VALENTINA ZAFFARONI CAORSI

REVISÃO TAXONÔMICA DE *MELANOPHRYNISCUS MACROGRANULOSUS* BRAUN, 1973 *E M. CAMBARAENSIS* BRAUN & BRAUN, 1979, DUAS ESPÉCIES AMEAÇADAS DE EXTINÇÃO DO EXTREMO SUL DA MATA ATLÂNTICA

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

ÁREA DE CONCENTRAÇÃO: BIOLOGIA COMPARADA

ORIENTADOR: PROF. DR. MÁRCIO BORGES MARTINS

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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Aprovada por:

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Prof. Dr. Santiago Castroviejo-Fisher

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Porto Alegre

2014

"A classificação por descendência não pode ser inventada por biólogos, ela pode apenas ser descoberta" (Theodosius Dobzhansky)

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SUMÁRIO

RESUMO

Melanophryniscus macrogranulosus Braun, 1973 foi descrita com base em nove exemplares procedentes do Morro da Gruta, Dom Pedro de Alcântara, Rio Grande do Sul. Sua distribuição foi ampliada com um registro para Barra do Ouro, Maquiné, RS. *Melanophryniscus cambaraensis* Braun & Braun, 1979 foi descrita com base em exemplares provenientes da Fortaleza dos Aparados, Cambará do Sul, RS, sendo a primeira espécie do gênero descrita com coloração dorsal verde. Posteriormente, foi registrada na Floresta Nacional de São Francisco de Paula (FLONA), RS. Ambas espécies são consideradas ameaçadas de extinção, devido a destruição e fragmentação de seu habitat. As duas espécies foram originalmente diagnosticadas pelo "diferente tipo de ambiente" que habitam e por sua coloração. Contudo, não é possível diferenciar as populações atualmente conhecidas por estes parâmetros, existindo um problema taxonômico. O objetivo do nosso trabalho foi esclarecer a identidade taxonômica delas, através de um estudo morfológico, incluindo a variação individual de padrão de coloração, genético e bioacústico. As atividades de campo foram desenvolvidas no Rio Grande do Sul no período entre 2012 e 2013. A fotoidentificação visual e automatizada (software) foram testadas na população da FLONA de São Francisco de Paula usando a amputação de falanges como método de controle. A maior acurácia foi registrada com a fotoidentificação visual (99.4%), seguido da amputação de falanges (95.3%) e, por último, com a utilização do software (90.9%). Quanto ao estudo taxonômico, as análises de morfologia e genética não evidenciaram a existência de caracteres diagnósticos para as duas espécies referidas. O estudo da morfologia desta espécie mostrou que os calos nupciais em machos de *M. macrogranulosus* estão presentes nos dedos I (polegar), II e III. As observações feitas também identificaram o primeiro registro destas estruturas dérmicas em fêmeas, dentro do gênero *Melanophryniscus*. O estudo bioacústico da espécie, mostrou que o canto de anúncio dela se inicia com o canto do tipo A, composto por pulsos únicos e é sempre seguida do canto do tipo B, um trinado extremamente longo. Foi encontrada uma alta variação em todos os níveis estudados: intra e inter-individual e populacional, não sendo possível a separação clara de grupos. Os aspectos morfológicos, genéticos e bioacústicos estudados não corroboraram o status taxonômico atual das duas espécies, reconhecidas atualmente como válidas. Portanto, *M. cambaraensis* Braun & Braun, 1979 foi considerado um sinônimo Junior de *M. macrogranulosus*. Utilizando todos os dados adquiridos ao longo do trabalho e seguindo os critérios da lista vermelha da IUCN, a espécie ficou enquadrada na categoria "Em Perigo".

INTRODUÇÃO E JUSTIFICATIVA

2.1. Grupo de estudo

O gênero *Melanophryniscus* Gallardo, 1961, pertence à família Bufonidae e inclui 26 espécies válidas (Frost, 2014). Sua distribuição geográfica é restrita ao sudeste da América do Sul, indo desde o Uruguai, até o sul do Brasil, cruzando o centro e norte da Argentina, o Paraguai e a Bolívia central (Kwet *et al.,* 2005). As espécies do gênero secretam toxinas, como alcalóides e bufadienolides para sua defesa (Daly *et al.,* 2008; Hantak *et al.,* 2013), sendo popularmente conhecidas como os sapinhos-de-barriga-vermelha, por apresentar coloração aposemática, junto com o comportamento de reflexo "unken", que serve para exibir a coloração vermelha e laranja do ventre quando são perturbados (Kwet *et al.,* 2005; Langone *et al.,* 2008; Santos & Grant, 2010).

Melanophryniscus macrogranulosus Braun, 1973 e *M. cambaraensis* Braun & Braun, 1979 encontram-se dentro do grupo de *Melanophryniscus tumifrons* (Cruz & Caramaschi, 2003), caracterizado pela presença de uma tumefação frontal, caráter sugerido por Baldo & Basso (2004) como uma sinapomorfia para o grupo. Estas duas espécies são endêmicas do extremo sul da Mata Atlântica, no Rio Grande do Sul, Brasil, e são consideradas ameaçadas de extinção (Garcia & Vinciprova, 2003).

Melanophryniscus macrogranulosus foi descrita com base em nove exemplares procedentes do Morro da Gruta, Município de Dom Pedro de Alcântara, estado do Rio Grande do Sul. Sua distribuição foi ampliada com dois registros, sem coordenadas geográficas específicas na Barra do Ouro, Município de Maquiné (Escobar *et al.,* 2006; Poli *et al.,* 2012), localizado na encosta da Serra Geral, RS. Ambas localidades estão inseridas dentro dos limites da Floresta Ombrófila Densa (IBGE, 2004). Apesar do seu status de conservação, não existem outros trabalhos realizados com a espécie posteriores a sua descrição.

A principal ameaça para *Melanophryniscus macrogranulosus* é a perda, descaracterização e fragmentação do habitat devido à ação antrópica (Garcia & Vinciprova, 2003). A espécie é considerada atualmente ameaçada em nível regional (Categoria Em Perigo - EN; Listas do Estado do Rio Grande do Sul e Brasil; Subirá *et al*., 2012) e global (Categoria Vulnerável - VU; IUCN, 2013). Tanto a área de Dom Pedro de Alcântara quanto a de Barra do Ouro, em Maquiné, encontram-se impactadas pela descaracterização, destruição e forte fragmentação do habitat. Atualmente, o único sítio de reprodução conhecido para esta espécie, segundo a literatura, a localidade-tipo no Morro da Gruta, encontra-se bastante impactada devido ao despejo inadequado de lixo no local.

Melanophryniscus cambaraensis foi descrita por Braun & Braun, 1979 como a primeira espécie do gênero com a coloração dorsal verde, baseando-se em 60 exemplares oriundos da região da Fortaleza dos Aparados, Município de Cambará do Sul, RS. Entretanto, esta população não é encontrada no local

desde 1990 (segundo registro da coleção científica do Museu de Ciências e Tecnologia da PUCRS). Posteriormente, foi registrada mais uma população na Floresta Nacional de São Francisco de Paula (FLONA SFP), no Município de São Francisco de Paula, RS, dentro dos limites da Floresta Ombrófila Mista (IBGE 2006). Atualmente, são conhecidas apenas essas duas populações do sapinho-verde-de-barriga-vermelha, que é considerado ameaçado de extinção no RS e no Brasil na categoria Vulnerável (Garcia & Vinciprova, 2003 Subirá et al., 2012). O principal fator de ameaça para esta espécie é a destruição do habitat, uma vez que sua distribuição geográfica é restrita, mesmo considerando que as duas populações conhecidas estão localizadas dentro de áreas de preservação. Recentemente, foram realizados trabalhos sobre a migração da população de *M. cambaraensis* da FLONA de São Francisco de Paula, porém não existem outras publicações.

2.2 Bioma Mata Atlântica

A distribuição geográfica de *Melanophryniscus macrogranulosus* e *M. cambaraensis* está inserida dentro do Bioma Mata Atlântica (Fig.1), o qual se estende ao longo do litoral brasileiro, do Rio Grande do Norte até o Rio Grande do Sul e abriga um conjunto de ecossistemas predominantemente florestais (IBGE, 2004).

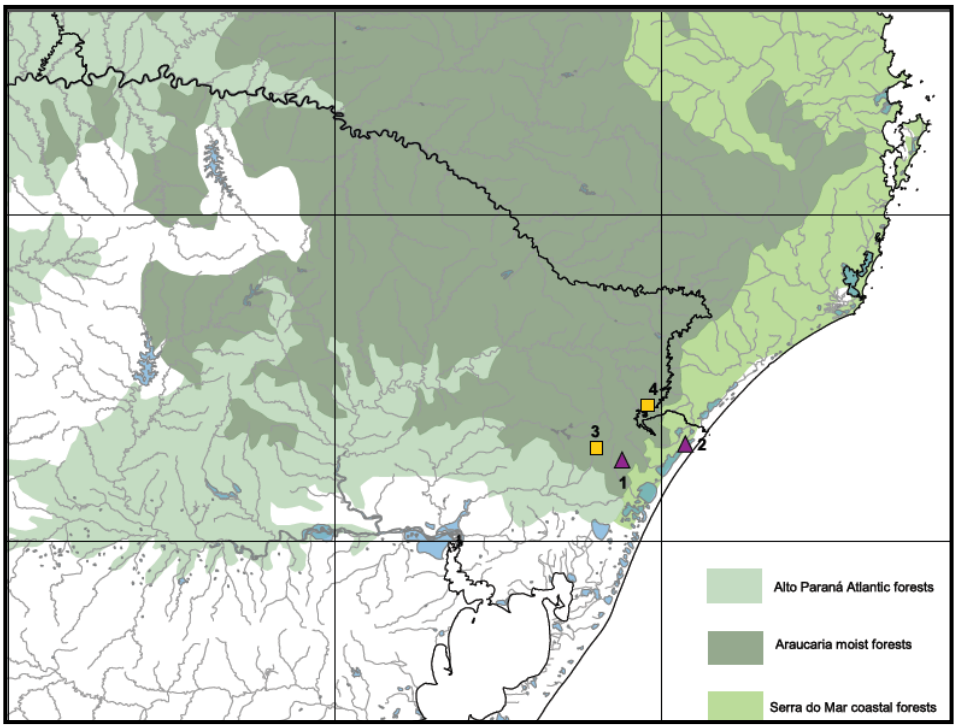

Figura 1. Distribuição das populações conhecidas de *Melanophryniscus macrogranulosus* (triângulos na cor roxa) em Barra do Ouro, Município de Maquiné (1) e no Morro da Gruta, Município de Dom Pedro de Alcântara (2) e de *M. cambaraensis* (quadrados na cor amarela) na Floresta Nacional de São Francisco de Paula, no Município de São Francisco de Paula (3) e no Parque Nacional da Serra Geral, Município de Cambará do Sul (4); todas localizadas no estado do Rio Grande do Sul, Brasil.

A Mata Atlântica é um dos biomas mais devastados e seriamente ameaçados do Brasil (Myers *et al.,* 2000). Dos 139.585.893 hectares originais, a floresta está restrita a apenas 16.377.472 ha, a maioria em pequenos fragmentos, o que representa apenas 11.73% da sua extensão original (Ribeiro *et al.,* 2009). Os remanescentes mais significativos desse bioma estão na Região Sul e Sudeste (Câmara, 2005). A Mata Atlântica caracteriza-se por possuir uma formação vegetal muito úmida, temperaturas ao redor de 25ºC e alta pluviosidade anual (2400 – 4000 mm), o que permite a manutenção de grande variedade de espécies animais e vegetais (Goerck, 1999).

A Mata Atlântica ocupa grande parte do estado do Rio Grande do Sul, onde ocorre a maioria dos ecossistemas integrantes deste bioma: Floresta Ombrófila Densa, na faixa costeira do litoral e nas encostas de Osório a Torres; a Floresta Ombrófila Mista e os Campos de Altitude na região do planalto; as Florestas Estacionais Deciduais e Semideciduais na encosta sul da Serra Geral e região do Alto Uruguai; e a vegetação de restinga, presente na maior parte do litoral gaúcho (Marcuzzo *et al*., 1998).

2.3. Fotoidentificação

Atualmente, o método de identificação individual mais utilizado é a marcação por amputação de falanges (Ferner, 2007; Phillott *et al.,* 2007, 2008). Este método permite o reconhecimento individual através de uma combinação única de dígitos amputados quando o animal é capturado pela primeira vez (Heyer *et al.,* 1994). Este método é preferido por muitos pesquisadores devido ao seu baixo custo, fácil implementação e resultados confiáveis (Funk *et al.,* 2005). Estudos sugerem que a amputação de falanges pode aumentar o risco de mortalidade dos indivíduos marcados (McCarthy & Parris, 2004), o que seria problemático no estudo de espécies ameaçadas. O impacto da amputação de falanges ainda não está claro (Funk *et al.,* 2005; Phillott *et al.,* 2007, 2008), mas uma técnica de identificação individual confiável, fácil de implementar e sem impacto físico seria preferida.

A identificação individual através de marcas naturais já é realizada em algumas espécies de anfíbios (p. ex. Golay & Durrer, 1994; Kenyon *et al.,* 2009; Miranda *et al.,* 2005), e alguns autores (Cairo & Di Tada, 2005; Cairo & Zalba, 2007) sugerem a utilização do padrão de coloração ventral (Fig.2 C-D) para reconhecer indivíduos do grupo dos sapinhos-de-barriga-vermelha (*Melanophryniscus*: Bufonidae). Entretanto, esse método nunca foi testado utilizando-se outro tipo de marcação como controle dentro do gênero *Melanophryniscus*.

2.4. Taxonomia

Braun & Braun (1979: 10) relatam que as diferenças entre as espécies *Melanophryniscus macrogranulosus* e *M. cambaraensis* são o "diferente tipo de ambiente" que habitam e a "coloração dorsal, gular e peitoral". O autor não explicitou as diferentes características ambientais, entretanto *Melanophryniscus macrogranulosus* era conhecido apenas no Morro da Gruta (Braun, 1973). Atualmente, já são conhecidas populações desta espécie em outras localidades e, apesar de uma delas, *M. macrogranulosus*, viver nas baixas altitudes e *M. cambaraensis* na serra, a mais de 900m, o ambiente onde elas reproduzem é muito semelhante.

