX **267C** and aPTX **323B**, which occurred in all 55 individuals and comprised the principle dietary alkaloids, we detected 35 additional alkaloids (Table 1), almost all of which are branched chain compounds and are likely derived from oribatid mites (Saporito et al. 2007, 2011). There was no relationship between the number and quantity of alkaloids detected, which reflects the fact that chemical defenses of *M. moreirae* are dominated by a few alkaloids that are present in large quantities. In their study of 52 toads collected 35 years ago at the same locality, Daly et al. (1984) found only PTX **267C** and aPTX **323B**. The composition of dietary alkaloids is known to vary among frogs in the same population over time, presumably in relation to variation in arthropod availability (Daly et al. 2007; Saporito et al. 2006, 2007), which might explain our different results; however, it is also likely that improvements in instrument sensitivity allowed us to detect alkaloids that were overlooked previously.

The causes and consequences of the extensive individual variation observed in the alkaloid composition of poison frog skin are poorly understood. Studies have shown that intraspecific alkaloid variation can be explained by frog sex and geographic and temporal variation in arthropod availability (reviewed by Saporito et al. 2012), but other potential causes, such as post-metamorphic age, fine-scale alkaloid abundance in different microhabitats, and genetic differences in alkaloid uptake capacity, remain to be studied. We

found that the ratio of bufotenine:alkaloid quantity decreased with increasing quantities of dietary alkaloids, suggesting that *M. moreirae* might regulate bufotenine synthesis in relation to sequestration of dietary alkaloids. In order to understand the biological significance of our observations, it must be determined if a regulatory mechanism indeed exists or if variation in the ratio of bufotenine:alkaloid quantity is due to some other underlying cause (e.g., individual age), and the effectiveness of bufotenine and dietary alkaloids in defending against predators, parasites, and pathogens must be evaluated.

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TABLE

Table 1. Alkaloids identified in *Melanophryniscus moreirae* arranged by structural class.

Structural Class	5,8-I	5,6,8-I	PTX	aPTX	hPTX	Tri	Unclass
1	225D	225L	251D	293K	281K	261J	237W
2	241K(1)	277E(1)^a	253F	305A(1)^f		265S(1)^h	251GG
3	297G	279F(3)^b	265D	323B(2)^g			281R
4		281H^c	267C(2)^d	337D			283H
5		295G	295F^e				
6		297H	323A				

Alkaloids that are **underlined** represent new alkaloids that have not yet been previously described (see *Appendix*).

The number of isomers detected for each alkaloid are indicated by parentheses.

^a Two new isomers of 5,6,8-I **277E** were identified, both represent new isomers.

^b Two new isomers of 5,6,8-I **279F** were identified.

^c One new isomer of 5,6,8-I **281H** was identified.

^d One new isomer of PTX **267C** was identified.

^e One new isomer of PTX **295F** was identified.

^f One new isomer of aPTX **305A** was identified.

^g One new isomers of aPTX **323B** were identified.

^h One new isomer of Tri **265S** was identified.

Abbreviations for alkaloid structural classes are as follows: 5,8-I (5,8-disubstituted indolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); PTX (pumiliotoxin); aPTX (allopumiliotoxin); hPTX (homopumiliotoxin); Tri (tricyclic); Unclass (unclassified as to structure).

FIGURES

Fig. 1 Chemical structures of the three most abundant defensive chemicals in *Melanophryniscus moreira*.

Fig. 2 nMDS plot of alkaloid composition between males and females of *Melanophryniscus moreirae*. Each circle represents an individual male or female frog, and the distance between symbols represents the difference in alkaloid composition. The diameter of each circle is proportional to the quantity of alkaloids present in that frog (μg per frog skin).

Fig. 3 Relationship between bufotenine quantity/alkaloid quantity (μg per frog skin) and the quantity of alkaloids (μg per frog skin) in males and females of *Melanophryniscus moreirae*. Filled circles indicate males and open circles indicate females. Graph axes are $\log_{10}$ -scaled.

Capítulo 2

AGE STRUCTURE AND LIFE-HISTORY TRAITS IN THE BRAZILIAN RED BELLIED TOAD *Melanophryniscus moreirae*

ADRIANA M. JECKEL¹ and TARAN GRANT¹

¹ Departamento de Zoologia, Instituto de Biociências, Universidade de
São Paulo, 05508-090 São Paulo, São Paulo, Brazil

Abstract - Information on age structure, longevity, age at maturity, and reproductive lifespan are important to answer questions in ecology and evolutionary biology. Toads of the South American genus *Melanophryniscus* have been studied extensively in recent years, but information on age is unavailable for any species in the clade. We used skeletochronology to infer the age of 62 juvenile and adult individuals of *Melanophryniscus moreirae*, a species that is endemic to the high elevation Serra da Mantiqueira plateau in southeastern Brazil. Periosteal growth marks were most evident in femora, with analyses of humeri and phalanges being either impossible due to poor definition of growth marks or underestimating ages by 1–2 years. Adult age varied from 5–7 years in females (median and mode = 6) and 4–8 years in males (median and mode = 5). Snout–vent length (SVL) was sexually dimorphic in adults (sexual dimorphism index = 0.13), with females (mean SVL = 26.2 ± 0.2 mm) being significantly larger than males (mean SVL = 23.2 ± 0.2 mm). Similarly, mean adult age was greater for females (6.1 ± 0.21 years) than for males (5.4 ± 0.13 years), suggesting that sexual size dimorphism might be a consequence of different age structures in female and male populations. However, adult females were larger than adult males of the same age, which shows that age structure merely exacerbates an effect that is already present within age cohorts. Age and mean size at maturity were greater for females (5 years, 26.5 ± 0.5 mm SVL) than males (4 years, 23.8 ± 0.2 mm SVL). Our sample included two juvenile females that were 5- and 6-years-old, respectively; nevertheless, these two adult-aged juveniles were considerably smaller than both the mean female size at maturity and the smallest adult female, showing that female sexual maturity is causally related to size, not age. Juvenile males were absent from our sample, so we are unable to determine if age and size at maturity are also decoupled in males of *M. moreirae*. Although both age and size at maturity are greater for females than males, females do not have increased longevity (female longevity = 7; male

longevity = 8); consequently, the potential reproductive lifespan of males (4 years) is twice that of females (2 years).

Keywords—Age at maturity, Anura, Amphibia, Bufonidae, LAG, Longevity, Reproductive lifespan; Skeletochronology.

ANSWERS to many questions in ecology and evolutionary biology require knowledge of the age of sampled individuals. Age at maturity, reproductive lifespan, and longevity are important life-history traits (e.g., Stearns 2000), and age structure is a key variable in population dynamics models (e.g., Coulson et al. 2000). Individual age can explain differences in size between species (e.g., Tessa et al. 2011), populations (e.g., Alcobendas and Castanet 2000; Miaud et al. 2001; Hasumi and Borkin 2012), and sexes (e.g., Monnet and Cherry 2002). Information on age can also have important implications for the conservation of threatened species (e.g., Yetman et al. 2012).

The age of individual amphibians cannot be reliably inferred from measurements of body length and mass because body size is affected by environmental and genetic factors that are unrelated to age (Halliday and Varrell 1988). The most accurate method is to capture, mark, and recapture (CMR) individuals over a prolonged period of time, which provides direct evidence of either the absolute or minimum age of recaptured individuals. However, this method is extremely labor-intensive, requiring that fieldwork be conducted over many years for long-lived species and that a very large number of recently metamorphosed individuals be marked so that at least a few of them can be expected to survive to be recaptured (Halliday and Verrell 1988).