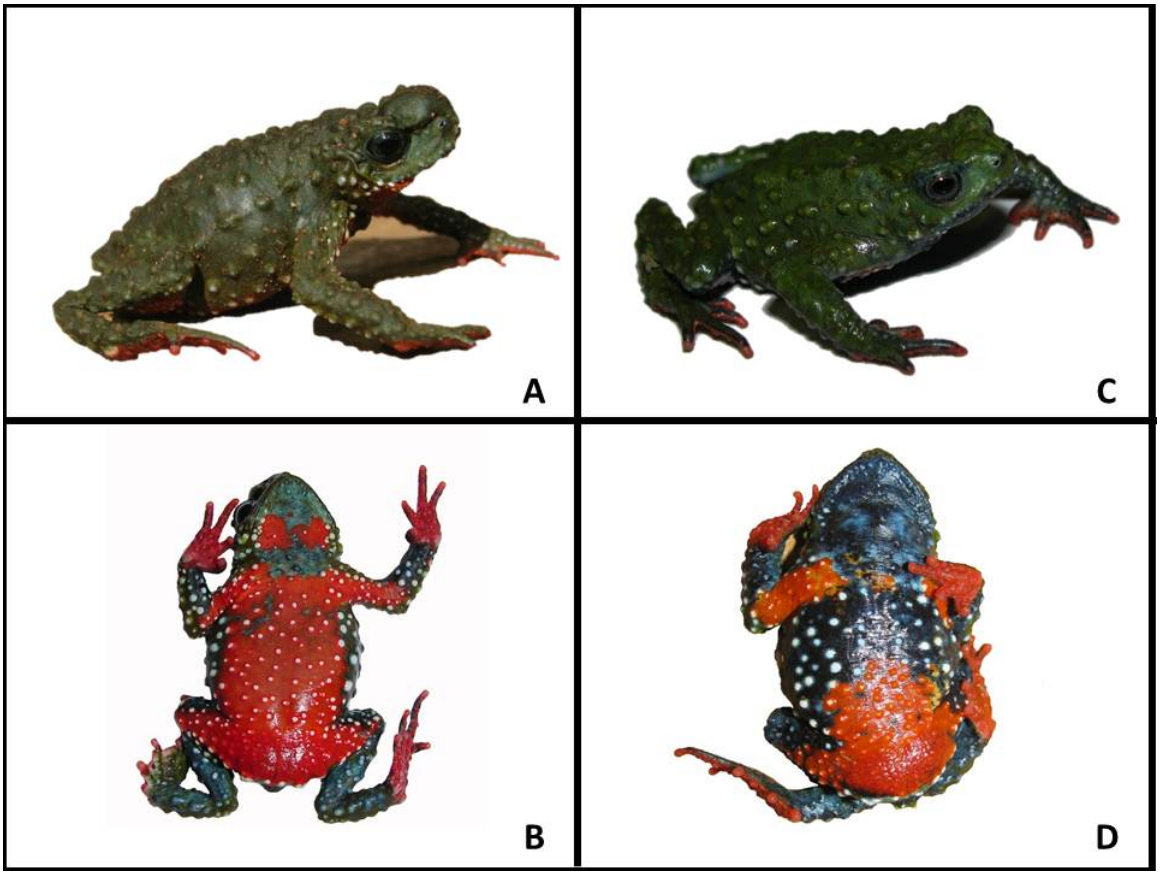

Figura 2., Aspecto geral, em vista lateral e ventral, de *Melanophryniscus cambaraensis* (esquerda) e *M. macrogranulosus* (direita); (A e B, exemplar da FLONA de São Francisco de Paula, RS; C e D, exemplar da localidade-tipo, Morro da Gruta, Dom Pedro de Alcântara, RS).

Aspectos da coloração da pele, descritas por Braun & Braun (1979) como características para diferenciar as duas espécies, dizem respeito à coloração da região dorsal verde, gular branca-amarelada e peitoral verde-amarelada com tons vermelhos em *M. cambaraensis* enquanto que em *M. macrogranulosus* essas regiões são uniformemente pretas. Porem, indivíduos observados recentemente na localidade tipo de *M. macrogranulosus* e outras localidades (e.g. Escobar *et al.,* 2006) concordam com a

descrição morfológica de Braun (1973) mas apresentam a coloração dorsal verde. A região gular e peitoral do animal têm coloração azul, entretanto alguns indivíduos de *M. cambaraensis* apresentam, também, esse padrão de coloração, misturado com o verde. Braun (1973) utilizou apenas nove exemplares, já depositados na coleção desde 1960 para descrever *M. macrogranulosus*. Nesta situação, a coloração se torna escura, devido ao uso do álcool para a conservação do material coletado. Entretanto, a descrição original apresenta os dados de coloração como sendo dos exemplares em vida. É possível que os dados tenham sido retirados das notas de campo tomadas em 1960 (Escobar *et al.,* 2004).

A Figura 2 mostra o padrão de coloração dorsal (A e C) e ventral (B e D) de dois indivíduos das espécies em estudo. Elas apresentam, visivelmente, diferentes tonalidades de verde, o que varia de indivíduo para indivíduo nas diferentes populações conhecidas. Assim como relatado para outras espécies do gênero (Cairo & Di Tada, 2005; Vaira, 2002), o caráter referente à coloração dos indivíduos, usado como diagnose para *Melanophryniscus macrogranulosus* de *M. cambaraensis*, é muito variável e se sobrepõe entre as espécies.

Considerando que as populações conhecidas até hoje encontram-se no bioma brasileiro mais ameaçado do Brasil (Myers *et al.,* 2000) e que ambas espécies apresentam distribuição restrita e são consideradas ameaçadas de extinção, o esclarecimento da identidade taxonômica das populações conhecidas como *Melanophryniscus cambaraensis* e *M. macrogranulosus* é fundamental para planejar e implementar estratégias de conservação para as mesmas.

2.5. Excrescências nupciais

Os anfíbios adultos podem possuir algumas variações morfológicas da epiderme, como calos nupciais, espinhos ou garras (Wells, 2007). Os calos nupciais são caracteres secundários influenciados por liberação hormonal e estão, geralmente, presente em machos adultos. Estas estruturas são formadas, basicamente, por um engrossamento da epiderme e, muitas vezes, uma derme com glândulas específicas associadas (Duellman & Trueb, 1986; Epstein & Blackburn, 1997). Elas podem variar de acordo com a espécie e exibir diferentes arquiteturas adaptadas para algum comportamento reprodutivo, como cortejo, amplexo, entre outros (Fujikura *et al.,* 1988; Kurabuchi, 1993). O calo nupcial (Fig. 1), geralmente, está presente no dedo polegar e, ocasionalmente, também ocorre no segundo e terceiro dedos ou podem, ainda, estar presente nos braços ou pés (Duellman & Trueb, 1986).

As excrescências nupciais são usadas durante o cortejo e o acasalamento (Wells, 2007), podendo ajudar o macho a segurar a fêmea durante o amplexo ou, inclusive, durante combates contra outros machos (Savage, 1961). Os calos são muito presentes em espécies que reproduzem em corpos d´água, especialmente, em córregos (Duellman & Trueb, 1986), e em algumas espécies, se reduzem fora da

temporada reprodutiva, porém nunca alcançam o estágio morfológico das fêmeas (Emerson, 2000; Wells, 2007). Foi observada a presença de excrescências nupciais no gênero *Melanophryniscus* (Anura: Bufonidae) em *M. alipioi* (Langone *et al.,* 2008)*, M. cambaraensis* (Santos *et al.,* 2010), *M. krauczuki* (Baldo & Basso, 2004), *M. pachyrhynus* (Baldo *et al.,* 2012), *M. rubriventris* (Vaira, 2005), e *M. stelzneri* (Cei, 1980), *M. devincenzii*, *M. moreirae*, *M. simplex*, e *M. tumifrons* (Peloso *et al*., 2012).

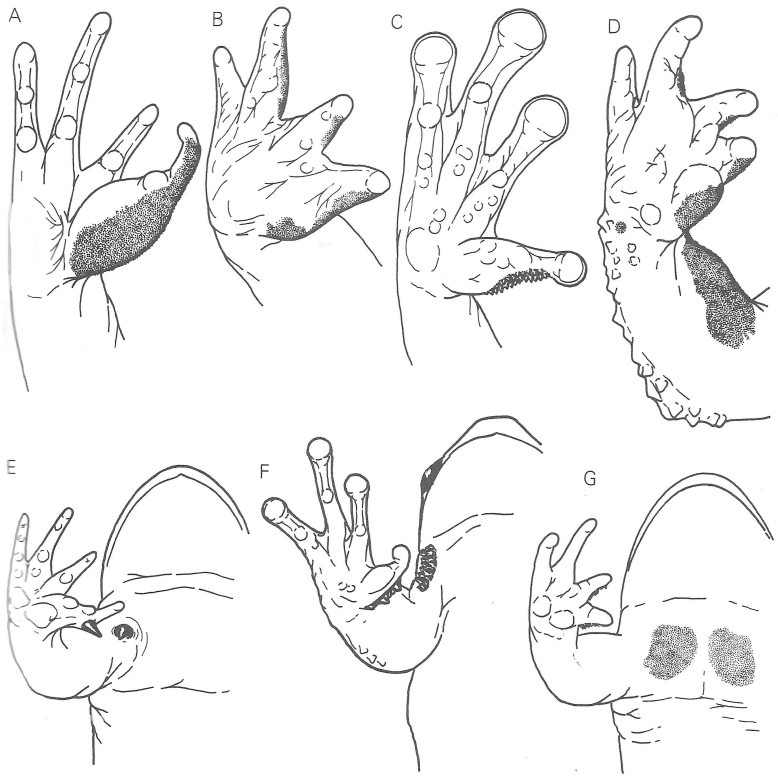

Figure 3. Vista ventral das excrescências nupciais de machos reprodutivos. Ilustração retirada de Duellman & Trueb, (1986).

A maioria das espécies apresenta calos no dedo I (polegar), e algumas também no dedo II e III, sempre em machos. Os autores consideram a presença de calos um dimorfismo sexual. Apesar de existirem alguns trabalhos, a maioria apenas cita a presença dos calos, mas não os descreve em detalhes (Peloso *et al.,* 2012). Muitas espécies ainda não foram estudadas, incluindo *Melanophryniscus cambaraensis* e *M. macrogranulosus.*

2.6. Bioacustica

A produção de sons é um método para advertir a presença de um indivíduo a outros e pode ser encontrada em diversos grupos de animais, incluindo, nos vertebrados, as aves, os mamíferos e os anfíbios, especialmente os anuros (Duellman & Trueb, 1986). Por definição, o som é uma energia

mecânica, formada pela oscilação de pressão em um meio material, e que se desloca de forma longitudinal. Este sinal emitido é recebido e processado pelo indivíduo que o escuta.

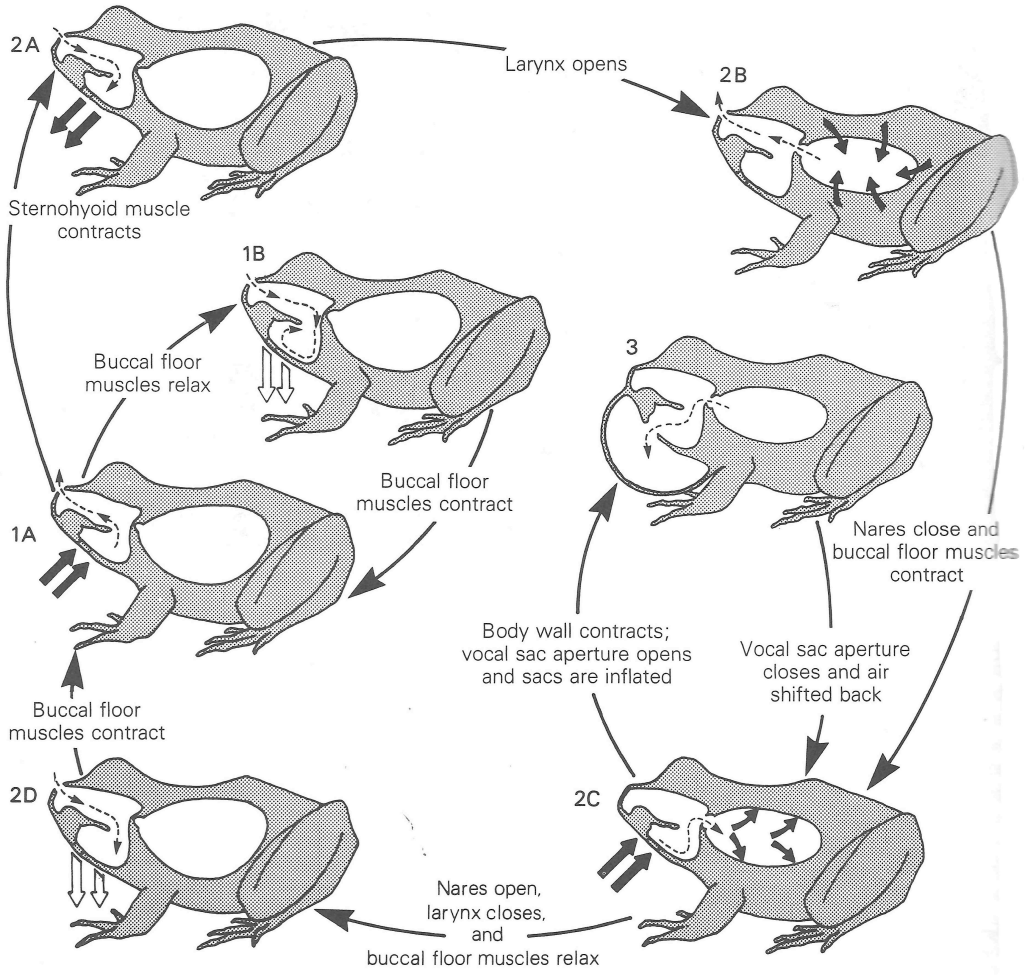

Figure 4. Esquema da movimentação do ar na vocalização de anuros. Ilustração de Duellman e Trueb, (1986), modificado de Gans, (1973).

As principais estruturas envolvidas na produção de som são as narinas, a cavidade bucal, o saco vocal, os pulmões, a laringe e a musculatura associada a eles. Os ciclos respiratórios oscilatórios (Fig. 4 – 1A-B) envolvem o deslocamento do ar atmosférico na cavidade bucal, através das narinas. O ciclo para levar o ar até os pulmões inclui dois estágios, o primeiro a partir do exterior para a cavidade bucal (Fig. 4 - 2A) e o segundo, após a expiração (Fig.4 – 2B), da cavidade bucal para os pulmões (Fig. 4 – 2C). Estes ciclos ventilatórios são alimentados pela atividade muscular da parede inferior da cavidade bucal (Duellman & Trueb, 1986).