As an alternative to CMR, the most widely used method of determining the age of individual amphibians is skeletochronology, which infers age from incremental skeletal growth marks that are correlated with predictable, periodic events (Sinsch 2015). During periods of reduced metabolic rates, such as hibernation or estivation, bones slowly produce dense layers of osteocyte matrix that are organized into discrete lines of arrested growth (LAGs; Rozenblut and Ogielska 2005). In bone formed during periods of greater metabolic activity, osteocytes deposit matrix quickly and blood vessels are produced. In species found in regions characterized by well-defined seasons, the alternating pattern of LAGs and loose matrix can be used to infer individual age. Although not without difficulties, especially in studies of long-lived species (Eden et al. 2007; Wagner et al. 2011; Sinsch 2015),

skeletochronology has also been shown to estimate the age of individual amphibians accurately (e.g., Matsuki and Matsui 2009; Sinsch 2015).

Melanophryniscus comprises 26 species distributed in tropical and subtropical South America between 22–39°S in Argentina, Bolivia, Brazil, Paraguay, and Uruguay (Zank et al. 2014). Most species have highly restricted distributions, making them especially vulnerable to geographic shifts in climate suitability (Zank et al. 2014) and environmental degradation (e.g., Caorsi et al. 2014). Four species of *Melanophryniscus* are currently listed as endangered or critically endangered, with another six listed as vulnerable or near threatened (IUCN Red List of Threatened Species 2015).

Since 2010 there has been a flurry of research on this genus, increasing knowledge on species diversity and relationships (e.g., Caramaschi and Cruz 2011; Baldo et al. 2012; Peloso et al. 2012), adult and larval morphology and development (e.g., Haad et al. 2011; Bidau et al. 2011; Bonansea and Vaira 2012; Baldo et al. 2014; Kurth et al. 2014), cytogenetics (Baldo et al. 2012), chemical defense (e.g., Garaffo et al. 2012; Grant et al. 2012; Hantak et al. 2013; Jeckel et al. 2015), activity and movement patterns (Santos et al. 2010; Santos and Grant 2011; Sluys and Guido-Castro 2011; Cairo et al. 2013; Sanabria et al. 2014), diet (Quiroga et al. 2011), vocalizations (Caldart et al. 2013; Kurth et al. 2013), species distribution models and climate change (Toranza and Maneyro 2013; Zank et al. 2014), and helminth parasite load (Hamann et al. 2014). Nevertheless, despite these major advances, the only age-related information available for any species in *Melanophryniscus* refers to the development of *M. klappenbachi*, in which hatching occurs within 48 h of oviposition and metamorphosis is completed within 10–28 d (Kurth et al. 2014). Information on the age of post-metamorphic individuals is unavailable for any species of *Melanophryniscus*.

In the present study, we used skeletochronology to infer the ages of juvenile and adult individuals of *Melanophryniscus moreirae*. This species is endemic to the high altitude (1800–2400 m) mountain range Serra da Mantiqueira (Bokermann 1967; Marques et al. 2006; Weber et al. 2007) where winter temperatures reach as low as -7 °C and dormant toads lie concealed in hibernacula 5–15 cm deep in soil and ravines (Carvajalino-Fernández et al. 2013). Given this seasonal behavior, we predicted that the LAGs would be conspicuous, with each one corresponding to one winter in the individual's life. We used individual ages to calculate age structure, age at maturity, and longevity for each sex.

MATERIALS AND METHODS

Sample Collection

A total of 63 *Melanophryniscus moreirae* (41 males, 15 females, 7 juveniles) were collected in Itatiaia National Park (Serra da Mantiqueira, Rio de Janeiro, Brazil, GPS coordinates: 22°23'05.88" S, 44°40'41.83" W) on November 30, 2013. All toads were measured for snout-vent length (SVL, nearest 0.1 mm) and sexed. Sex was determined by examination of gonads. Males with vocal slits and nuptial pads were scored as adults and those lacking these structures were scored as juveniles. Females with enlarged, differentiated ova and convoluted oviducts were scored as adults and those with undifferentiated ova and narrow, straight oviducts were scored as juveniles. Specimens were fixed in 10% formalin, preserved in 70% ethanol and deposited in the amphibian collection of the Museu de Zoologia da Universidade de São Paulo under voucher numbers MZUSP 154089–154151.

Skeletochronology

To determine the most appropriate long bone to use to infer individual age, we carried out a preliminary histological study of mid-diaphyseal sections of femora, humeri, and phalanges taken from 12 specimens. Based on those results (see below), we used femora to infer the age of all specimens. Bones were decalcified in a 15% formic acid solution for 24–96 h, depending on size, and embedded in paraffin wax. A rotary microtome was used to cut 7 µm sections that were subsequently stained with Mayer's haematoxylin and eosin (modified from Kusrini and Alford 2006). Sections were analyzed and photographed in a Nikon Eclipse 80i light microscope equipped with digital camera Nikon DS-Ri1. We selected three sections from the mid region of the diaphysis and with the smallest medullar cavity of each individual and photographed for measurements using ImageJ 1.48v.

To avoid under-estimation of age due to endosteal resorption, a back calculation must be applied by comparing LAGs with the first marks of bone growth observed in juveniles (Hemelaar 1985; Rozenblut and Ogielska 2005). Following Piantoni et al. (2006), we performed a quadratic regression of the mean perimeter of the inner periosteal perimeter (IPP; medullar cavity or line of resorption) and LAGs on snout–vent length (SVL) to determine the existence of endosteal resorption and concomitant loss of LAGs. The number of resorbed LAGs at a given SVL was calculated as the number of LAG regression curves beneath the respective IPP (Fig. 1); if the IPP of a given individual did not exceed the estimated LAG perimeters for LAG, we concluded that endosteal resorption did not result in loss of LAGs. Individual age was then calculated as the number of observed LAGs plus the number of resorbed LAGs. Because all juveniles in our sample were females, we pooled both sexes for this analysis. It is important to note that this assumes that the growth rate of males and females is the same during the first 2 years.

For each sex we calculated age at maturity (age of the youngest adult), mean SVL at age of maturity, longevity (oldest individual), potential reproduction lifespan (longevity – age at maturity), and median age.

Statistical Analyses

We used the Shapiro-Wilk test to test the normality of distribution of analyzed variables. Bone outer perimeter, IPP, SVL, and age were related by correlation analyses. To compare males and females, we used the Mann Whitney U test for comparing ranges. We also assessed sexual size dimorphism with the Lovich and Gibbons (1992) sexual dimorphism index (SDI). We used Spearman's rank correlation to test for the relationship age and SVL. We were unable to estimate growth curves (e.g., Miuad et al. 2000) due to limited samples sizes. Instead, we performed *t*-tests comparing male and female SVL for each age class and applied a Bonferroni correction for multiple tests; finding that males and females of the same age have significantly different SVLs shows that sexual size dimorphism is not due to different adult age structures for each sex (at least not exclusively). All error is reported as ± 1 S.E. and $P < 0.05$ was considered significant. All test were performed using the statistical software SigmaPlot version 13 (Systat Software, Inc) and R Project 3.1.1.

RESULTS

Skeletochronology

The femur was the most suitable bone for skeletochronological studies in *Melanophryniscus moreirae*. The number of LAGs observed in humeri was the same as observed in femora for six (50%) of the specimens; compared to femora, the number of LAGs was underestimated by one in four specimens (33%) and two in one specimens (8%), and we were unable to reliably identify any humeral LAGs in one specimen (8%). We had more difficulty interpreting the LAGs in phalanges because the lines were not as clear as in the other long bones. The number of phalangeal and femoral LAGs matched in only two individuals (16%); compared to femora, the number of LAGs was underestimated by one in seven specimens (60%) and two in one specimens (8%), and we were unable to reliably identify any phalangeal LAGs in two specimens (16%). Consequently, we used the femur to determine the age of all other specimens in this study.