A estrutura do canto é reconhecida nos anuros como espécie-específica (Gerhardt, 1994). Uma revisão feita por Wells (2007) dividiu o canto dos anuros em seis tipos: 1) O canto de anúncio, emitido

por machos durante o evento reprodutivo, que pode ter tanto a função de atrair fêmeas conspecíficas, como de avisar o posicionamento de um macho para os outros, ajudando-os a manter uma distância para vocalizar no sítio reprodutivo ou, ainda, de saber o tamanho corporal do macho vocalizando e seu potencial para disputar uma fêmea ou um sítio de vocalização. 2) O canto nupcial é emitido por machos para chamar a atenção das fêmeas quando estão por perto, podendo ser emitido em resposta a aproximação de uma fêmea ou, por exemplo, na intenção de tornar seu canto o mais evidente dentro de um coro. 3) O canto de reciprocidade é observado apenas em algumas espécies e é emitido por fêmeas receptivas em resposta ao canto emitido por um macho da mesma espécie. 4) Canto agressivo, emitido por machos durante qualquer forma de interações agonísticas. 5) Canto de soltura, um sinal acústico usado para avisar que foi agarrado um par inapropriado para a reprodução. 6) Canto de angústia, alerta e defesa, um som chamativo produzido por indivíduos de ambos os sexos em resposta a distúrbios e, possivelmente, para alertar vizinhos de algum perigo ou assustar um predador.

O padrão temporal de reprodução tem implicações sobre a maneira como os machos irão investir seu tempo e sua energia durante o evento (Wells, 1977). A reprodução explosiva, característica das espécies *Melanophryniscus cambaraensis* e *M. macrogranulosus*, ocorrer em um curto período de tempo, e permite que os machos invistam sua energia em um curto prazo, porém limita a possibilidade de escolha do parceiro. Atualmente, apenas oito das 26 espécies reconhecidas para o gênero tem seu canto descrito e apenas uma delas é pertencente ao grupo *M. tumifrons*. A estrutura do canto destas espécies mostra um padrão que se repete dentro do gênero, o canto é dividido em dois fragmentos: o canto A, composto por pulsos únicos, e o canto B, formado por uma série de pulsos contínuos com um espaçamento curto, formando um trinado. Entretanto, este padrão tem algumas peculiaridades espécie-específicas. Alguns autores (Bee *et al.,* 2001; Castellano *et al.,* 2002) já observaram que podem ser encontradas variações dos parâmetros acústicos, tanto ao nível de espécie, como entre e dentro de indivíduos e populações. Esta ferramenta bioacústica pode ser usada para o reconhecimento de novas espécies que não podem ser distinguidas por caracteres morfológicos (e.g. Heyer *et al.,* 1996).

2.7. Conservação

As espécies *Melanophryniscus macrogranulosus* e *M. cambaraensis*, incluídas em listas de espécies ameaçadas nacional e regionalmente, foram abordadas durante o Plano de Ação Nacional para a Conservação dos Anfíbios e Répteis Ameaçados da Região Sul do Brasil, realizado na FLONA de Ipanema, Município de Pirassununga, São Paulo, Brasil durante o período de 17-21 de outubro de 2011. Durante a reunião foram identificadas ações necessárias para a conservação destas duas espécies (ICMBio, 2012) das quais nós ficamos responsáveis. Os estudos propostos por este projeto visam

contemplar as ações propostas no Plano de Ação para a conservação destes sapinhos-de-barriga-vermelha, endêmicos do Rio Grande do Sul.

OBJETIVO GERAL

A fim de contribuir com conhecimento sobre a diversidade e conservação de anuros do sul da Mata Atlântica, propõem-se testar a identidade taxonômica das populações de *Melanophryniscus* atualmente referidas a *M. cambaraensis* e *M. macrogranulosus*.

3.1. Objetivos específicos

- Testar a validade da fotoidentificação como marcação individual na população de *Melanophryniscus cambaraensis* da FLONA SFP.
- Definir a identidade taxonômica das diferentes populações conhecidas, através da análises genética e morfológica.
- Descrever as excrescências nupciais das espécies *Melanophryniscus* em estudo.
- Descrever o canto de anúncio das espécies de *Melanophryniscus* em estudo.
- Tentar localizar a população de *Melanophryniscus cambaraensis* da localidade-tipo, na Fortaleza dos Aparados, nunca mais vista desde a década de 90.
- Localizar novas populações das espécies em estudo.

RESULTADOS

4.1. Capítulo 1: Avaliação dos métodos de amputação de falanges e fotoidentificação para a identificação individual do sapinho-de-barriga-vermelha, *Melanophryniscus cambaraensis*

A eficácia da fotoidentificação foi testada em 492 imagens, que correspondiam a 147 indivíduos, usando como métodos a fotoidentificação visual e a automatizada (WildID software) e amputação de falanges como técnica de controle. A maior acurácia foi registrada com a fotoidentificação visual (99.4%), seguido da amputação de falanges (95.3%) e, por último, com a utilização do software (90.9%). A diferença da eficácia dos métodos utilizados foi significativa para todas as comparações. Os erros dos métodos de fotoidentificação foram sempre falsos negativos, considerando recapturas como capturas novas, enquanto que os erros cometidos com a amputação de falanges foram todos de identificação cruzada, referenciando uma recaptura ao indivíduo equivocado. Uma vez que os erros de identificação

ocorreram em todos os métodos de formas diferentes, os resultados sugerem que estudos que requerem uma alta acurácia devem utilizar pelo menos dois métodos diferentes para permitir a validação cruzada. O desempenho de cada método depende do organismo de estudo, para avaliar as condições de trabalho, os impactos trazidos aos indivíduos e a taxa de erros nas inferências. Sendo assim, os pesquisadores devem avaliar cuidadosamente as vantagens e desvantagens de cada método de identificação individual antes de investir recursos e tempo na coleta de dados em campo.

4.2. Capítulo 2: Status taxonômico de *Melanophryniscus macrogranulosus* Braun, 1973 e *M. cambaraensis* Braun & Braun, 1979 (Anura: Bufonidae), sapinhos-de-barriga-vermelha do extremo sul do Brasil

O resultado das análise da morfologia (17 medidas do corpo) demostraram que existe uma variação geográfica das populações conhecidas de *Melanophryniscus cambaraensis* e *M. macrogranulosus*, porém nenhum caráter permitiu separar grupos bem definidos. As análises moleculares (parte do gene citocromo b) mostraram uma distância genética inferior a 0,5% entre as populações estudadas, enquanto que com a espécie *M. simplex* foi encontrada uma variação superior a 3%. As análises da morfologia e da genética não evidenciaram a existência de caracteres diagnósticos para as duas espécies referidas. Portanto, *Melanophryniscus cambaraensis* Braun & Braun, 1979 foi considerado um sinônimo Junior de *Melanophryniscus macrogranulosus*.

4.3. Capítulo 3: Calos nupciais em *Melanophryniscus macrogranulosus* (Anura: Bufonidae) e a presença de uma inesperada estrutura dérmica para o gênero

Foram encontrados calos nupciais em machos de *Melanophryniscus macrogranulosus* nos dedos I (polegar), II e III. As observações feitas mostraram também a presença destas estruturas nas espécies *M. dorsalis*, *M. sanmartini* e *M. simplex.* Adicionalmente, nossos resultados identificaram o primeiro registro destas estruturas dérmicas em fêmeas, dentro do gênero *Melanophryniscus*. Contudo, neste caso essas estruturas são encontradas apenas no dedo I e em menor densidade que nos machos.

4.4. Capítulo 4: Canto de anúncio do sapinho-de-barriga-vermelha, *Melanophryniscus macrogranulosus,* do extremo sul da Mata Atlântica*,* Brasil: Codificando a variação dos sinais acústicos em diferente níveis

Indivíduos de *Melanophryniscus macrogranulosus* foram observados cantando nos sítios reprodutivos, pequenos riachos d'água formados após intensas chuvas. Os machos cantaram, geralmente,

dentro da rasa lâmina de água formada no sítio, lugar onde também foram observados machos procurando ativamente por fêmeas, amplexos formados e desovas. O canto de anúncio da espécie é composto por duas partes: canto A e canto B. Ele se inicia com o tipo A, composto por pulsos únicos de frequência modulada, separado por intervalos longos e durando, aproximadamente, 0,44 - 6 segundos. Esta parte é sempre seguida do canto B, um trinado extremamente longo de pulsos contínuos não modulados emitidos a uma taxa de 32,8 – 42,6 pulsos por segundos e durando entre 9 – 32,1 segundos. O pico de frequência observada para a espécie foi em média 2318.8 (Hz), com desvio padrão de 235, mínima de 1968 e máxima de 3000. Foi encontrada uma alta variação em todos os níveis estudados, intra e inter-individual e populacional, assim como observados para outras espécies do gênero e de outros anuros estudados. Esta ferramenta mostrou resultados similares as análises morfológicas e genéticas do capítulo anterior, corroborando a hipótese de que *M. cambaraensis* e *M. macrogranulosus* são sinônimos.

Nota:

O Programa de Pós-graduação em Biologia Animal estabelece como norma para dissertação de Mestrado apresentadas em forma artigos, que estes sejam colocados nas normas editoriais exigidas para publicação direta nos periódicos científicos escolhidos e que as normas sejam incluídas no trabalho. A fim de facilitar a leitura, algumas normas não foram contempladas na dissertação. As legendas e figuras foram incluídas ao longo do texto e não ao final, como é usual na formatação de submissão das revistas escolhidas.

CAPÍTULO 1: Clip or Snap? An Evaluation of Toe-Clipping and Photo-Identification Methods for Identifying Individual Southern Red-Bellied Toads, *Melanophryniscus cambaraensis*

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CLIP OR SNAP? AN EVALUATION OF TOE-CLIPPING AND PHOTO-IDENTIFICATION METHODS FOR IDENTIFYING INDIVIDUAL SOUTHERN RED-BELLIED TOADS, *MELANOPHRYNISCUS CAMBARAENSIS*

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Abstract. The most common method for identifying individual amphibians is toe-clipping (TC), whereby captured individuals are marked by a unique combination of amputated phalanges that corresponds to a unique alphanumeric code. However, ethical and methodological objections to this method have been raised and there is broad interest in developing alternative methods. One alternative is to use photo-identification methods (PIMs) to identify individuals based on their natural markings. We tested the efficacy of TC and two PIMs — visual matching (VM) and computer-assisted matching (CAM) using the software Wild-ID — in identifying individual adults of the endangered southern red-bellied toad, *Melanophryniscus cambaraensis.* We collected data over 5 mo at Floresta Nacional de São Francisco de Paula, Rio Grande do Sul, Brazil. All specimens were toe-clipped and photographed. The total dataset included 492 captures of 147 individuals. VM was most accurate (99.4%), followed by TC (95.3%) and CAM (90.9%); VM was significantly more accurate than TC and CAM and TC was significantly more accurate than CAM. CAM accuracy diminished as dataset size increased but was considerably faster than VM. All CAM and VM errors were false negatives but involved different images; all TC errors were cross-identifications. Given that misidentifications occurred using both PIMs and TC, our results suggest that studies that require high accuracy should employ at least two methods to allow cross-validation. The performance of each method and the impacts of different kinds and rates of error on inferences depend on the organisms, field conditions, dataset sizes, and study questions. As such, researchers must carefully evaluate the trade-offs of each method before investing significant time and resources in collecting field data.

Keywords. Individual identification; mark-recapture; visual matching; computer-assisted matching; misidentification; Amphibia; Anura; Bufonidae.

Introduction

Many wildlife studies require that individuals be identified in order to draw inferences from repeated observations or eliminate pseudoreplicates prior to analysis. For amphibians, the most widely used method of individual identification is toe-clipping, whereby captured individuals are marked by a unique combination of amputated phalanges that corresponds to a unique alphanumeric code (Donnelly *et al.,* 1994). Toe-clipping is quick, easy, inexpensive, and has become established through decades of use. Although ethical and methodological objections have been raised (Perry *et al.,* 2011), other methods of artificially marking amphibians (*e.g.,* external tags, passive internal transponder tags, brands, tattoos, subcutaneous elastomers) are afflicted by similar or worse problems. In the absence of a clearly superior method, the conservation importance of the ecological and demographic information obtained from toe-clipping studies clearly outweighs their potential negative impacts (Funk *et al.,* 2005). Nevertheless, there is broad interest in developing quick, easy, inexpensive, and reliable individual identification methods that avoid artificial marking.

Photo-identification methods (PIMs), which use photographs of unique natural markings to identify individuals (Bradfield, 2004), avoid most of the ethical objections to artificial marking methods, and it can be quick, easy, and inexpensive to generate images in the field. However, PIM utility also depends on the researcher's ability to quickly and accurately identify individuals. Assuming persistent natural markings occur, visual matching can be highly effective for small datasets but becomes increasingly onerous and inaccurate as image databases grow, which has led to the development and application of a variety of image matching algorithms (Kelly, 2001; Arzoumanian *et al.,* 2005; Speed *et al.* 2007; Gamble *et al.,* 2008; Hastings *et al.* 2008; Hiby *et al.* 2009; Sherley *et al.,* 2010). Recently, Bolger *et al.* (2011) released the software Wild-ID, which combines both approaches by scoring the pairwise similarity of all images and presenting the user with the 20 top-ranked matches for visual match confirmation. Few studies have compared toe-clipping and PIMs, and none has compared the accuracy of toe-clipping, visual matching, and computer-assisted matching. As such, the primary objective of this study was to compare the accuracy of toe-clipping and two PIMs — visual matching and computer-assisted matching using Wild-ID — to identify adult individuals of the endangered southern red-bellied toad, *Melanophryniscus cambaraensis* Braun and Braun, 1979. To better assess the trade-offs associated with these two PIMs, we also compared the time required for visual and computer-assisted matching and evaluated the numerical performance of Wild-ID.

Materials and Methods

Data collection

Melanophryniscus cambaraensis is a small (*ca.* 35 mm snout-vent length), poisonous toad that migrates diurnally (Santos and Grant, 2011). Dorsal coloration is bright green and varies little among individuals. Ventral coloration is predominantly red with highly variable green, grey, or black blotches and white tubercles (Fig. 1; Braun and Braun, 1979). Coloration and tuberculation are not sexually dimorphic; however, recently metamorphosed individuals lack bright coloration (Fig. 2) and the ontogeny of pigmentation is unknown. As such, we focused exclusively on adults.

We studied *Melanophryniscus cambaraensis* at Floresta Nacional de São Francisco de Paula, southern Brazil (29°25'41.3"S, 50°23'44.5"W, 866 m above sea level), from October 2008-February 2009 (139 d from first to last sampling day). The study site and capture methods are described in Santos *et al.* (2010) and Santos and Grant (2011). All captured specimens were first weighed to 0.1 g and examined for overall health. Digits were removed according to Waichman's (1992) alphanumeric system using surgical scissors sterilized by flaming and cleaning in 100% ethanol, and 1% silver sulfadiazine antibiotic cream was immediately applied to the wound. Amputated digits were preserved in 100% ethanol. Previously toe-clipped specimens were examined for digit regeneration (Ursprung *et al.,* 2011) and their unique alphanumeric code was immediately recorded.

All specimens were subsequently photographed with a digital camera (Sony DSC-H1 5.1 MP, Sony DSC-W210 12.1 MP, or Sony DSC-W90 8.1 MP) using the built-in flash. We photographed entire venters by placing specimens on their backs on white paper next to a ruler (0.5 mm precision) for scale. Previous studies highlighted the importance of obtaining high quality images (Forcada and Aguilar, 2000; Gowans and Whitehead, 2001), so we ensured animals were clean (*i.e.,* free of debris that could obscure natural marks), dry (to avoid flash reflections), and in a position that did not conceal ventral markings, and we took 2-3 images per specimen to ensure proper focus and framing. Individuals were observed for at least 5 min prior to release. Images were later screened for quality and lighting and a single image was selected from each capture event. Selected images were cropped to eliminate as much of the background as possible and were saved in a new directory for individual identification.

Individual identification

Toe-clipping identification was based on the alphanumeric code recorded in the field. PIM identification was performed without knowledge of the specimens' alphanumeric codes. Visual image matching was accomplished by comparing each image to all others and examining the coloration of

the belly, throat, arms, and legs. For computer-assisted image matching we used the Java program Wild-ID (Bolger *et al.,* 2011), which uses the scale invariant feature transform algorithm (SIFT; Lowe, 2004) for pattern extraction, compares the geometric arrangement of the SIFT features of each pair of images, and calculates a match score. The software then shows the 20 top-ranked matches for visual confirmation.

Given that no identification method is necessarily error-free, we used cross-validation to definitively establish specimen identity. To measure the accuracy of each method, we scored each identification as correct or incorrect relative to the cross-validated identification. We performed a X^2 test to determine if the accuracy of the three methods differed significantly, assuming a significance level of 0.05 and Bonferroni correction for multiple comparisons. We also classified each error as (1) false negative (misidentification of a recaptured individual as a previously uncaptured individual), (2) false positive (misidentification of a previously uncaptured individual as a recaptured individual), or (3) cross-identification (misidentification of one previously captured individual as another previously captured individual).

Because all specimens were toe-clipped and photographed simultaneously, we were unable to compare field-processing times for the two methods. However, we compared the time required to perform visual and computer-assisted (Dell Inspiron N5110, Intel Core i5-2410M 2.3 GHz 2.30 GHz CPU, 6 GB RAM, Windows 7) matching of 100 randomly selected images. To better understand the performance of the Wild-ID software, we examined the rank of the correct matches among the 20 top-matches and (Bolger *et al.,* 2011) and assessed the accuracy of computer-assisted analyses of reduced datasets by analyzing the first 25%, 50%, and 75% of captures.

Results

The total dataset included 492 images of 147 individuals. We observed neither digit regeneration nor indication of infection, necrosis, or deterioration of health attributable to toe-clipping. However, we did observe several apparently unrelated injuries and malformations, including partial absence of unclipped digits, recently injured and infected unclipped digits, and inability to use the right leg of a previously uncaptured individual.

The greatest accuracy was achieved by visual matching (VM), which correctly identified all but three captures (99.4%). Toe-clipping (TC) was second, with 95.3% accuracy, followed by computer-assisted matching (CAM), with 90.9% accuracy for the entire dataset. The differences in accuracy for the analyses

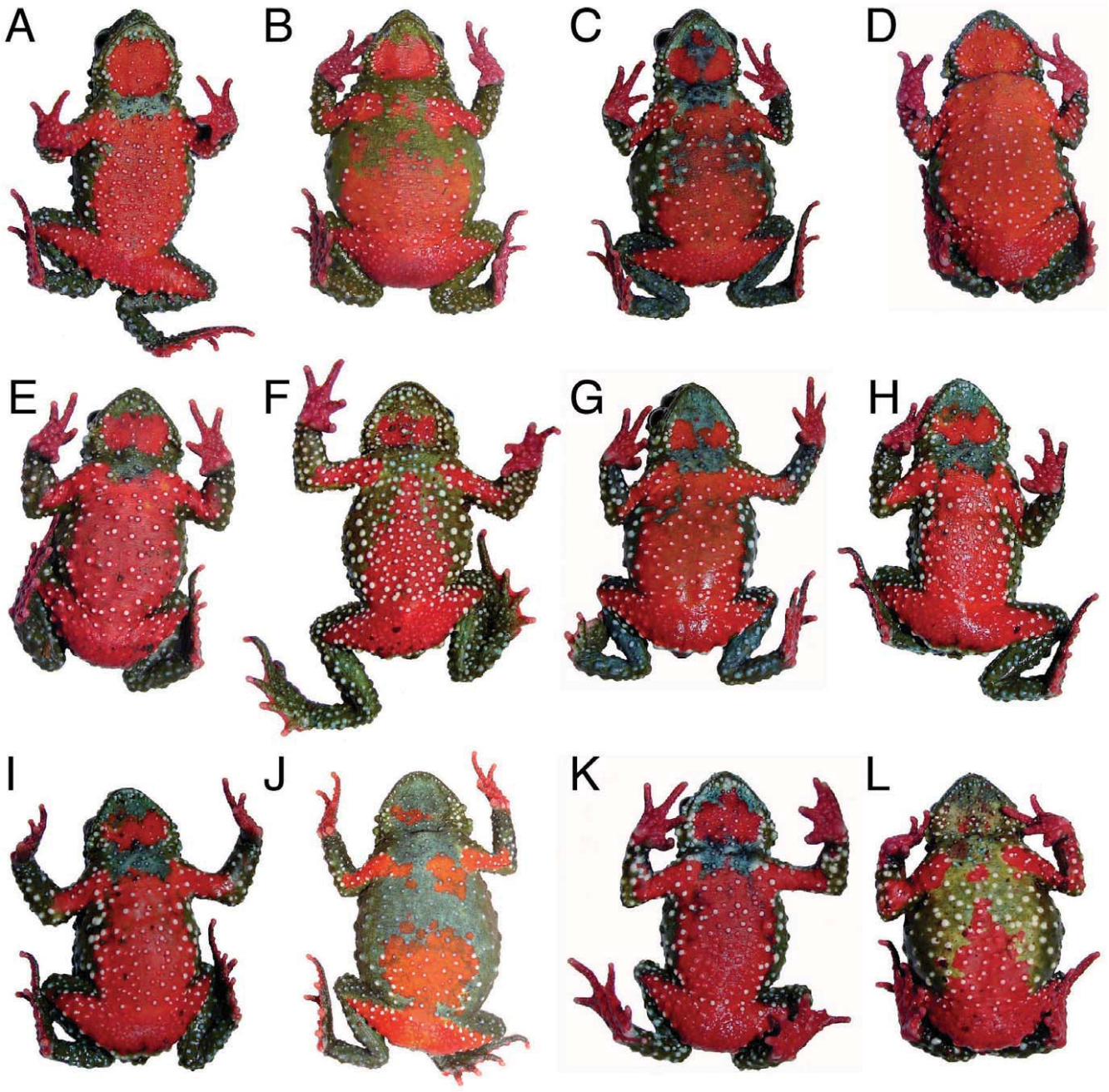

FIGURE 1. Ventral view of a sample of 11 adult individuals of Melanophryniscus cambaraensis from Floresta Nacional de São Francisco de Paula, RS, Brazil. (A) A1 C2 (male, SVL = 29.2 mm). (B) A2 D3 (female, SVL = 32.4 mm). (C) A3 D3 (female, SVL = 31.6 mm). (D) B3 C1 (female, SVL = 34.7 mm). (E) C1 D3 (male, SVL = 28 mm). (F) C1 D4 (male, SVL = 27.9mm). (G) C3 D5 (male, SVL = 29.6 mm). (H, I) C4 (male, SVL =29.9 mm). (J) B3 D1 (female, SVL = 31.8 mm). (K) C4 D2 (male, SVL = 29.1 mm). (L) D1 (male, SVL = 31.3 mm).

of the entire dataset were highly significant (VM-CA: $X^2 = 38.635$, $p = 0.0001$; VM-TC: $X^2 = 15.802$, $p = 0.0001$; CA-TC: $X^2 = 7.646$, $p = 0.0057$; corrected significance level = 0.017). All CAM and VM errors were false negatives but involved different images. All TC errors were cross-identifications. Choice-rank in CAM was high, with > 90% of the correct matches ranked in the top 3 (74% ranked first, 10% second, 5% third). The time required to match 100 randomly selected images was 180 min for VM and 20 min for CAM, including the time required to visually confirm each match. CAM accuracy was 95% for the 25% ($n = 123$ captures), 50% ($n = 246$ captures) datasets and 93% for the 75% ($n = 369$ captures) dataset.

Discussion

None of the tested methods was error-free, but visual image matching (VM) was significantly more accurate than toe-clipping (TC) and computer-assisted image matching (CAM), and TC, in turn, was significantly more accurate than CAM. Our finding that VM was significantly more accurate than TC differs from that of Kenyon *et al.* (2009), who reported that TC was considerably, albeit not significantly, more accurate than VM. This difference probably owes to differences in the conspicuousness of the natural markings in the two species. Indeed, Kenyon *et al.* (2009) underscored the difficulty in visually identifying individuals that lacked distinctive dorsal hourglass patterns, which constituted the majority of their sample, whereas the bright ventral patterns of *Melanophryniscus cambaraensis* were well defined in all sampled individuals.

Although both photographic identification methods (PIMs) exhibited the same class of error (viz., false negatives), the specific images that were misidentified differed. As such, by combining the two PIMs all errors were eliminated. TC errors were exclusively cross-identifications caused by human errors when recording alphanumeric codes in the field. Given that misidentifications occurred using both PIMs and TC, we recommend that studies that require high accuracy employ at least two methods to allow cross-validation.

The performance of Wild-ID (Bolger *et al.,* 2011) in identifying individual *Melanophryniscus cambaraensis* was similar to that of previously studied programs and organisms (*e.g.,* Kelly, 2001; Arzoumanian *et al.,* 2005; Speed *et al.,* 2007; Gamble *et al.,* 2008; Hastings *et al.,* 2008; Hiby *et al.,* 2009; Sherley *et al.,* 2010). Importantly, correct matches were ranked in the top 3 in the vast majority of comparisons, which greatly facilitated visual confirmation. Although CAM accuracy diminished as dataset size increased and was significantly lower than for either VM or TC, CAM image matching was considerably faster and remained > 90% accurate.

We did not observe any variation in ventral coloration during the course of our study, which is consistent with most previous studies of adult anurans (Stephenson and Stephenson, 1957; Denton and Beebee, 1993; Kenyon *et al.,* 2010; but see Kenyon *et al.,* 2009). Nevertheless, we caution that the bright ventral pigmentation of *Melanophryniscus cambaraensis* is lacking at metamorphosis and is acquired over time. Insofar as we exclusively targeted migrating adults, we did not assess ontogenetic variation in ventral coloration or its effect on PIM accuracy. Similarly, although we did not observe digit regeneration in adults, we did not assess the potentially extensive regeneration in juveniles (*e.g.,* Richards *et al.,* 1975), which could also confound individual identification.

No method of individual identification can be guaranteed to be completely error-free, and the overall performance of each method and the impacts of different kinds and rates of error on inferences will depend on the organisms, field conditions, dataset sizes, and study questions. Toe-clipping has the ancillary advantages of generating tissue samples for DNA analysis and skeletochronology of phalanges, and, although accuracy was high in the present study, PIMs appear to be less effective for species with less conspicuous natural markings. As such, selection of the optimal method of individual identification is a scientific problem — not a legal or political one — that requires researchers to carefully evaluate the trade-offs of each method before investing significant time and resources in collecting field data.

FIGURE 2. Ventral view of a metamorphosing individual (8.4 mm SVL) of *Melanophryniscus cambaraensis*.

Resumo

O método mais utilizado para a identificação individual de anfíbios é a marcação por amputação de falanges (AF), no qual cada indivíduo capturado é marcado através de uma combinação única de falanges amputadas de um ou mais dígitos de acordo com um código alfanumérico. Entretanto, alguns questionamentos éticos e metodológicos tem sido levantados a respeito deste método de marcação e existe um grande interesse em desenvolver métodos alternativos. Os métodos de fotoidentificação (MFI) são uma alternativa que permite identificar indivíduos a partir de padrões de coloração e marcas naturais dos animais estudados. Neste estudo, nós testamos a eficácia da AF e dois tipos de MFI — através da identificação visual (IV) e outro com o auxílio de computador (AC) usando o software Wild-ID — na identificação individual de adultos do sapinho-de-barriga-vermelha, *Melanophryniscus cambaraensis*. Os dados foram coletados durante um estudo de cinco meses realizado na Floresta Nacional de São Francisco de Paula, Rio Grande do Sul, Brasil. Todos os espécimes coletados foram marcados pelo método de AF e posteriormente fotografados. O banco de dados teve um total de 492 capturas correspondentes a 147 indivíduos. O método de IV foi o método mais acurado (99,4%), seguido de AF (95,3%) e AC (90,9%); IV foi significativamente mais acurado que os outros dois métodos, enquanto AC foi significativamente menos acurado. A acurácia de AC diminuiu conforme aumentou o banco de dados a ser analisado, entretanto, seu processamento foi consideravelmente mais rápido que IV. Todos os erros cometidos com AC e IV foram falsos negativos, porém envolveram diferentes imagens; já os erros da AF foram identificações cruzadas. Uma vez que os erros de identificação ocorreram tanto nos métodos MFI como AF, os resultados sugerem que estudos que requerem uma alta acurácia devem utilizar pelo menos dois métodos diferentes para permitir a validação cruzada. O desempenho de cada método, seus impactos e taxa de erros nas inferências dependem do organismo estudado, condições do trabalho em campo, tamanho do banco de dados e os objetivos do estudo. Sendo assim, os pesquisadores devem avaliar cuidadosamente as vantagens e desvantagens de cada método de identificação individual antes de investir recursos e tempo na coleta de dados em campo.