We observed well-defined femoral LAGs (Fig. 2) in 62 of the 63 specimens; we were unable to unambiguously delimit LAGs in one adult male (MZUSP 154131), which we excluded from subsequent analyses. All 55 adults included in the study possessed some degree of endosteal resorption and 78% also exhibited endosteal deposition. Lines of resorption were clearly distinguishable from LAGs, with lines of resorption having the same

shape as the medullar cavities and being more irregular than LAGs (Fig. 2). According to the back-calculation method, two LAGs were resorbed in all adults and two large juveniles.

Body Length, Age Structure and Growth Pattern

SVL was sexually dimorphic in adults (SDI = 0.13), with females being significantly larger than males ($P < 0.001$; Table 1). Adult age varied from 5–7 years in females and 4–8 years in males (Fig. 3), with females being, on average, older than males (mean adult female age = 6.1 ± 0.21 years; mean adult male age = 5.4 ± 0.13 years; $P = 0.01$). The mean SVL of same-aged males and females differed significantly for 5- and 6-year-old specimens ($t = 5.6889$, $df = 23$, $P = 8.592e-06$ and $t = 5.3142$, $df = 17$, $P = 5.709e-05$, respectively; significance level following Bonferroni correction = 0.017) but was not significant for 7-year-old individuals due to small sample size ($t = 3.1973$, $df = 5$, $P = 0.02407$). Age at maturity, mean SVL at age of maturity, longevity, potential reproduction lifespan, and median age for males and females are presented in Table 1.

All seven juveniles were females aged 2–6 years. Despite the overlap in age between adult and juvenile females, there was no overlap in SVL; the oldest and largest juvenile was 6 years old and measured 22.2 mm SVL, whereas the smallest adult female, a 7-year-old (one of the oldest females sampled), measured 24.8 mm SVL. The four 5-year-old adult females were 25.1–27.5 mm SVL and included the largest female sampled. Spearman's rank correlation of SVL with adult age was not significant with either sexes pooled ($r = 0.258$, $P = 0.06$) or analyzed separately (females: $r = -0.254$, $P = 0.36$; males: $r = 0.013$, $P = 0.94$).

DISCUSSION

Skeletochronology

The present study demonstrates that skeletochronology can be successfully used to infer individual age in species of *Melanophryniscus*. LAGs were distinct in all but one specimen, and it was possible to relate each periosteal line to an annual winter, because of their habit to hibernate during cold months (Carvajalino-Fernández et al. 2013). Our results are in agreement with other studies that is possible to obtain age structure and growth pattern information with skeletochronology in anurans from sub-tropical climates, specially those from high altitude areas with marked seasonal differences in temperature (e.g., Guarino et al. 1998; Lin and Hou 2002; Morrison et al. 2004; Lai et al. 2005).

Because skeletochronology has not been used previously in *Melanophryniscus*, we carried out a preliminary analysis to determine which long bone is best suited for inferring individual age. Most studies use phalanges for these kinds of studies because there is no need to sacrifice the animal (Castanet and Smirina 1990). However, the challenges of

skeletochronology can be greater when using phalanges than other bones due to their smaller size and less pronounced LAGs, resulting in age underestimation (e.g., Rozenblut and Ogielska 2005). In *M. moreirae*, calculations based on phalanges also underestimated age when compared to femora. We selected femora because humeri presented more resorption and endosteal remodeling. In some species of salamanders the humerus are more suitable for skeletochronology because it had undergone less remodeling than femur (Wake and Castanet 1995). Bone mineral resorption can differ in different bones of the body due to differences in function (Castanet and Smirina 1990; Leclair 1990). Due to differences in bone remodeling, we suggest that a preliminary study of long bones are conducted in species that skeletochronology was never used, to avoid miscounting and increase reliability in skeletochronological data. The animals used in this study were used for other ecological questions and the animals had to be collected and sacrificed (see Jeckel et al. 2015), allowing us to use the different long bones for comparison.

There are a few methods used in amphibian skeletochronology studies to avoid underestimating number of LAGs due to endosteal resorption (Hemelaar 1985; Sagor et al. 1998; Piantoni et al. 2006; Guarino et al. 2008). All of them use graphical and sometimes statistical analysis of the patterns and perimeter or diameters of LAGs. We used the method proposed by Piantoni et al. (2006), because they based the back-calculation on measurements of juveniles and needed relatively fewer samples compared to other methods. Sagor et al. (1998) back-calculated the number of LAGs using only the distribution of frequency of the first two LAGs observed in adults because they did not have access to juveniles. In skeletochronology studies for age determination, it is important to use the best back-calculation method that fits the sample data, and not ignore that some LAGs might have been resorbed. In this way, the underestimation of age is avoided or at least minimized.

Rapprochement of lines deposited late in life and bone heterochrony are two of the challenges that can lead to underestimation of individual age (Castanet et al. 1988; Eden et al. 2007; Wagner et al. 2011). The individuals we analyzed did not exhibit accumulation of peripheral LAGs in any semaphoront or long bone, so it is unlikely that rapprochement would prevent a clear observation of lines. In addition to that, rapprochement represents a significant problem only in species that live considerably longer than *M. moreirae* (Sinsch 2015), and there is no evidence that bone heterochrony occurs in this species.

Our results showed that adult age and SVL are not significantly related. Individual differences in growth rate until the first-breeding age also explains the diversity of sizes in a specific age class (Halliday and Varrell 1988; Wake and Castanet 1995). Consequently, body

size data is not a reliable data to indicate age or maturity in *M. moreirae*, as shown in many other amphibians (e.g. Halliday and Verrell 1988; Castanet and Smirina 1990; Ento and Matsui 2002; Morrison et al. 2004; Bruce and Castanet 2006; Yamamoto et al. 2011).

Age and Size at Maturity

Our results indicate that female sexual maturity in *M. moreirae* is causally related to size, not age. Female age of maturity was achieved at 5 years, but our sample also included two juvenile females that were 5- and 6-year-olds, respectively. Nevertheless, these two adult-aged juveniles were considerably smaller than both the mean female size at maturity and the smallest adult female. Our sample did not include any juvenile males, so we are unable to determine if age and size at maturity are also decoupled in males of *M. moreirae*.

Age of maturity is generally believed to be proportional to longevity, whereby females that are older than males when breeding for the first time tend to have greater longevity than males (e.g., Miaud et al. 2000; Monnet and Cherry 2002). In contrast, although both age and size at maturity are greater for female *M. moreirae* than for males, females do not appear to have increased longevity; indeed, our data suggest that longevity might even be greater in males than in females. The net effect is that the potential reproductive lifespan of males is twice that of females, which appears to be unique among anurans. Nevertheless, these results might be a sampling artifact and should be tested using increased sample sizes.

Sexual Size Dimorphism

In most species of anurans females are, on average, larger than males (Shine 1979). Given that amphibians undergo indeterminate growth (Halliday and Verrell 1988), different age structures in adult male and female populations would result in sexual size dimorphism. To test this hypothesis, Monnet and Cherry (2002) performed a meta-analysis of 51 populations representing 30 species and concluded that sexual size dimorphism in Anura can be explained by differences in age structure between sexes in breeding populations. However, their analysis necessarily assumes that males and females of the same age are also the same size. If males and females of the same age are not the same size, then age structure fails to explain sexual size dimorphism.

As in most other anurans, adult females of *M. moreirae* were significantly larger than adult males. Both the mean and median adult ages were also greater for females than males, which is consistent with Monnet and Cherry's (2002) findings. However, we found that adult females were significantly larger than adult males of the same age, which shows that age structure merely exacerbates an effect that is already present within age cohorts.

Although our results show that age structure is not responsible for sexual size dimorphism in *M. moreirae*, the mechanism remains unknown. For example, fecundity increases with body size in females but not males (e.g., Trivers 1972; Crump 1974), which suggests that the larger female size might be due to increased growth rate in females. Alternatively, female-biased dimorphism could also be caused by loss of energy by males during territorial defense, advertising behavior, and physical combat that require massive amounts of energy that might otherwise be used for growth (Woolbright 1983). Similarly, migration is an additional energy expense; although both sexes must migrate between terrestrial habitats and aquatic breeding sites, Santos et al. (2010) found that individual males of *M. cambaraensis* migrated more frequently than individual females.

Anuran body size and sexual size dimorphism might be too complex to only one trend respond to this life-history strategy that maximizes reproductive success. Amphibian growth is related to age at maturity because growth rate is higher prior to reaching maturity (Turner 1960; Halliday and Varrell 1988). Differences in age at maturity could be the explanation most fitted to explain SSD in *M. moreirae*. Males reach maturity sooner than females, so growth rate is expected to decrease sooner in males than females (Monnet and Cherry 2002). To test this hypothesis, we would require juvenile males to estimate prematurity growth rate and/or 4-year-old females to compare to 4-year-old males (the age at maturity for males), both of which were lacking in our sample.

Age structure and conservation

Studies on age structure are important for conservation strategies of rare, endemic or threatened species (Driscoll 1999; Khonsue et al. 2002; Yetman et al. 2012). *M. moreirae* is endemic to Serra da Mantiqueira, where the Itatiaia National Park is located. Itatiaia was the first National Park in Brazil, founded in 1937. Since then, the location is protected from any kind of human land use. Since the first description of the species, in 1920 by Miranda-Ribeiro, the number of individuals in breeding seasons is abundant (Bokermann 1967; Sluys and Guido-Castro 2011). Guix et al. (1998) claimed that population of *M. moreirae* declined in comparison to observations of Bokermann, in 1967. The reason for this reported decline is unknown and it could have been a part of a natural cycle in abundance, common in anuran populations. The recruitment of each year is important for the stability of populations, because the fluctuation and the dominant age class depend on the new metamorphosed individuals (Driscoll 1999). The longevity of individuals can also be informative because allow us to understand the impact of failed recruitment in a year (Driscoll 1999). In addition to that, older individuals are usually larger than younger individuals (Monnet and Cherry

2002), and larger females produce larger eggs and more clutches during a breeding season, also influencing the population stability. Our study provides important information on age structure of *M. moreirae* for population stability studies. From our personal observation, there were hundreds of breeding individuals Itatiaia National Park at the time of collection. The decline observed by Guix et al. (1998) might have been result of a difficult year in recruitment, that could have influenced the next few years of the population.

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TABLE

Table 1. *Melanophryniscus moreirae* age structure. The values for median and mode were equal in both sexes. AM: age at maturity, age of the youngest adult; PRLS: potential reproductive lifespan; size: snout–vent length.

Sex	N	Mean Size ± SE (mm)	AM (yr)	Mean Size at AM ± SE (mm)	Longevity (yr)	Median, Mode Age (yr)	PRLS (yr)
Male	41	23.2 ± 0.2	4	23.8 ± 0.2	8	5	4
Female	15	26.2 ± 0.2	5	26.5 ± 0.5	7	6	2
Juvenile	7	16 ± 1.3	-	-	-	-	-

FIGURES

Figure 1. Estimation of the endosteal resorption. Quadratic relationship between medullar perimeter (grey regression line), LAGs (black regression lines), and snout-vent length in *Melanophryniscus moreirae* are indicated. The number of resorbed rings at a certain SVL (vertical dotted line) corresponds to the number of regression curves (lines of arrested growth) that are under the medullar radius of that SVL (horizontal grey line).

Figure 2. Cross-section in the mid-diaphyseal region of the femur of a 6-year old male adult *Melanophryniscus moreirae*, showing four lines of arrested growth. Black arrows = lines of arrested growth; white arrow = Line of resorption; mc = marrow cavity.

Figure 3. Population age structure of *Melanophryniscus moreirae*.

Capítulo 3

VARIATION IN SEQUESTERED AND SYNTHESIZED CHEMICAL DEFENSES IN THE BRAZILIAN POISON FROG: AGE EXPLAINS RICHNESS, SIZE EXPLAINS QUANTITY, SEX EXPLAINS NOTHING

ADRIANA M. JECKEL¹, RALPH A. SAPORITO², AND TARAN GRANT¹

¹ *Departamento de Zoologia, Instituto de Biociências, Universidade de
São Paulo, 05508-090 São Paulo, São Paulo, Brazil*

² *Department of Biology, John Carroll University, University Heights, Ohio 44118, USA*

Abstract – Amphibians have chemical compounds in their skin for protection against predators and pathogens. These compounds can be synthesized endogenously or sequestered from the diet. Independently of the origin, variation in defensive chemical composition is common between species, among individuals of the same population and even among life stages. The variation in chemical composition has been widely studied in many anuran species, but the causes of this variation are not completely understood. We hypothesize that the age of the sampled individual might have an important role in the diversity of compounds detected. Here, we test our hypothesis using *Melanophryniscus moreirae*, a bufonid toad that was recently reported to have both sequestered and biosynthesized defensive compounds in its skin. Age, size and sex of each individual were related with alkaloid richness, alkaloid quantity and bufotenine quantity, to test which of the individual traits would explain the chemical compounds diversity. Age was significantly related to alkaloid richness, size explained both quantities of alkaloid and bufotenine, but sex was not related to compounds diversity. What remains to be understood is if richness and quantity of defensive chemical compounds provide a better protection against predators and pathogens.

INTRODUCTION

Amphibians possess a wide diversity of chemical compounds in their skin, stored in granular glands (1). These chemicals are known to have a role of protecting the individual from predators and pathogens. Some of these compounds are known to be biosynthesized endogenously like biogenic amines, proteins, peptides and steroids, and some are sequestered

from an arthropod-based diet, like lipophilic alkaloids (2, 3). Independently of the origin, these chemical compounds vary in types and quantities between populations, among individuals and even between life stages (e.g. 4, 5, 6, 7, 8). The ecological importance and the cause of variation are still not completely understood. Different predation and pathogen pressures in different localities might explain ecological causes of variation (6), and possible mimicry among individuals could explain individual variation (9).

Intra-specific variation in diversity of alkaloids has been reported in many species. Biomodification of sequestered compounds (10, 11), genetics for sequestration (12) and ability to synthesize alkaloids (13) are known to cause some of the interspecific variation. However, as alkaloids are sequestered from dietary arthropods, the availability through time and space of these arthropods has a major role in the overall diversity of a population (3, 7). Although individuals of the same population have more similar alkaloid composition than individuals from different populations (14), intra-population differences have been reported for many poison frogs (e.g. 15, 16, 17, 18). Some of the variation was explained by different sex (16, 17), but the cause of the high variation is not completely understood.