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CAPÍTULO 2: Taxonomic status of the endangered red-bellied-toads *Melanophryniscus macrogranulosus* Braun, 1973 and *M. cambaraensis* Braun & Braun, 1979 (Anura: Bufonidae), from southern Atlantic Rainforest in Brazil

(Artigo nas normas de submissão do periódico *Zootaxa*)

Taxonomic status of the endangered red-bellied-toads *Melanophryniscus macrogranulosus* Braun, 1973 and *M. cambaraensis* Braun & Braun, 1979 (Anura: Bufonidae), from southern Atlantic Rainforest in Brazil

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Abstract: *Melanophryniscus macrogranulosus* was described in 1973 by Braun and nowadays is known in Dom Pedro de Alcântara and Maquiné, Rio Grande do Sul (RS) state, Brazil. Six years latter, *M. cambaraensis* was described by Braun and Braun as the first species of the genus with green skin pattern coloration. Geographic distribution of this species extends for Cambará do Sul and São Francisco de Paula, RS, Brazil. The veracity of this two species has been questioned for their extremely similar morphology. Aspects of pattern coloration described as different by the author varied so much that they overlap. Reproductive sites known for the species are very similar, the opposite described in the description article. Considering that both species are endemic from the state and are categorized as endangered, the main goal of our study is to review the taxonomic status of *M. macrogranulosus* and *M. cambaraensis* and looked up to extend the range of distribution for the species. We collected data in four different municipalities from 2011-2013. We analyzed 17 different measurement of external morphology and partial sequences of *Cytochrome b* of some populations. Morphology and molecular data did not

show evidence to separate groups, excluding them as properties capable of explaining the status of the last species as valid. Therefore, we refer to all as *M. macrogranulosus*. Under IUCN Red List criteria (IUCN 2013), all these impacts associated with the small extent of occurrence known for the species justify the category as Endangered.

Key-words: endangered species, morphology, genetics.

Introduction

Melanophryniscus belongs to the family Bufonidae and includes 26 recognized species (Frost, 2014) endemic to South America, distributed in Argentina, Brazil, Bolivia, Paraguay and Uruguay. Most species have a dark coloration with bright ventral spots or patches, from which is derived their common names − red-bellied-toads. These toads perform the "unken" reflex behavior, used to exhibit their aposematic coloration when disturbed (Kwet *et al.,* 2005; Langone *et al.,* 2008; Santos & Grant, 2011). The bright coloration could be associated with the presence of toxic skin alkaloids sequestered from diet that prevent predation, infections and parasites (Saporito *et al.,* 2009, 2012). Such substances have been found in other species of the genus (e.g. Daly *et al.,* 2008; Garraffo *et al.,* 1993; Grant *et al.,* 2012). The hypothesis is that younger individuals uptake these alkaloids gradually over lifetime (Saporito *et al.,* 2009) and concomitantly evolve bright coloration and/or frontal swelling (Saporito *et al.,* 2010).

This genus is arranged in three different groups (Cruz & Caramaschi, 2003) weakly defined based on morphological characters (Baldo *et al.,* 2012): *M. stelzneri*, *M. moreirae* and *M. tumifrons* species group. This last one is characterize by the presence of a frontal skin gland (Naya *et al.,* 2004), which is presumed to be a synapomorphy of this group (Baldo & Basso, 2004). The *M. tumifrons* species group currently includes eight species: *M. cambaraensis* Braun & Braun, *M. devincenzii* Klappenbach, *M. macrogranulosus* Braun, *M. pachyrhynus* (Miranda-Ribeiro), *M. simplex* Caramaschi & Cruz, *M. spectabilis* Caramaschi & Cruz, *M. tumifrons* (Boulenger) and *M. peritus* (Caramaschi and Cruz).

Melanophryniscus macrogranulosus was described by Braun (1973) based in nine individuals from Morro da Gruta, municipality of Dom Pedro de Alcântara, State of Rio Grande do Sul (RS), Brazil.

Latter, Escobar *et al*. (2004) extended the known distribution to Barra do Ouro, municipality of Maquiné, RS. *Melanophryniscus cambaraensis* was described by Braun & Braun (1979) as the first species of the genus with green coloration. This species was originally found in the Serra Geral National Park (PARNA da Serra Geral), municipality of Cambará do Sul, RS and latter discovered in the São Francisco de Paula National Forest (FLONA SFP), municipality of São Francisco de Paula, RS, Brazil. This species has not been seen in the type locality since 1990. Another three localities were reported in Caorsi *et al*., (unpublished data, Fig. 2 – number 1, 4 and 5).

The extremely similar morphology of *Melanophryniscus macrogranulosus* and *M. cambaraensis* has led some herpetologist to question the validity of this species Braun and Braun (1979: 10), reported as differences between the "environment" they inhabit and their "dorsal, throat and pectoral pattern of coloration". The current known distribution and reproduction sites of this species are very similar (personal observation). Braun & Braun (1979) reported a green dorsum, yellowish throat and yellowish green pectoral region for *M. cambaraensis*; while these regions are supposedly uniformly black in *M. macrogranulosus*. However, all individuals found in the type locality are actually green (personal observation). Braun (1973) reported that the description of the species was based on nine individuals collected in 1960 and deposited in the Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul. Escobar et al. (2004) suggested that the original description of color pattern was possibly made after field notes from the collector (Thales de Lema).

The pattern of coloration in the genus *Melanophryniscus* has been reported to be especially variable within species (Baldo *et al,.* 2012; Cairo & Di Tada, 2005; Vaira, 2002). Our data corroborates those previous reports. Color pattern is highly variable within and between populations putatively attributed to *M. macrogranulosus* and *M. cambaraensis* (Fig. 1). However, despite the variation observed, all live specimens of *M. macrogranulosus*, recently collected, suggest that the ground color is green. Besides, the variation observed extensively overlaps between populations. Actual data indicates absence

of diagnostic characters for these two taxa. Consequently, species identification has been done solely on the basis of altitude and geographic localities (personal observation).

Figure 1. *Melanophryniscus macrogranulosus* from: A, Morro da Gruta, Dom Pedro de Alcântara; B, Lower Garapiá, Maquiné; and *M. cambaraensis* C, São Francisco de Paula National Forest, São Francisco de Paula; and D, Serra Geral National Park, Cambará do Sul (specimen from the type locality; photograph: Arno Lise, 1970´s).

Both, *Melanophryniscus macrogranulosus* and *M. cambaraensis,* are listed as threatened at regional (Garcia & Vinciprova, 2003; Subirá *et al*., 2013) and global levels (Garcia *et al*., 2004; Silvano & Garcia, 2010). The distribution of both species is also endemic to the southern extreme of the endangered Atlantic Rainforest. Bioacoustic analyses of this two species did not show any evidence to define groups by this characters (Caorsi *et al*., unpublished data).

Considering this scenario, the main goal of our study was to review the taxonomic status of *Melanophryniscus macrogranulosus* and *M. cambaraensis*, testing the possible conspecificity of the populations currently identified under these names. Furthermore, we have attempted to record new occurrence sites along potential areas, and to locate the vanished population from the Serra Geral

National Park, RS, Brazil. Finally, considering our taxonomic conclusions, we reevaluated the conservation status of the taxon according to the IUCN criteria.

Material and methods

Sample treatment and Data Collection

Given the lack of known diagnosable characters for *Melanophryniscus macrogranulosus* and *M. cambaraensis*, and to test the possible conspecificity of recorded populations, putative species were not *a priori* defined as groups in the analyses; each reproductive site was considered as a population for the purposes of the morphological and genetic analyses.

We collected data in 20 field trips to previously known or potential localities looking for *Melanophryniscus macrogranulosus* and *M. cambaraensis* in Rio Grande do Sul, Brazil (Tab.1). Seven field trips were undertaken to Morro da Gruta, type locality of *M. macrogranulosus*, municipality of Dom Pedro de Alcântara; four to Barra do Ouro, municipality of Maquiné; three to São Francisco de Paula National Forest (FLONA SFP), municipality of São Francisco de Paula; and six to Serra Geral National Park (PARNA da Serra Geral), municipality of Cambará do Sul, type locality of *M. cambaraensis*. All the expeditions were conducted after heavy rains (ranging between 40-180mm in the fewer days of the reproductive event, according to the Instituto Nacional de Meteorologia (www.inmet.gov.br) and to a pluviometer implanted in the Serra Geral National Park by our team). In the field, we collected material to morphological and molecular analyses and made efforts to find new distribution records for the species. All the material collected during the study was deposited in the Coleção Herpetológica do Museu de Ciência e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul (MCP) and Coleção Herpetológica do Departamento de Zoologia da Universidade Federal do Rio Grande do Sul (UFRGS).

Morphological analyses

For the morphological analyses, we examined 115 specimens (84 males and 31 females) collected along this study or from three herpetological collections from Rio Grande do Sul: (MCP), (UFRGS) and

Coleção de anfíbios do Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul (MCN) (Anexo 1: Specimens examined). The following 17 measurements of each specimens were taken with digital calipers (0.01mm) under a dissecting microscope: snout-vent length (SVL), head width (HW), head length (HL – measured from the snout to the end of the jaw), straight head length (HLS - measured parallel to the longitudinal axis of the body), eye diameter (ED), eye-nostril distance (END), snout-nostril distance (SND), internarial distance (IND), interorbital distance (IOD), upper eyelid length (UEL), upper eyelid width (UEW), thigh length (TL), tibia length (TI), tarsus length (TAL), foot length (FL), forearm length (FAL), hand length (HAL). Frontal swelling measures, common in taxonomic studies involving *M. tumifrons* group species, were excluded from the analyses due to the deformation observed in this macrogland after preservation. We have obtained adequate samples, for morphological analyses, from five localities (2, 5, 7, 8 and 9) for males and four localities (2, 5, 7 and 9) for females (see Tab.1).

Student´s t-test was used to search for sexual dimorphism in selected measurements. Since we have found differences between sexes, all analyses were run separately for males and females. Descriptive statistics for both sexes included mean, standard deviation and range of all measurements for each population. We also performed a Canonical Variate Analysis as an exploratory tool. For this analysis, we removed the effect of overall size by replacing raw variables for their relative proportion in relation to SVL. The small sample size for females did not allow robust analysis, so only males were used to make comparisons between sites.

Molecular analyses

For molecular analyses, we used tissue samples from material collected during field trips and deposited in scientific collection (Anexo 1: Examined specimens – Molecular analyses). We used a total of 33 individuals, from six localities (2, 5, 6, 7, 8 and 9; see Tab.1). Samples were stored in 95% ethanol at -20°C and DNA was extracted using CTAB Method (adapted from Doyle and Doyle, 1987) and DNeasy kit (QIAGEN). Molecular samples from the type locality of *M. cambaraensis* were obtained

from specimens deposited in 70% ethanol since 1973 at MCN. We were able to extract DNA from only one individual, using extracting PureLink® Genomic DNA Mini Kit (Invitrogen). Partial sequences of *Cytochrome b* were obtained from some individuals of *Melanophryniscus macrogranulosus* and from *M. simplex,* a very close relative species of the genus, also belonging to *M. tumifrons* species group. Primers used for this specific region were MVZ 15-L (Moritz et al., 1992) and H15149 (Kocher et al., 1989), forward and reverse respectively. The standard PCR program consisted of a initial denaturing step of 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C, followed by a final extension step of 3 min at 72°C. PCR-amplified products, with approximately 385 pb, were sequenced in Macrogen Inc, South Korea. Sequences were edited and aligned with Geneious software and molecular analyses were conducted using MEGA version 5 (Tamura *et al.* 2011), performing the analysis *Net between group means*, this net is given by the average distance between groups and the mean within-group distance.

Table 1. Geographical data from known records of *Melanophryniscus macrogranulosus*. All localities in Rio Grande do Sul, Brazil.

Municipality	**Locality**	**Coordinates/Altitude**
Dom Pedro de Alcântara	**1** - *Mato dos Macacos* (next to Morro do Forno lagoon)	29°19'37.6" S, 49°51'00" W / 32m asl
	2 - *Morro da Gruta* (Type locality)	29°24'21.16" S, 49°51'1.48" W / 31m asl
Maquiné	**3** - *Morro do Cantagalo*	29°42'44" S, 50°09'00" W / 100m asl
	4 – *Vale do arroio Carvão, Barra do Ouro*	29°32'29.95" S, 50°13'40.56" W / 370m als
	5 – *Lower Garapiá waterfall, Barra do Ouro*	29°30'33" S, 50°14'45" W / 200m als
	6 - *Upper Garapiá waterfall, Barra do Ouro*	29°30'19.00"S, 50°14'29.03" W / 230m als
São Francisco de Paula	**7** - *FLONA SFP (road site)*	29◦25′41.3”S, 50◦23′44.5”W / 866m als
	8 - *FLONA SFP (forest site)*	29º25´43.3” S, 50º23´47,4 W/ 900m asl
Cambará do Sul	**9** - *PARNA da Serra Geral*	29°4'43,58" S, 49°59'13,05" W / 1008m asl

Results and Discussion

Morphological and genetic data corroborates our *a priori* hypothesis of lack of diagnosable characters for *Melanophryniscus macrogranulosus* and *M. cambaraensis*, indicating that *M. cambaraensis* is a junior synonym of *M. macrogranulosus.* In the following sections, we present the analyses of morphological and genetic variation that supports this taxonomic arrangement, as well as a

redescription of *Melanophryniscus macrogranulosus.* In addition, the new taxonomic status groups together two officially endangered species, rendering obligatory to review its conservation status.

Our effort to find new reproductive sites resulted in three new localities for *Melanophryniscus macrogranulosus* (Fig. 2 – number 3, 6 and 7). Our records add to other six previously reported, resulted in nine localities, distributed in four municipalities in the State of Rio Grande do Sul, Brazil (Tab.1). All records are restricted to the southern limits of the Atlantic Rainforest.

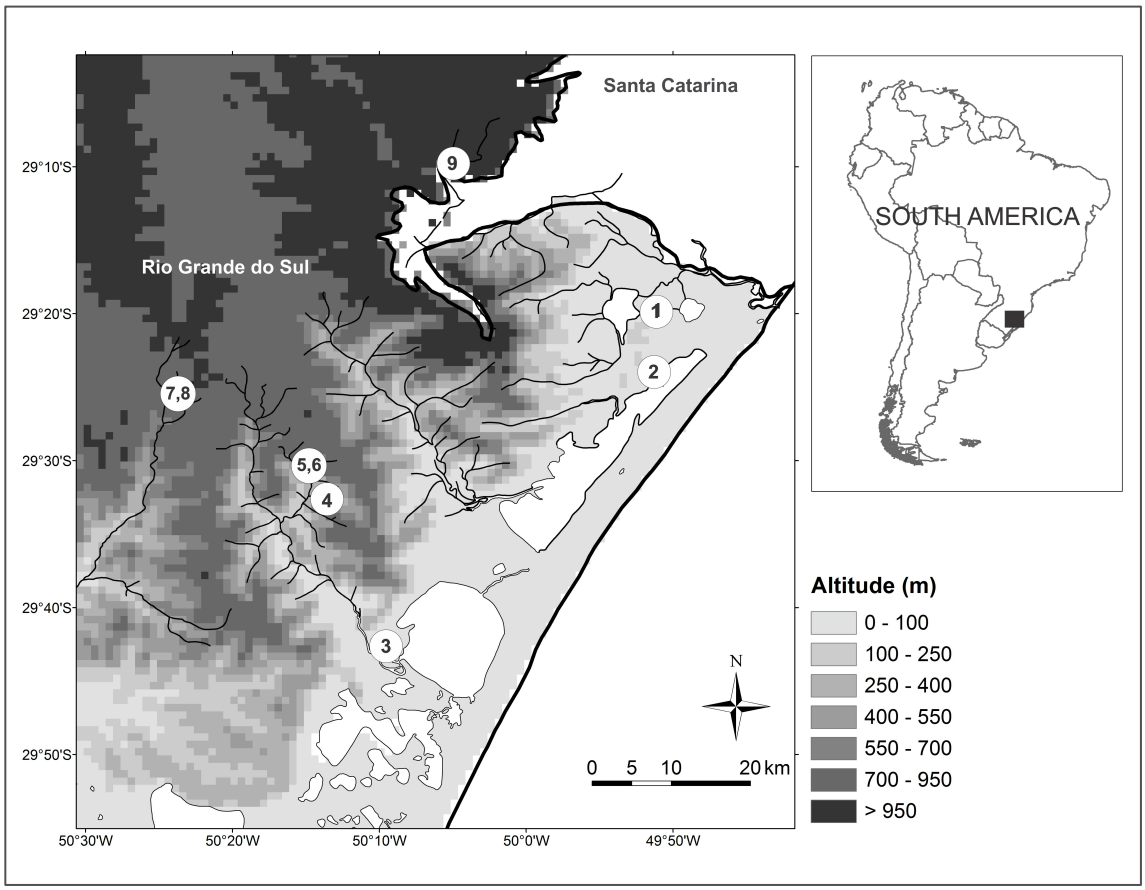

Figure 2. Geographic distribution of *Melanophryniscus macrogranulosus*. Localities (all in the State of Rio Grande do Sul, Brazil): **1** - Mato dos Macacos (next to Morro do Forno lagoon); **2** - Morro da Gruta (Type locality); **3** - Morro do Cantagalo; **4** – Vale do arroio Carvão, Barra do Ouro; **5** – Lower Garapiá waterfall, Barra do Ouro; **6** - Upper Garapiá waterfall, Barra do Ouro; **7** - FLONA SFP (road site); **8** - FLONA SFP (forest site); **9** - PARNA da Serra Geral (see Table 1 for additional details).

Morphological analyses

Variation and Sexual Dimorphism: some measurements showed differences between males and females (Table 2 and 3). Sexual dimorphism in snout-vent length was significant (Student´s t-test, t= -5,406, p<0,05), showing that females are larger than males. However, males have longer and wider heads (Fig. 3A-B) and larger legs and feet in proportion to its SVL (Fig. 3C-F). Nuptial pad, composed by many minute spines, is present in fingers I, II and III on males and only in finger I on females, but reduced and not visible under a dissecting microscope.

Table 2. Variation in 17 morphometric characters of *Melanophryniscus macrogranulosus* from different localities. Acronyms according to material and methods. Values are Mean ± Standard Deviation (Range) from raw data.

	Males (n=84)					Females (n=31)			
Measurement (mm)	Morro da Gruta (n=18)	Lower Garapiá (n=7)	FLONA road (n=16)	FLONA forest (n=8)	PARNA Serra Geral (n=35)	Morro da Gruta (n=4)	Lower Garapiá (n=3)	FLONA road (n=9)	PARNA Serra Geral (n=15)
SVL	32,8 ± 1,6	28,6 ± 1,5	30 ± 1,5	32 ± 1,6	32,8 ± 1,4	37,5 ± 1,1	30 ± 2,8	33,1 ± 1,7	35 ± 0,9
	(30 - 34,7)	(25,8 - 30,5)	(26,6 - 32,1)	(30 - 35,5)	(30 - 35,8)	(36,5 - 39,1)	(27,2 - 32,8)	(31,5 - 37)	(33,1 - 36,3)
HW	9,1 ± 0,6	7,9 ± 0,5	8,4 ± 0,5	8,6 ± 0,4	8,7 ± 0,4	9,5 ± 0,4	8,2 ± 0,8	8,7 ± 0,5	8,9 ± 0,3
	(7,9 - 10,2)	(7,3 - 8,8)	(7,7 - 9,5)	(7,9 - 9,1)	(7,8 - 9,5)	(9 - 9,86)	(7,5 - 9,1)	(7,9 - 9,5)	(8,2 - 9,3)
HL	7,7 ± 0,5	7,1 ± 0,4	7,3 ± 0,4	7,2 ± 0,2	7,8 ± 0,4	8,2 ± 0,2	7,1 ± 0,4	7,6 ± 0,3	8 ± 0,3
	(6,7 - 9)	(6,4 - 7,5)	(6,6 - 8)	(7 - 7,5)	(6,8 - 8,5)	(8 - 8,45)	(6,86 - 7,65)	(7,2 - 8)	(7,3 - 8,5)
HLS	6,3 ± 0,3	6,1 ± 0,4	6,3 ± 0,4	6,3 ± 0,2	6,8 ± 0,5	7,1 ± 0,3	6,1 ± 0,3	6,2 ± 0,7	6,9 ± 0,4
	(5,5 - 6,8)	(5,5 - 6,7)	(5,5 - 6,8)	(5,9 - 6,6)	(5,9 - 7,7)	(6,7 - 7,28)	(5,8 - 6,42)	(4,8 - 7)	(6,3 - 7,7)
ED	2,9 ± 0,2	2,8 ± 0,1	2,9 ± 0,2	3 ± 0,2	3 ± 0,2	3,1 ± 0,2	2,8 ± 0,2	3 ± 0,3	3 ± 0,2
	(2,7 - 3,5)	(2,6 - 3)	(2,6 - 3,2)	(2,7 - 3,2)	(2,6 - 3,4)	(3 - 3,4)	(2,7 - 3)	(2,6 - 3,3)	(2,7 - 3,2)
END	2,8 ± 0,2	2,5 ± 0,1	2,8 ± 0,2	2,8 ± 0,1	2,9 ± 0,2	3,1 ± 0,2	2,6 ± 0,1	2,8 ± 0,1	3 ± 0,2
	(2,3 - 3)	(2,4 - 2,8)	(2,4 - 3)	(2,6 - 3)	(2,3 - 3,2)	(2,91 - 3,3)	(2,5 - 2,6)	(2,6 - 3)	(2,4 - 3,3)
SND	1,5 ± 0,1	1,3 ± 0,1	1,5 ± 0,2	1,5 ± 0,2	1,7 ± 0,1	1,4 ± 0,1	1,3 ± 0,01	1,6 ± 0,2	1,8 ± 0,1
	(1,2 - 1,7)	(1,3 - 1,6)	(1,1 - 1,7)	(1,3 - 1,8)	(1,3 - 2)	(1,3 - 1,54)	(1,28 - 1,31)	(1,2 - 1,9)	(1,7 - 2)
IND	2,4 ± 0,2	2,3 ± 0,2	2,5 ± 0,1	2,6 ± 0,2	2,6 ± 0,2	2,7 ± 0,4	2,3 ± 0,1	2,5 ± 0,3	2,7 ± 0,2
	(2,1 - 2,7)	(2 - 2,6)	(2,3 - 2,7)	(2,2 - 3)	(2,3 - 2,9)	(2,2 - 3,1)	(2,2 - 2,4)	(1,8 - 2,9)	(2,4 - 3)
IOD	4,1 ± 0,5	3,7 ± 0,5	4,6 ± 0,5	4,6 ± 0,4	4,6 ± 0,5	5,4 ± 0,4	3,9 ± 0,2	4,9 ± 0,6	4,8 ± 0,6
	(3,4 - 4,9)	(2,9 - 4,5)	(3,8 - 5,6)	(4,1 - 5,4)	(3,6 - 5,7)	(4,9 - 5,77)	(3,7 - 4,1)	(4 - 5,8)	(3,8 - 5,9)
UEL	3,7 ± 0,5	3,4 ± 0,4	3,5 ± 0,3	3,4 ± 0,3	3,8 ± 0,3	4,2 ± 0,2	3,8 ± 0,1	4 ± 0,4	3,7 ± 0,2
	(2,9 - 4,7)	(3 - 4,1)	(2,9 - 4,1)	(3,1 - 4)	(3 - 4,4)	(4 - 4,4)	(3,7 - 3,9)	(3,5 - 4,8)	(3,4 - 4,2)
UEW	2,7 ± 0,2	2,6 ± 0,4	2,5 ± 0,3	2,4 ± 0,3	2,7 ± 0,2	2,5 ± 0,4	2,6 ± 0,3	2,6 ± 0,3	2,7 ± 0,2
	(2,2 - 3,1)	(2,1 - 3)	(1,79 - 2,9)	(2 - 2,8)	(2,4 - 3,3)	(2 - 2,9)	(2,3 - 2,8)	(2,1 - 3)	(2,2 - 3)
TL	13,3 ± 1,4	11,5 ± 0,7	12,7 ± 0,8	12,7 ± 0,9	13,4 ± 0,6	13,4 ± 0,5	12,5 ± 0,8	12,5 ± 0,5	13,4 ± 0,6
	(10,2 - 14,9)	(10,7 - 12,4)	(10,1 - 13,7)	(10,9 - 13,9)	(12,2 - 14,7)	(12,8 - 13,9)	(11,8 - 13,4)	(11,6 - 13,3)	(12,4 - 14,6)
TI	12,2 ± 0,4	10,8 ± 0,4	11,3 ± 0,8	11,8 ± 0,5	12,8 ± 0,6	14 ± 0,2	11,3 ± 0,2	11,6 ± 0,6	13,2 ± 0,4
	(11,6 - 13)	(10,1 - 11,2)	(9,3 - 12,8)	(10,8 - 12,2)	(11,6 - 14,1)	(13,72 - 14,1)	(11 - 11,4)	(10,6 - 12,5)	(12,3 - 14)
TAL	8,3 ± 0,3	6,9 ± 0,6	7,5 ± 0,4	7,8 ± 0,4	8,4 ± 0,4	0,2 ± 0,8	7,5 ± 0,1	7,3 ± 0,3	8,4 ± 0,5
	(7,6 - 8,8)	(5,7 - 7,7)	(6,7 - 8,4)	(7,3 - 8,5)	(7,3 - 9,3)	(8,1 - 9,8)	(7,4 - 7,6)	(6,8 - 7,9)	(7,4 - 9,6)
FL	12,4 ± 0,7	10,8 ± 0,8	11,8 ± 0,8	12 ± 0,9	13,2 ± 0,7	13,6 ± 0,2	11,5 ± 0,4	11,9 ± 0,6	13,5 ± 0,7
	(11,2 - 13,5)	(9,6 - 12)	(9,5 - 12,6)	(10,9 - 13,1)	(12,2 - 14,7)	(13,29 - 13,8)	(11,1 - 11,8)	(10,8 - 12,8)	(12,2 - 14,6)
FAL	8,2 ± 0,5	7,1 ± 0,5	7,3 ± 0,6	7,9 ± 0,4	(7,7 ± 0,3)	9,5 ± 0,5	7,6 ± 0,6	7,6 ± 0,6	8,1 ± 0,5
	(7,4 - 9,5)	(6,5 - 8,1)	(6,0 - 8)	(7,1 - 8,5)	(7 - 8,5)	(8,93 - 10)	(7 - 8)	(6,6 - 8,5)	(7,4 - 9)
MA	8,1 ± 0,4	6,8 ± 0,5	7,6 ± 0,6	7,5 ± 0,4	(8,4 ± 0,4)	8,0 ± 0,3	7,4 ± 0,3	8 ± 0,6	9,2 ± 1,4
	(6,9 - 8,5)	(5,9 - 7,3)	(6 - 8,2)	(6,8 - 8)	(7,7 - 9,2)	(8,4 - 9,1)	(7,1 - 7,7)	(6,8 - 8,8)	(8,3 - 13,9)

Despite some differences of parameters mean values observed among samples sites, we found large variation and the extremes overlapped. Canonical Variance Analysis, using 16 morphologic characteristics (excluding SVL), showed little discrimination between groups, indicating the characters of different samples site studied are not enough to separated concretized units (Fig. 4 and 5).

Table 3. Variation in 17 morphometric characters of *Melanophryniscus macrogranulosus* from different localities. Acronyms according to material and methods. Values are means of proportions of morphometric variables in relation to snout-to-vent length (expressed as % of SVL).

	Morro da Gruta	Lower Garapiá	FLONA road	FLONA forest	PARNA Serra Geral	Total	Morro da Gruta	Lower Garapiá	FLONA road	PARNA Serra Geral	Total
	Males (n=84)						Females (n=31)				
HW	0,28	0,28	0,28	0,27	0,27	0,27	0,25	0,27	0,26	0,25	0,26
HL	0,24	0,24	0,24	0,23	0,24	0,24	0,22	0,24	0,23	0,23	0,23
HLS	0,19	0,21	0,21	0,2	0,21	0,2	0,19	0,2	0,19	0,2	0,2
ED	0,09	0,1	0,1	0,09	0,09	0,09	0,08	0,09	0,09	0,08	0,09
END	0,08	0,09	0,09	0,09	0,09	0,09	0,08	0,09	0,08	0,09	0,08
SND	0,04	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,05	0,05	0,05
IND	0,07	0,08	0,08	0,08	0,08	0,08	0,07	0,08	0,08	0,08	0,08
IOD	0,13	0,13	0,15	0,14	0,14	0,14	0,14	0,13	0,15	0,14	0,14
UEL	0,11	0,12	0,11	0,11	0,12	0,11	0,11	0,13	0,12	0,11	0,12
UEW	0,08	0,09	0,08	0,08	0,08	0,08	0,07	0,09	0,08	0,08	0,08
TL	0,41	0,41	0,42	0,4	0,41	0,41	0,36	0,42	0,38	0,38	0,38
TI	0,37	0,38	0,37	0,37	0,39	0,38	0,37	0,38	0,35	0,38	0,37
TAL	0,25	0,24	0,25	0,25	0,25	0,25	0,25	0,25	0,22	0,24	0,24
FL	0,38	0,38	0,39	0,38	0,4	0,39	0,36	0,38	0,36	0,38	0,37
FAL	0,25	0,25	0,24	0,25	0,24	0,24	0,25	0,26	0,23	0,23	0,24
HL	0,24	0,24	0,25	0,24	0,26	0,25	0,24	0,25	0,24	0,26	0,25

Results of morphometric analyses showed some variation in values recorded for different localities. However, we could not find a pattern in the variation that could discriminate well-defined groups. The highest difference in head width was observed between Morro da Gruta and Lower Garapiá. When observing straight head length, PARNA da Serra Geral is the most dissimilar sample. Nevertheless, when removing the effect of size (Tab.4), Morro da Gruta and Cambará do Sul are the most distinct groups. The same pattern was detected for the ratios straight head length/head width, eye-nostril-distance/head width and snout-nostril distance/head length. Analyzing all these ratios for head measurements, we observed that low values are concentrated in low altitudes and highest values in high altitudes, however there is a gradient between them and a large overlap between localities. Individuals

from Morro da Gruta tend to have wider heads than individuals from PARNA da Serra Geral. The opposite happens with ratios of eye-nostril/head width, internarial distance/head width, snout-nostril distance/head length where the proportion increases with altitude. Summarizing, a subtle geographic variation between localities is observed, where individuals from lowland tend to have smaller and straighter snouts and as altitude increases, it gets bigger and rounder. However, the large variation of these characters, within and between localities, did not allow to discretize any groups (Fig.5).