Recently, Jeckel et al. (19) reported the co-occurrence of high quantities of biosynthesized indolealkylamine bufotenine and sequestered lipophilic alkaloids in the Brazilian poison frog *Melanophryniscus moreirae* (Bufonidae), with bufotenine quantity, alkaloid quantity and richness (number of alkaloid) varying among individuals. They did not find a relationship between number of alkaloids and quantity of alkaloids, or skin mass, but they did find a relationship between quantity of alkaloids and skin mass. Skin mass is usually used as an estimate of individual size but in *M. moreirae*, it cannot be an indicator of individual age (Chapter 2). Our hypothesis is that this variation can be explained by individual age. If the compounds detected in an individual frog are the result of a lifespan of uptake/synthesis and accumulation, older frogs might have more diversity (quantity and number of alkaloids, and quantity of bufotenine) of compounds in their skin than younger frogs. To test our hypothesis, we determined the chemical compounds information of seven juveniles of different sizes and ages of the same population, added to the known information of adults (19) and compared them to possible explanatory factor like age, sex and size of each individual.

MATERIALS AND METHODS

Samples

Chemical and morphological data for adults of *Melanophryniscus moreirae* were taken from Jeckel et al. (19). We added the chemical profiles of 7 juveniles, collected in the

same location (Itatiaia National Park, Serra da Mantiqueira, Rio de Janeiro, Brazil, GPS coordinates: 22° 23' 05.88" S, 44° 40' 41.83" W) and same time of the adults (November 30, 2013), to test for ontogenetic changes in chemical compound diversity. All juveniles were measured for snout–vent length (nearest 0.1 mm), sexed, and weighed (nearest 0.1 mg). Skin samples were stored in individual 4 mL glass vials with Teflon-coated lids, containing 100% methanol. Ages of all 62 (40 males, 15 females and 7 juveniles) individuals were determined in Jeckel and Grant (Chapter 2) by skeletochronology.

Chemical analyses

Alkaloids and bufotenine were isolated from individual methanol extracts using an acid-base extraction (following reference 20). In brief, 10 µg of nicotine ((-)-nicotine ≥ 99%, Sigma-Aldrich) in a methanol solution (internal standard) and 50 µL of 1 N HCl were added to 1 mL of the original methanol extract. This combined methanol extract was concentrated with nitrogen gas to 100 µL and then diluted with 200 µL of deionized water. This solution was then extracted four times, each time with 300 µL of hexane. The aqueous layer was then treated with saturated NaHCO₃, followed by extraction 3 times, each time with 300 µL of ethyl acetate. The combined ethyl acetate fractions were dried with anhydrous Na₂SO₄, evaporated to dryness, and then reconstituted with methanol to 100 µL.

Gas chromatography-mass spectrometry (GC-MS) analysis was performed on a Varian Saturn 2100T ion trap MS instrument coupled to a Varian 3900 GC with a 30 m 0.25 mm i.d. Varian Factor Four VF-5ms fused silica column. GC separation was achieved by using a temperature program from 100 to 280 °C at a rate of 10 °C per minute with helium as the carrier gas (1 mL/min). Alkaloid/bufotenine fractions were analyzed with both electron impact MS (EI-MS) and chemical ionization MS (CI-MS) with methanol as the CI reagent. Vapor phase Fourier-transform infrared spectral data (GC-FTIR) were obtained using a Hewlett-Packard model 5890 gas chromatograph, with an Agilent J&W DB-5 capillary column (30m, 0.25 mm i.d., 0.25 µm), using the same temperature program as above, coupled with a model 5965B (IRD) narrow band (4000-750 cm⁻¹) infrared detector.

Individual alkaloids were identified by comparison of the observed MS properties (and FTIR properties for bufotenine) and GC retention times (R_t) with those of previously reported anuran alkaloids (21). Identification of bufotenine was based on comparison to reference standard: bufotenine solution, B-022, Cerilliant, Sigma-Aldrich. Isomers of previously characterized alkaloids were tentatively identified based on comparisons of EI mass spectral data and GC retention times. Individual frog skin extracts were analyzed in triplicate and the average quantity of defensive compounds was determined by comparing the

observed alkaloid peak areas to the peak area of the nicotine internal standard, using a Varian MS Workstation v.6.9 SPI.

Statistical Analyses

We performed Shapiro-Wilk to test for normality of the variables. As some of the variables did not present a normal distribution, we performed non-parametric multiple regression analyses using 9,999 permutations of the data to estimate the significance (two-tailed tests) of the regression coefficients using package *ape* (22) in R Project 3.1.1 (23). We performed multiple regression analyses using alkaloid richness per individual skin, alkaloid quantity per individual skin, and bufotenine quantity per individual skin as response variables and age, skin mass, and sex as the explanatory variables.

RESULTS

All seven *Melanophryniscus moreirae* juveniles were female. Their skin mass varied from 32.4 mg to 281.4 mg (average: 87.5 ± 93.9 mg). GC/MS analysis resulted in the detection of 18 alkaloids from seven different structural classes. We detected the indolealkylamine bufotenine in only three of the seven juveniles. Alkaloid richness (max: 12; min: 1 alkaloid per individual skin), quantity of alkaloid (max: 331.1 μ g; min: 1.5 μ g per individual skin) and quantity of bufotenine (max: 121.3 μ g; min: 0.0 μ g per individual skin) varied among individuals. Only allopumiliotoxin (aPTX) **323B** was detected in all samples, and the second most common alkaloid was aPTX **337D**, present in four juveniles. Pumiliotoxin (PTX) **267C** and PTX **265D** were present in three individuals, and all other alkaloids (5,8-I, 5,6,8-I, Tricyclic, hPTX and unclassified) were present in only one or two juveniles. All alkaloids found in juveniles and its quantity is shown in Table 1 of *Supplementary Material*.

The results of the multiple regression analyses are summarized in Table 1. Alkaloid richness had a significant relationship with age, but not skin mass, whereas both quantity of alkaloids and quantity of bufotenine were explained by skin mass, but not age. Sex was not significant in any of the analyses.

DISCUSSION

We combined adult and juvenile chemical compound data in this study to understand the possible causes of variation in chemical diversity in the Brazilian poison frog *Melanophryniscus moreirae*. Age explained alkaloid richness present in each individual. Alkaloids are sequestered from the diet (7), and the longer the frog lives, more diversity of alkaloid-containing arthropod it is expected to have encountered, and more diversity of alkaloid it might have in its skin. Variation has been reported in many poison frog species,

and relationship between individual size and alkaloid richness has been tested in most studies with diverging results (e.g 16; 17). However, body size is not a reliable factor to estimate age in many anuran species (24) because of differences in individual growth pattern. The body size explained the quantity of alkaloids and bufotenine present in the skin. It is known that granular glands of a poison frog increase allometrically through ontogeny (25). The individual body size could represent the storage capacity of chemical compounds, explaining the relationship between quantity of synthesized and sequestered chemical to the individual skin mass.

Previous studies in *Melanophryniscus* did not find differences between males and females (15, 18) and our results confirm the lack of relationship between sex and alkaloid diversity. However, differences in richness and quantity of alkaloids have been reported for a mantellid (16) and a dendrobatid frog (17), with female presenting a higher richness than males. Differences in diet, by preference or availability based on behavior, may explain this difference. In both species, the males tend to be territorial during reproduction season, which may limit the foraging range, when compared to females that probably have larger home ranges and may encounter more diversity of alkaloid-containing arthropods (16, 17). On the other hand, reproduction in *Melanophryniscus* is explosive, in which many individuals migrate simultaneously to breeding ponds and males do not have territorial behavior because they actively search for females. Therefore, males and females from *Melanophryniscus* species may have similar home range and foraging behavior, explaining the alkaloid diversity similarity.

Diet composition is known to vary among life stages (26, 3) and may account for the differences in alkaloid types we found between juveniles and adults (8). It was remarkable that the most abundant alkaloid in adult (PTX **267C**) was present in only three juveniles and the second most abundant alkaloid in adults (aPTX **323B**) was the one found in all seven juveniles and unique in three of the smallest of them. Variation in alkaloid composition was also reported for dendrobatid poison frogs (8). Differences in diet, due to body size, microhabitat and arthropod availability may probably be the main variation cause (27).

The small amount of bufotenine found in the youngest juveniles was intriguing, considering that it was the second most abundant compound in adults, representing on average 22% of the total quantity of defensive chemicals. Four of the smallest juveniles, with two and three years old, did not have detectable amounts of bufotenine in their skin. The youngest juvenile with relatively significant amount of bufotenine has also three years old, but larger than the juveniles lacking bufotenine. Ontogenetic changes in biosynthesized

chemicals are known in *Rhinella marina*, also a bufonid toad (6). Post-metamorphic toads had significantly lower richness and less quantity of bufadienolides, a steroid toxin common in species of Bufonidae family, than adults, older juveniles and freshly laid eggs. The amount of toxin present was related to palatability and toxicity of toads, showing that post-metamorphic toads may be more susceptible to predation (6). *Melanophryniscus moreirae* juveniles lacking bufotenine were not post-metamorphic toads, but had already two or three years old and were collected at the same environment than adults. This means that juveniles rely only on alkaloids to protect them in the first years of life. The question, which could not be answered in this study, is if there is some kind of endogenous trigger that starts the production of bufotenine in *M. moreirae*.

Our study reports for the first time the relationship between age and diversity of defensive chemical compounds in the skin of the poison frog *M. moreirae*. The alkaloid richness increases as individuals get older, as a possible consequence of a lifetime of arthropod consuming. Nevertheless, there is no information if the richness of compounds in the skin has an influence on predator or pathogen avoidance. Mina et al. (28) reported that alkaloid cocktails (i.e. naturally occurring alkaloid mixtures) has an important role in inhibiting microbial growth, and that the combination of many alkaloids have a different response than when alkaloids are analyzed alone (29). In their analysis, the individuals that had higher richness and quantity of alkaloids were most effective against the microbes tested, suggesting that potential synergistic effects between alkaloids may be important. Further studies are needed to address the same kind of questions with predators defense. Variation in chemical compounds in different life stages was also reported with juveniles possessing significantly less diversity of defensive compounds in their skin. Further studies should explore the ecological consequences of the ontogenetic shifts in alkaloid diversity and production of bufotenine in the predation avoidance capacity.

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TABLE

Table 1. Multiple regression results for *M. moreirae* defensive compounds diversity.

Significant p-values are in bold ($p < 0.05$). Abbreviation: Per., permutational; SM, skin mass.

	Bufotenine Quantity			Alkaloid Quantity			Alkaloid Richness		
F-statistic _{3, 58}	9.9			10.8			9.4		
Per. p-value	< 0.001			< 0.001			< 0.001		
	<u>Sex</u>	<u>SM</u>	<u>Age</u>	<u>Sex</u>	<u>SM</u>	<u>Age</u>	<u>Sex</u>	<u>SM</u>	<u>Age</u>
Coefficient	13.56	0.63	1.42	107.3	1.62	38.44	-0.78	0.01	1.52
p-value	0.468	< 0.001	0.874	0.083	0.004	0.206	0.404	0.330	0.002

FIGURE

Figure 1. Gas chromatographs showing alkaloid variation in three individuals of *Melanophryniscus moreirae* of different ages. Peaks that are not identified represent nonalkaloid compounds (i.e., plasticizers, fatty acid methyl esters, etc. that were not removed during the fractionation process), as determined by analysis of mass spectrograms. (A) Juvenile *M. moreirae* of 2 years (one alkaloid), (B) adult male *M. moreirae* of 4 years (seven alkaloids and bufotenine), (C) adult male *M. moreirae* of 7 years (13 alkaloids and bufotenine).

Conclusão

Esta dissertação teve como objetivo principal testar a hipótese de que a variação das toxinas encontradas na pele de uma determinada espécie de anfíbio poderia ser explicada pela idade do indivíduo. Para isso, determinamos a diversidade de toxinas (capítulo 1) e a idade de cada indivíduo (capítulo 2) de uma população de *Melanophryniscus moreirae*, para finalmente testarmos a nossa hipótese (capítulo 3). Apesar de atingirmos o nosso objetivo principal apenas na terceira parte da dissertação, os dois primeiros capítulos foram imprescindíveis para que pudesse existir o terceiro capítulo. Na primeira parte, concluímos que havia uma grande diversidade de toxinas (quantidade, riqueza e composição) entre adultos de uma mesma população. Esta variação não pôde ser totalmente explicada pelas variáveis que tínhamos até o momento, as quais eram o comprimento rostro-cloacal (*snout-vent length*, SVL), a massa da pele e o sexo dos indivíduos. Apenas a quantidade de toxinas foi explicada pelo tamanho do animal. Além disso, corroboramos a presença de grandes quantidades de dois tipos diferentes de toxinas na pele de *M. moreirae*: a amina biogênica biossintetizada bufotenina e os alcaloides lipofílicos sequestrados da dieta.

Na segunda parte da dissertação, determinamos a idade de cada indivíduo da amostra e concluímos que, para *M. moreirae*, o SVL não está correlacionado com a idade. Desta forma, o tamanho do indivíduo não pode ser usado como um fator indicativo da idade do animal. Ademais, identificamos diferenças na idade e no tamanho médio de maturação entre os sexos, que podem explicar o dimorfismo sexual do SVL. Para determinação da estrutura etária a população utilizamos o método de osteocronologia, bastante utilizado em anfíbios. Foi necessário fazer um estudo prévio do osso longo mais confiável para estimar a idade em *M. moreirae*, já que este método nunca foi utilizado para nenhuma espécie deste gênero. A história natural da espécie nos permitiu confiar que cada linha de parada de crescimento representasse realmente um ano, já que é sabido que eles hibernam durante os meses frios.

Finalmente, com as informações de diversidade de toxinas e de idade, foi possível testar a relação entre fatores da diversidade (riqueza de alcaloide, quantidade de alcaloide, quantidade de bufotenina) com fatores como o sexo, a idade e o tamanho dos indivíduos. Com a regressão múltipla, foi possível quantificar a relação de cada variável, controlando a interferência das outras. A nossa hipótese, de que a idade dos indivíduos explica a diversidade, não foi rejeitada para pelo menos uma variável, a riqueza de alcaloides. A quantidade de alcaloides e de bufotenina, continuaram sendo explicadas pela massa da pele, o que significa que a capacidade de armazenamento das toxinas em um indivíduo influencia a

quantidade de toxinas na pele. A diversidade de toxinas na pele, então, é explicada por pelo menos duas variáveis, a idade e o tamanho do animal. O sexo do indivíduo não tem relação nenhuma com a diversidade de toxinas, pelo menos em *M. moreirae*.

Durante o nosso estudo, outras perguntas sobre a defesa química de *M. moreirae* surgiram, tais como: qual a importância da bufotenina, que exige gasto energético de biossíntese, se a defesa química poderia ser realizada somente pelos alcaloides lipofílicos sequestrados? Existe algum tipo de controle do animal sobre a produção de bufotenina relacionada com a quantidade de alcaloides sequestrados? Por que os juvenis mais novos não possuem bufotenina na pele para defesa? Existe algum tipo de gatilho endógeno ou exógeno que inicia a produção de bufotenina? Uma espécie que tem a capacidade de produzir bufotenina e também consegue sequestrar alcaloides, como *M. moreirae*, fornece uma oportunidade de responder estas perguntas e compreender melhor muitos fatores da defesa química e a sua evolução na ordem Anura.