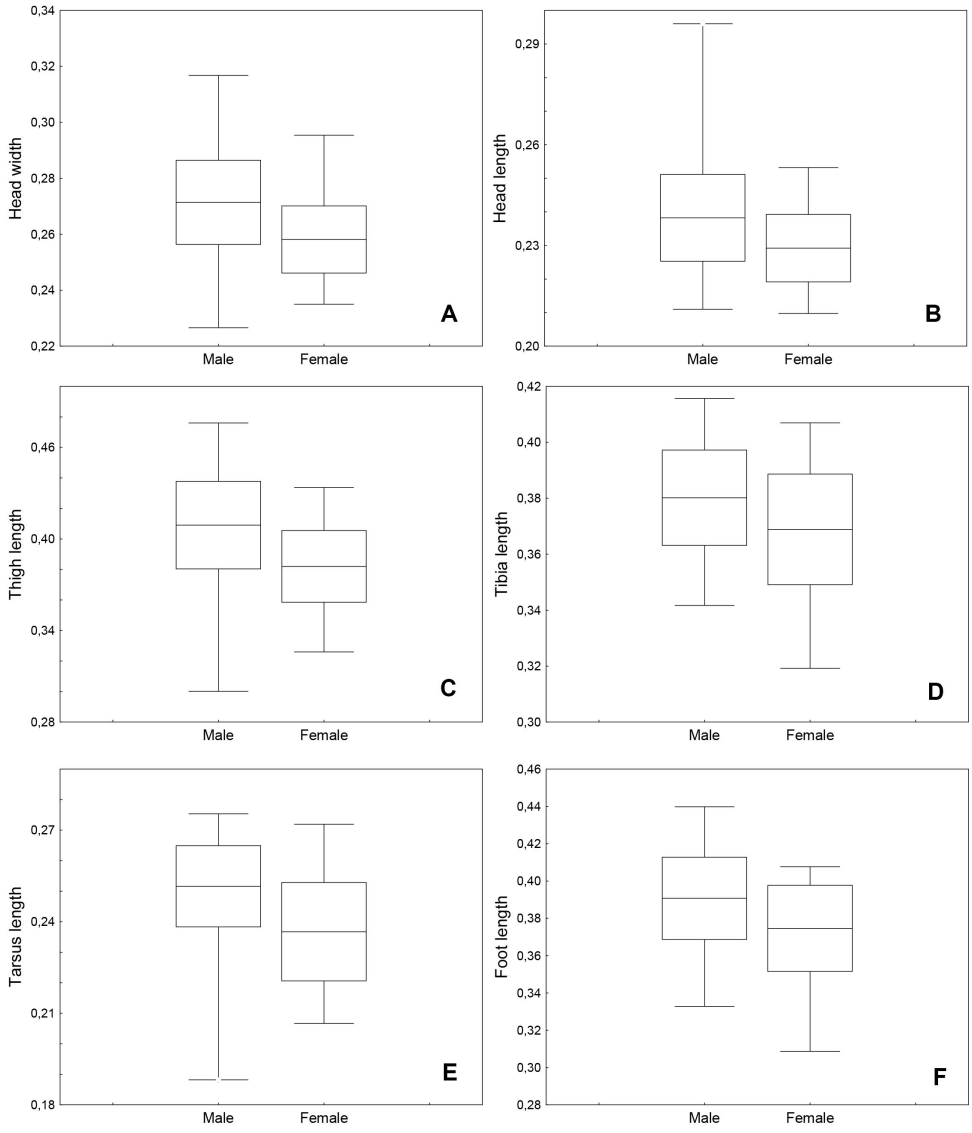

Figure 3. Sexual comparison of variation in head and limb morphology, (expressed as the ratio: character/SVL) of *Melanophryniscus macrogranulosus*. Measurements are summarized in box spanning the standard deviation and the mean indicated by a line inside the box and whisker plots with highest and lowest observations.

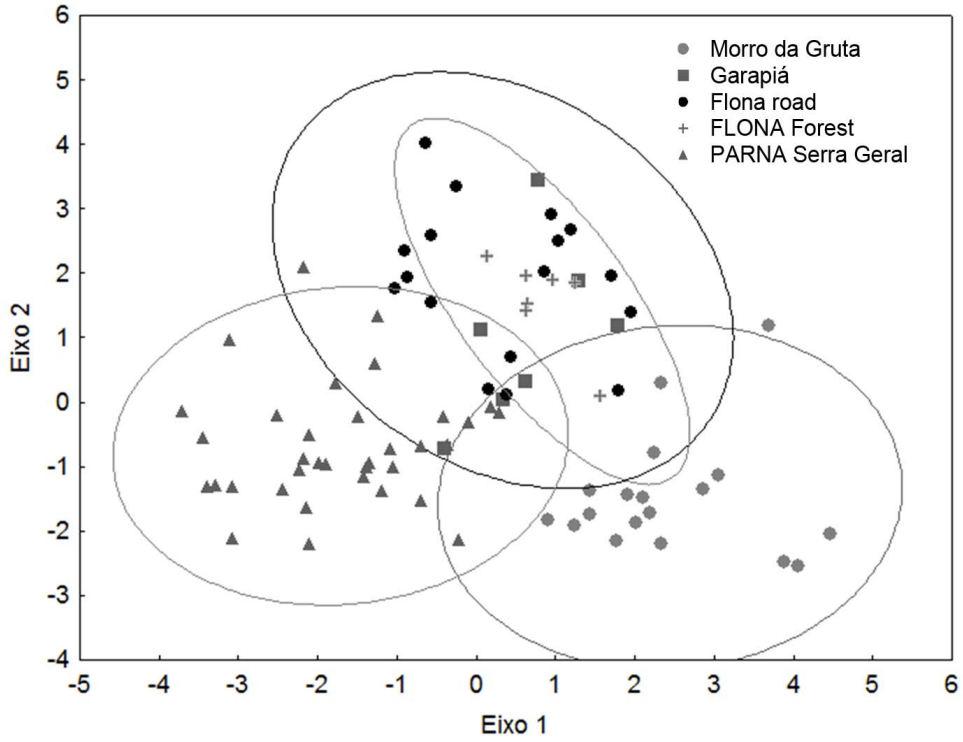

Figure 4. Bivariate plot of the first two axes scores of Canonical Variate Analysis of morphometric data of males *Melanophryniscus* macrogranulosus, from different localities.

Table 4. Selected ratios of head measurements of *Melanophryniscus macrogranulosus* from different localities. Acronyms according to material and methods. Values underlined were the lowest observed; values in bold, the highest.

	Morro da Gruta	Lower Garapiá	FLONA road	FLONA forest	Cambará	TOTAL
HL/HW	0,85	0,89	0,88	0,83	**0,9**	0,88
HLS/HW	0,7	0,76	0,75	0,73	**0,78**	0,75
END/HW	0,31	0,32	0,33	0,33	**0,33**	0,32
END/HL	0,36	0,36	0,38	**0,39**	0,37	0,37
IND/END	0,88	0,92	0,91	**0,96**	0,93	0,92
SND/END	0,53	0,53	0,52	0,55	**0,6**	0,56
SND/HL	0,19	0,19	0,2	**0,22**	**0,22**	0,21
IND/HW	0,27	0,29	**0,3**	0,31	**0,3**	0,3
IND/HL	0,32	0,33	0,34	**0,38**	0,34	0,34

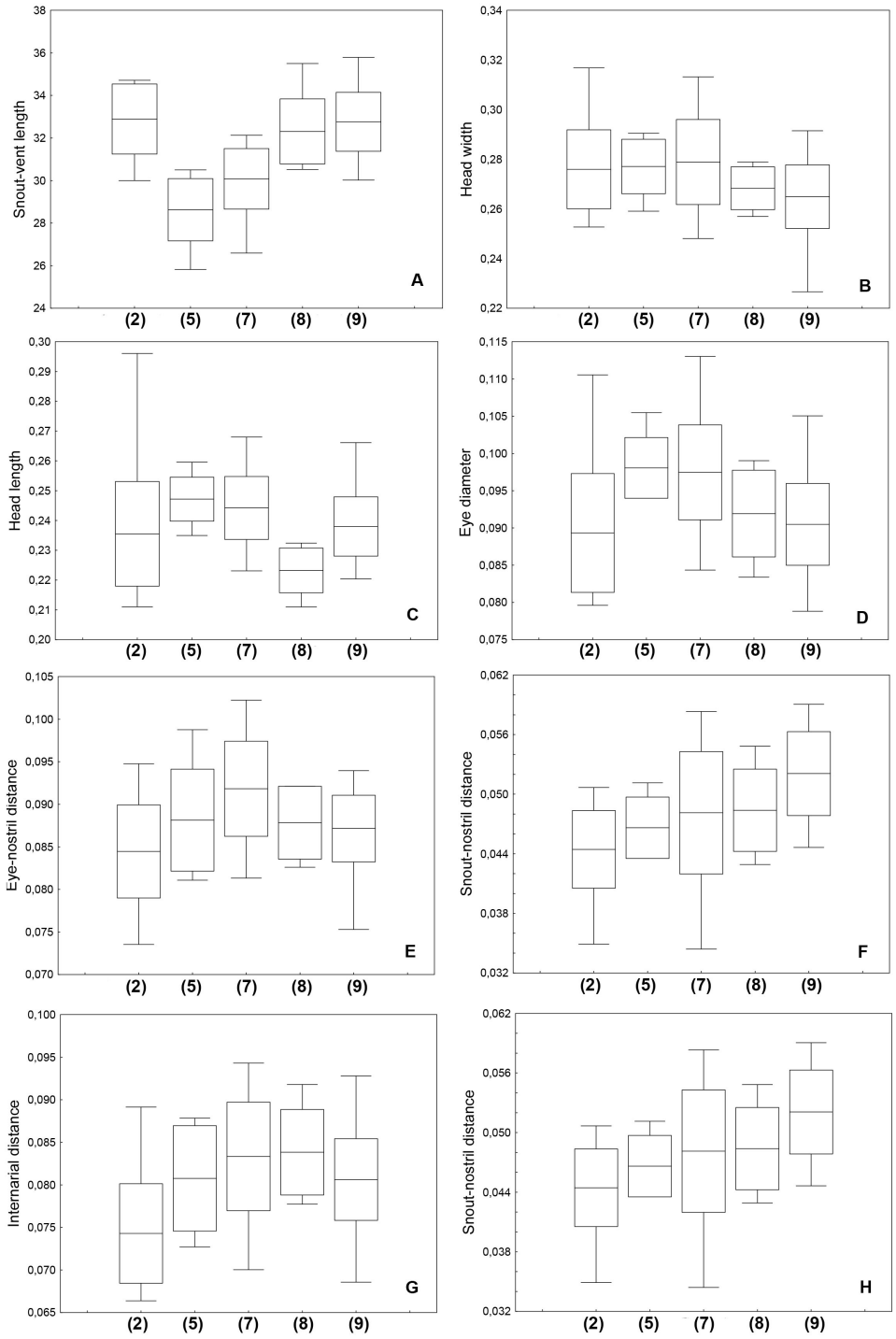

Figure 5. Variation of snout-vent length and head morphology (expressed as the ratio character/SVL) of *Melanophryniscus* macrogranulosus from different localities. Localities: **2** - Morro da Gruta (Type locality); **5** – Lower Garapiá waterfall, Barra do Ouro; **7 -** FLONA SFP (road site); **8 -** FLONA SFP (forest site); **9 -** PARNA da Serra Geral. Measurements are summarized in box spanning the standard deviation and the mean indicated by a line inside the box and whisker plots with highest and lowest observations. References for number in parentheses in horizontal axis are indicated according to Figure 1.

Color in life: It is possible to find a large variation in the pattern of color between and within populations (Fig. 6). Dorsum varies from light to dark green, some individuals are deep dark green. Unusually, it is possible to find toads with a blue pattern. Ventral surface exhibits a green or grayish blue coloration with a red patch in abdominal region, throat, pectoral and axils with white spots covering all surface. A high number of individuals from Morro da Gruta tend to be bluer on ventral surface, while in FLONA São Francisco de Paula, the most common pattern found is green. In Garapiá it is possible to find both patterns in quantity. Palmar and plantar surfaces present orange reddish coloration and the iris is black.

Figure 6. Color pattern of ventral surface in adults of *Melanophryniscus* species from Morro da Gruta (A-C; vouchers: UFRGS 6452, UFRGS 6448, UFRGS 6454), Lower Garapiá (D-G; unvouchered specimen, UFRGS 6585, UFRGS 6586, UFRGS 6587), FONA road (H-J; unvouchered specimens. Photograph: R. Rocha Santos), FLONA forest (K-M; unvouchered specimens) and PARNA da Serra Geral (N-O; Photograph: A. Lise, slides from 1970´s).

Molecular analyses

Results from genetic distance analysis showed very low levels of divergence between *Melanophryniscus macrogranulosus* sample sites (Tab. 5). All populations of *M. macrogranulosus* diverge less than 0,1%, some being 100% identical. Similarity between them was higher than with *M. simplex*, always more than 3%. Negative values on table indicate those samples where the mean divergence within populations was higher than between them. Few differences were found for PARNA da Serra Geral and the rest of *M. macrogranulosus* localities. This may be due to the single sequence used for this population.

Table 5. Genetic distance (%) between groups from partial sequences of *cytochrome b* of six sample sites (first lines) of *Melanophryniscus macrogranulosus* and one from *M. simplex* (the last line).

Sample site	1	2	3	4	5	6	7
Morro da Gruta (n=11)	-						
Upper Garapiá (n=4)	0,000	-					
Lower Garapiá (n=3)	0,000	-0,058	-				
FLONA road (n=5)	-0,013	0,000	0,000	-			
FLONA forest (n=7)	0,017	0,017	0,017	0,017	-		
PARNA Serra Geral (n=1)	0,000	0,000	0,000	0,000	0,016	-	
M. simplex (n=2)	3,386	3,458	3,470	3,308	3,369	3,451	-

Taxonomic arrangement

Morphometric analyses showed some variation in values recorded for different samples sites of *Melanophryniscus macrogranulosus*. Males CVA showed little discrimination between groups, indicating the characters of different sample sites studied are not enough to separated concretized units. We observed some geographic variation between localities, especially when analyzing them in an altitude gradient. Individuals from lowland tend to have smaller and straighter snouts, while as altitude increases individuals have bigger and rounder snouts. Pattern coloration also has large variation inter and intra-populations. Although some patterns are more characteristic in some localities, we have found individuals with every color pattern in all populations. This variation was already reported in studies with the genus,

including FLONA road population, where the authors reported an individual coding of this ventral coloration, useful in studies with mark recapture techniques (Cairo & Di Tada, 2005; Cairo & Zalba, 2007; Caorsi *et al.,* 2012). Molecular analyses showed very low levels of divergence between *M. macrogranulosus* sample sites, with less than 0,1% of differences and some identical.