Em suma, a diversidade em número e quantidade das toxinas presentes na pele de anfíbios tem uma relação direta com a idade e o tamanho do animal, respectivamente, porém o papel ecológico desta variedade ainda não está totalmente compreendida, enfatizando a necessidade de investigações futuras.

Resumo

Anfíbios possuem uma grande diversidade de toxinas na pele que os defendem contra predadores e patógenos. Essas substâncias podem ser produzidas endogenamente ou sequestrados da dieta composta por artrópodes. Independentemente da origem, os compostos químicos podem variar muito entre espécies, entre populações da mesma espécie e até mesmo entre indivíduos de uma mesma população. Diferenças entre espécies e entre diferentes populações podem ser explicadas por diferenças na capacidade de produção ou sequestro de toxinas, por pressões ecológicas diferentes e por presença de artrópodes que contêm alcaloides no ambiente. As variações entre indivíduos de uma mesma população são comuns, porém a causa ainda não foi totalmente compreendida. Nossa hipótese é que parte dessas variações ocorrem pela diferença de idade entre indivíduos da mesma população. A diversidade de toxinas presente na pele de um indivíduo representa o balanço entre o tempo de vida, a produção e/ou sequestro dos compostos e a liberação dos mesmos para proteção. Para testar a nossa hipótese, escolhemos a espécie *Melanophryniscus moreirae*, um anuro da família Bufonidae, endêmico da Serra da Mantiqueira, Brasil. Esta espécie faz parte do único gênero da família capaz de sequestrar alcaloides lipofílicos da dieta, e existem estudos que detectaram compostos biossintetizados. As análises através de cromatografia a gás acoplada a um espectrômetro de massas resultaram em grandes quantidades de alcaloides e de bufotenina, uma amina biogênica, com grande variância entre indivíduos. A determinação da estrutura etária da população foi feita através de osteocronologia. Determinando a idade de cada indivíduo, foi possível identificar diferenças na idade de maturidade entre os sexos, e supor uma explicação para o dimorfismo sexual encontrado. Testamos nossa hipótese aplicando três regressões múltiplas com idade, sexo e massa da pele como variáveis explicativas, e número de alcaloides, quantidade de alcaloides e quantidade de bufotenina como as variáveis resposta de cada regressão. O número de alcaloides foi explicado pela idade, demonstrando que quanto mais velho é o animal, mais ele se alimentou, e maior é a probabilidade de ter se alimentado de diferentes fontes de alcaloides. Nosso estudo explica uma parte da variação de toxinas em anuros, porém, mais estudo será necessário para compreender esse sistema complexo de defesa química.

Abstract

Amphibians have a great diversity of toxins in their skin to defend them against predators and pathogens. These compounds can be either produced or sequestered from an arthropod diet. Independently of origin, these chemical compounds vary in composition between different species, between populations of the same species and even individuals of the same species. Differences between species and populations could be explained by differences in production or sequestration capacity, ecological pressures and alkaloid-containing arthropods availability. Variations among individuals of the same population are common, but the cause of this variation is not very well understood. We hypothesize that part of this variation is explained by differences in age of the individuals. The diversity of toxins in the skin represents the balance between lifespan, production and/or sequestration of compounds and their use for protection. To test our hypothesis we used the species *Melanophryniscus moreirae*, a bufonid toad, and endemic from Serra da Mantiqueira, Brazil. This species belongs to the only genus of this family that has the ability to sequester alkaloids from the diet, and other studies had also detected biosynthesized compounds. The Gas-Chromatography/Mass Spectrometry analysis resulted in high amounts of alkaloids and bufotenine, a biogenic amine, with high variance among individuals. We determined the age structure of this population by skeletochronology. By determining their age, we could find differences in maturation age between the sexes, and assume possible explanations for the size sexual dimorphism. We applied three multiple regression analysis to test our age hypothesis, with age, sex and skin mass as explanatory variables, and number of alkaloids, quantity of alkaloids and quantity of bufotenine as response variable of each regression. Age explained number of alkaloids, showing that the older the animal is, more it ate and the probability of eating from different alkaloid source is higher. Our study explains part of the chemical compound variation, but more study is needed to understand this complex system of chemical defense.

Anexos

Capítulo 1- Supplementary figures

Supplementary Figure 1. Mass spectral data for the 11 new alkaloids detected in *Melanophryniscus moreirae*. The Rt for each alkaloid is included, along with an approximate “corrected Rt” to match the retention times in the alkaloid library of Daly et al. (2005). Following the methods of Garraffo et al. (2012), based on comparisons of Rt’s for previously identified alkaloids in the present study with Rt’s from Daly et al. (2005), alkaloids in the present study eluted approximately 0.34 sec faster than times listed in Daly et al. (2005).

Abbreviations for alkaloid structural classes are as follows: 5,8-I (5,8-disubstituted indolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); aPTX (allopumiliotoxin); Tri (tricyclic); Unclass (unclassified as to structure).

Capítulo 1 - Supplementary Tables

Supplementary Table 1. Retention times for tentatively new isomers. The Rt for each alkaloid is included, along with an approximate “corrected Rt” to match the retention times in the alkaloid library of Daly et al. (2005). Following the methods of Garraffo et al. (2012), based on comparisons of Rt’s for previously identified alkaloids in the present study with Rt’s from Daly et al. (2005), alkaloids in the present study eluted approximately 0.34 sec faster than times listed in Daly et al. (2005).

Alkaloid	Rt	Corrected Rt	Rt of Previously Identified Isomers (data from Daly et al. 2005)
Tri 265S	12,88	12,54	12,21
PTX 267C	13,84	13,50	13.24, 13.98
5,6,8-I 277E	12,67	12,33	10.82, 11.33
5,6,8-I 277E	13,17	12,83	
5,6,8-I 279F	12,62	12,28	12.52, 12.91
5,6,8-I 279F	13,47	13,13	
5,6,8-I 281H	13,47	13,13	11.64, 12.68
PTX 295F	14,68	14,34	14,69
aPTX 305A	16,38	16,04	16,11
aPTX 323B	17,69	17,35	16.85, 17.20

* Multiple times represent different isomers.

Abbreviations for alkaloid structural classes are as follows: 5,6,8-I (5,6,8-trisubstituted indolizidine); PTX (pumiliotoxin); aPTX (allopumiliotoxin); Tri (tricyclic).

Supplementary Table 2. Defensive chemicals ranked by quantity in individual skins of *Melanophryniscus moreirae*. Chemicals are color-coded and their quantities (μg per individual) are reported in each cell. Abbreviations: 5,6,8-I, 5,6,8-trisubstituted indolizidine; 5,8-I, 5,8-disubstituted indolizidine; aPTX, allopumiliotoxin; hPTX, homopumiliotoxin; iso, isomer; PTX, pumiliotoxin; TRI, tricyclic; Unclass, unclassified as to structure.

MZUSP No.	Rank Order by Quantity (μg per individual)																		Color Code
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
154089	310	71	21	13	5	3	2	0.8	0.7										Bufotenine
154090	442	201	159	29	18	11	9	9	8	6	6	5	3	3	2	2	1	1	5,6,8-I 225L
154091	286	57	22	13	7	3	0.9												5,6,8-I 277E
154093	639	94	34	19	15	3	3	1	0.7										5,6,8-I 277E iso 1
154094	653	112	33	16	12	4	1												5,6,8-I 279F
154095	351	68	45	17	13	13	12	5	4	3	2	2	2						5,6,8-I 279F iso 1
154096	155	33	11	5	4	3	1	1	0.5										5,6,8-I 279F iso 2
154097	78	58	3	2	2	0.6													5,6,8-I 279F iso 3
154098	361	219	22	13	9	2	0.8	0.8											5,6,8-I 281H
154099	316	242	18	11	8	6	1	1	0.6										5,6,8-I 295G
154100	365	166	27	11	8	1	1												5,6,8-I 297H
154101	518	395	31	29	21	15	14	10	5	5	3	3	1	0.9					5,8-I 225D
154102	671	74	45	33	18	11	10	6	3	3	0.9								5,8-I 241K
154103	409	130	26	9	9	4	0.7	0.6	0.6	0.5									5,8-I 241K iso 1
154104	52	51	11	3	3	3	3	2	2	1	0.7	0.7	0.7	0.6	0.5				5,8-I 297G
154105	50	30	21	9	7	5	5	4	4	4	3	2	2	2					aPTX 293K
154106	494	198	29	18	13	11	6	4	3	2									aPTX 305A iso 1
154109	1234	352	77	34	25	5	3	2											aPTX 305A iso 2
1541010	245	47	16	11	9	5	4	2	2										aPTX 323B
1541011	430	32	32	13	12	5	4	4	3	3	3	3	3	2	2	2	0.9	0.5	aPTX 323B iso 1
1541012	138	98	34	10	5	5	4	3	3	2	2	1	1	0.8					aPTX 323B iso 2
1541013	325	97	18	12	11	10	2	1											aPTX 323B iso 3
1541014	72	43	42	10	7	4	3	3	2	1	0.5								aPTX 337D
1541015	120	89	52	5	5	3	3	2	2	1	1	0.9	0.8	0.7	0.7				hPTX 281K

1541016	268	74	51	21	15	9	7	7	5	4	3	1	1	0.5	PTX 251D		
1541017	143	25	18	9	4	2	1								PTX 253F		
1541018	120	45	21	7	4	4	2	1							PTX 265D		
1541019	308	110	21	13	10	6	0.9	0.9							PTX 267C		
1541020	138	31	8	5	4	3	2	1							PTX 267C iso 1		
1541021	138	90	29	5	3	2	2	1	0.9	0.9					PTX 267C iso 2		
1541022	109	86	51	5	3	3	2	2	1	1	1		0.7		PTX 295F		
1541023	157	102	13	8	4	0.7	0.7								PTX 323A		
1541024	172	48	41	8	5	4	4	4	2	1	0.8				TRI 261J		
1541025	244	92	80	18	13	8	7	5	3	3	2	1	1	1	0.5	TRI 265S	
1541026	157	47	21	8	4	4	3	0.9								TRI 265S iso 1	
1541027	89	30	17	3	2	2	1	0.7								UNCLASS 237W	
1541028	197	92	70	32	19	11	8	7	5	4	4	4	3	3	2	0.8	UNCLASS 251GG
1541029	68	50	11	3	2	1	0.9										UNCLASS 281R
1541030	192	70	35	10	5	4	4	3	2.85								UNCLASS 283H
1541031	67	58	20	19	6	6	4	1	1	1	0.9	0.8	0.6				
1541032	215	105	16	6	4	3											
1541033	261	91	16	6	5	5	3	0.8									
1541034	510	180	30	12	11	8	1										
1541035	144	95	32	9	9	8	3	2	2								
1541036	127	58	44	9	4	4	3	3	3	3	2	1	1	0.9	0.6		
1541037	252	103	92	18	8	7	6	5	4	4	3	2	2	2	2	1	1
1541038	486	53	28	24	13	4	4	4	3	2	0.8	0.5					
1541039	912	235	56	22	17	12	2	1	0.9	0.7	0.5						
1541040	304	165	134	81	18	16	6	6	5	4	4	3	2				
1541041	510	191	37	15	14	10	3	0.7	0.6								
1541042	157	145	17	10	10	6	6	4	2	2	2	1	1	0.5			
1541043	91	20	10	4	1	0.8	0.5										
1541044	90	60	45	6	6	4	2	2	2	2	2	1	0.9				
1541045	412	88	59	24	17	10	6	4	4	2	2						
1541046	229	112	56	13	11	11	9	5	5	5	3	3	2	2	2	0.8	

Capítulo 3 – Supplemental Material

Supplementary Table 1. Defensive chemicals ranked by quantity in individual skins of *Melanophryniscus moreirae* juveniles. Chemicals are color-coded and their quantities (µg per individual) are reported in each cell. Abbreviations: 5,6,8-I, 5,6,8-trisubstituted indolizidine; 5,8-I, 5,8-disubstituted indolizidine; aPTX, allopumiliotoxin; hPTX, homopumiliotoxin; iso, isomer; PTX, pumiliotoxin; TRI, tricyclic; Unclass, unclassified as to structure.

MZUSP	Rank Order by Quantity (µg per individual)													Color Code
	1	2	3	4	5	6	7	8	9	10	11	12	13	
154107	257	27	22	16	15	10	4	2	2	2				Bufotenine
154108	195	121	35	11	4	4	4	3	3	3	1	1	0	5,6,8-I 277E
154147	2													5,6,8-I 279F
154148	62													5,6,8-I 279F iso 1
154149	20	5	2											5,6,8-I 295G
154150	34	22	9	4	2	1	1	1						5,8-I 241K
154151	7													5,8-I 297G
														aPTX 323B
														aPTX 323B iso 1
														aPTX 337D
														hPTX 281K
														PTX 265D
														PTX 267C
														PTX 267C iso 1
														PTX 295F
														PTX 323A
														UNCLASS 237W
														TRI 265S
														UNCLASS 251GG

Biografia

Adriana Moriguchi Jeckel se formou bacharel em Ciências Biológicas no ano de 2011 e licenciada em Ciências Biológicas no ano de 2012 pela Pontifícia Universidade Católica do Rio Grande do Sul. De janeiro de 2008 a dezembro de 2009, foi bolsista do Programa de Educação Tutorial - SeSU/MEC. Durante a graduação, fez parte como iniciação científica do Laboratório de Sistemática de Vertebrados sob orientação do prof. Taran Grant (junho/2007 - junho/2008; junho/2010 - dezembro/2012) e do Laboratório de Plasticidade no Sistema Nervoso sob orientação da profa. Monica Ryff Moreira Vianna (julho/2008 - dezembro/2009). O primeiro semestre da graduação de 2010 foi cursado na University of Regina, em Regina, Canadá, como participante do programa de Mobilidade Acadêmica da PUCRS, onde cursou as seguintes disciplinas: Vertebrate Animal Biology, Vascular Plants, Biogeochemistry e Evolutionary Biology of Reproduction. Em fevereiro de 2011, estagiou no Laboratório de Biologia Celular do Instituto Butantan, sob orientação do prof. Carlos Jared e da profa. Marta Antoniazzi. Durante o mestrado na Pós-Graduação em Zoologia do Instituto de Biociências da Universidade de São Paulo, fez parte da sua pesquisa na John Carroll University, sob orientação do prof. Ralph Saporito, financiado pela Bolsa de Estágio em Pesquisa no Exterior, da Fundação de Amparo à Pesquisa do Estado de São Paulo, a qual financiou também parte do seu mestrado. Os primeiros seis meses de mestrado foram financiados pelo Conselho Nacional de Pesquisa do Brasil.

Durante o mestrado, publicou os seguintes artigos:

1. Carvajalino-Fernández J. M., Jeckel A. M., Indicatti R. P. (2013). *Melanophryniscus moreirae* (Amphibia, Anura, Bufonidae): Dormancy and hibernacula use during cold season. *Herpetologia Brasileira*, 2(3), 61–62.

2. Jeckel A. M., Grant T., Saporito R. A. (2015). Sequestered and synthesized chemical defenses in the poison frog *Melanophryniscus moreirae*. *Journal of Chemical Ecology*. doi:10.1007/s10886-015-0578-6