Morphology and molecular data did not show evidence of distinct groups, so we suggest that both taxa are synonyms. Therefore, we provide a redescription of *Melanophryniscus macrogranulosus*.

Melanophryniscus macrogranulosus Braun, 1973

Melanophryniscus macrogranulosus Braun, 1973. Type locality: Morro da Gruta, Dom Pedro de Alcântara, RS, Brazil (Fig. 7 A-B)

Melanophryniscus cambaraensis Braun & Braun, 1979. Type locality: PARNA da Serra Geral, Cambará do Sul, RS, Brazil (Fig. 7 C-D).

Diagnosis: A medium size *Melanophryniscus* diagnosed by the combination of the following character states: (1) SVL 25,8-35,8 (male) and 27,2-39,1 (female); (2) head slightly wider than long (HL/HW = 0,88) with the presence of frontal snout extending between the eyes to middle of the length of upper eyelid; (3) Body skin with big rounded granular warts in dorsum and lateral sides; (4) Absence of metatarsal gland on metatarsus; (5) Pattern coloration of dorsum varying between light to dark green, and less common to blue. Ventral surface exhibiting a green to grayish blue coloration pattern with a red patch in abdominal region and very common in throat, pectoral and axils with white spots covering all surface. Palmar and plantar surfaces present orange reddish coloration. (6) Presence of nuptial pad composted by many minute, keratinized spines in finger I, II and III on males and reduced presence of this structure in finger I (thumb) on females.

Holotype: MCN 1694, adult female (Fig. 7 A-B) from Morro da Gruta, municipality of Dom Pedro de Alcântara (29°24'21.16"S, 49°51'1.48"W, 31m asl), Rio Grande do Sul, Brazil. Specimen collected on

October 30 of 1960 by Thales de Lema and deposited in Museu de Ciências Naturais, in RS, Brazil (Fig. 7 A-B).

Measurements of Holotype: SVL 37.5, HW 9.9, HL 8.0, HLS 7.3, ED 3.1, END 3.2, SND 1.5, IND 2.6, IOD 5.8, EUL 4.0, EUW 2.8, TL 12.8, TI 13.7, TAL 9.3, FL 13.7, FAL 9.7, HAL 9.1.

Color of the Holotype in Preservative: Dorsum dark brown, ventral surface also dark with cream patches in abdominal region and limbs and soft white spots covering ventral surface; palmar and plantar surfaces also cream.

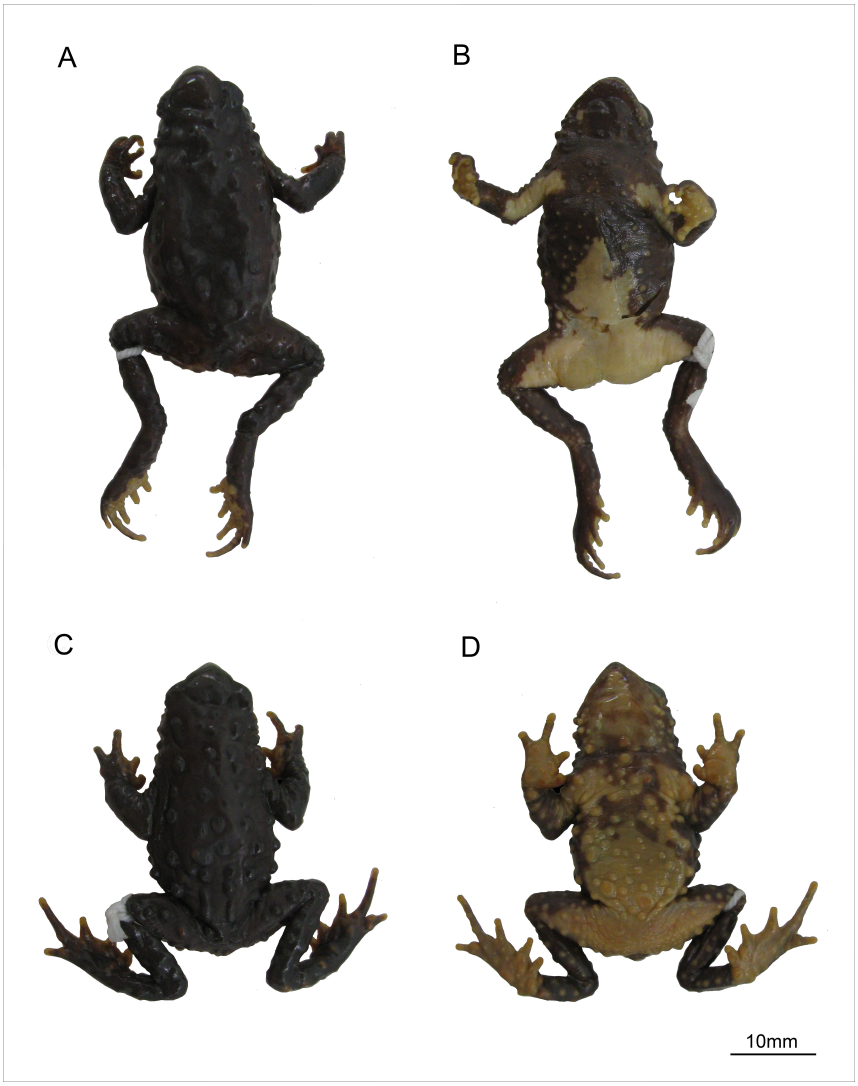

Figure 7. Dorsal and ventral view of the holotypes of *Melanophryniscus macrogranulosus* (A-B, MCN 1694) and *M. cambaraensis* (C-D, MCN 9797).

Comparisons with Other Species: The presence of a frontal macrogland includes *Melanophryniscus macrogranulosus* in the *M. tumifrons* group and allows to distinguish it from members of *M. stelzneri* group: *M. atroluteus* (Miranda-Ribeiro), *M. cupreuscapularis* Céspedez & Álvarez, *M. dorsalis* (Mertens), *M. estebani* Céspedez, *M. fulvoguttatus* (Mertens), *M. klappenbachi* Prigioni & Langone, *M. krauczuki* Baldo & Basso, *M. montevidensis* (Philippi), *M. paraguayensis* Céspedez & Motte, *M. rubriventris* (Vellard), and *M. stelzneri* (Weyen- bergh); and *M. moreirae* group: *M. langonei* Maneyro *et al.*, *M. moreirae* (Miranda-Ribeiro), and *M. sanmartini* Klappenbach; and three ungrouped species, *M. admirabilis* Di-Bernardo *et al.*, *M. alipioi* Langone *et al.*, and *M. vilavelhensis* Steinbach-Padilha.

Melanophryniscus macrogranulosus differs from all remaining species in the *M. tumifrons* group by dorsal skin smooth with big rounded glandular warts, instead of small to medium-size warts with keratinized spicules in *M. pachyrhynus* (Baldo *et al.,* 2012), *M. spectabilis* (Caramaschi & Cruz, 2002) and *M. peritus* (Caramaschi & Cruz, 2011) and longitudinal lines formed by fused glands in *M. devincenzii* (Klappenbach, 1968). The dorsal green coloration differs *M. macrogranulosus* from the rest of *M. tumifrons* species group, which are brown to gray in *M. pachyrhynus* and *M. devincenzii*, black to dark brown in *M. simplex* and *M. tumifrons* and marbled pattern of irregular pale yellow strips on a dark background in *M. spectabilis*. Ventral pattern of the present studied species varies from other species, which shows reticulated orange pattern (*M. pachyrhynus* and *M. peritus*), background dark brown to black with a red patched on abdomen and red or/and yellow spots on throat (*M. simplex* and *M. tumifrons*), a big orange/red splotch in abdominal region under a brown to black surface (*M. devincenzii*) and dark background with red/orange patches on the abdominal region with small orange spots on pectoral surface and three well distributed yellow/orange spots on throat (*M. spectabilis*).

Natural history: Reproductive sites known for this species are shallow temporaries streams formed after heavy rains and most of them are close to or inside a forest.

Conservation status: *Melanophryniscus cambaraensis* was described in 1979 with individuals collected in 1976 in the actual PARNA da Serra Geral, Cambará do Sul, RS, Brazil, when the area was not yet

legally protected. This population has not being seen in this locality since 1990 (according to a record of the Museu de Ciências e Tecnologia da PUCRS). Researches did field trips to investigate the vanished population in PARNA da Serra Geral, after heavy rains, before the year 2000 and some expeditions after that.

In our study, efforts to find this lost population were divided into "finding historic information" and "making field trips". We had access to Braun and Braun´s slides from field trips in the area from about 30 years ago and also we contact some researchers that joined them on the expeditions to find more information about the reproductive site. One field trip to the type locality was realized with one of their colleagues, Arno Lise, naturalist and arachnologist from PUCRS (results from this field trip are in ICMBio em foco, Edição 252, Ano 6, 05 de julho de 2013). Besides that, we did extensive searches in the appropriate habitat, all over the year, after heavy rains (from 30-180mm) and searched a total of 2km^2 inside the limits of PARNA da Serra Geral. Over all expeditions, we could not find any individual or even traces of reproduction (tadpoles or egg masses), at the two possible reproductive sites mentioned by Braun's field colleagues, nor at the rest of the area searched (Fig 8).

Figure 8. Potential reproductive site at the type locality of *Melanophryniscus cambaraensis* in Serra Geral National Park, Cambará do Sul, RS, Brazil (present day record). In the insert, an image from the 1970s´ of some individuals at the historic reproductive site (Photograph: Arno Lise).

Population of *Melanophryniscus macrogranulosus* from PARNA da Serra Geral, RS, Brazil, continues disappeared, completing now 24 years since the last record. This pattern of known species dwindling or disappearing in protected areas has been observed for other amphibians, as gastric brooding frogs in Australia and golden toad in Costa Rica (Georgia Department of Natural Resources, 2000; Pounds *et al*., 2004). The cause of most amphibian declines remains unknown, even thought the primary contributing factors usually are habitat loss, introduced species, overexploitation, and pollution (Mahony *et al*., 1999). Although many things changed at the protected area in 24 years, we did not identify the reason for the vanishing of this population. However, we detected some changes that could have affected the reproductive site where the toads used to live. That included changes that modified the habitat, such as reduction of Araucaria Forest to lowlands, presence or absence of fire and grazing, that used to be common and now is unusual. The last modify register at the area is a dirt road with a bridge built, after the area became protected, right next to one of the possible historic reproductive sites (identified by Arno Lise). The cause of *Melanophryniscus macrogranulosus* disappearing from PARNA da Serra Geral remains unknown.

Some new records were added to *Melanophryniscus macrogranulosus* distribution, however all records are inside the limits of the Atlantic Rainforest (IBGE, 2006), considered one of the most endangered and devastated environment of Brazil (Myers *et al.,* 2000). Only the populations from FLONA are inside the limits of a protected area, the other one is disappeared since 1990. This led us to highlight the concern with the conservation of this species even in protected areas. The remaining areas known for the species suffer from several threats; forests are reduced to few, small and discontinuous remnants mainly due to the constant deforestation. Even the few lasting areas are surrounded by crop plantations and livestock. The type locality, apart from these threats, also was affected by the recent duplication of the Federal Highway BR-101, that can be considered as another possible impact, because it might have isolated the type population and increased noise pollution (produced by cars and trucks) and road-kills, a problem found in other *Melanophryniscus* species that breed close to the road (Cairo &

Zalba, 2007). The extent of occurrence, calculated using Geocat® software, including all localities is 1903.37 km^2. Excluding the historic record to the PARNA Serra Geral, the extent reduces to 1096.5 km^2. Under the IUCN Red List criteria (IUCN, 2013), all observed impacts, associated with the small extent of occurrence (B1 <5000 km^2) known for *Melanophryniscus macrogranulosus* justify the species listing as Endangered (EN) – B1ab(i, ii, iii).

Anexo 1.

Material examined

Examined specimens - Morphology

Melanophryniscus cambaraensis – Brazil, Rio Grande do Sul: Cambará do Sul, PARNA da Serra Geral: MCN 9752-53, MCN 9755, MCN 9757, MCN 9758-60, MCN 9762-64 MCN 9765-84, MCN 9786-88, MCN 9791-92, MCN 9794-95, MCN 9797 (Holotype), MCN 9799-04, MCN 9806, MCN 9808-12; São Francisco de Paula, Floresta Nacional de São Francisco de Paula: MCN 13456-62, MCN 13466, MCN 13467-70, MCN 13472, MCN 13475-77, MCP 2770-71, MCP 2784, MCP 11239, UFRGS 1841, UFRGS 4817, UFRGS 6319-28, UFRGS 6426.

Melanophryniscus macrogranulosus – Brazil, Rio Grande do Sul: Dom Pedro de Alcântara, Morro da Gruta: MCN 1693, MCN 1694 (holotype), MCN 1695, MCN 1696, MCN 1699, MCN 1702, MCP 10319, MCP 10320, MCP 11139-41, MCP 11576, MCP 11936-37, UFRGS 2502, UFRGS 4702, UFRGS 6448, UFRGS 6449, UFRGS 6450, UFRGS 6451, UFRGS 6452, UFRGS 6454; Maquiné, Barra do Ouro: MCP 8104, MCP 8105, UFRGS 2476, UFRGS 2832, UFRGS 2830-31, UFRGS 6584-88.

Melanophryniscus devincenzii - (UFRGS 4196)

Melanophryniscus pachyrhynus (UFRGS 5828)

Melanophryniscus simplex – Brazil, Rio Grande do Sul, (UFRGS 4313)

Melanophryniscus spectabilis (UFRGS 4308)

Melanophryniscus tumifrons (UFRGS 2590)

Examined specimens – Molecular analysis

Melanophryniscus macrogranulosus: Morro da Gruta: MCP 11576-78, MCP 11937, UFRGST 3687-93. Lower Garapiá: UFRGST 3930-32. Upper Garapiá: MCP 11935, UFRGST 3586, UFRGST 3614-15. FLONA road: UFRGST 3321-22. FLONA forest: UFRGST 3324-30. PARNA Serra Geral: UFRGST 3941 (individual: MCN 8278).

Melanophryniscus simplex: Nova Roma do Sul: MCP 12567. São José dos Ausentes: MCP10844.

Acknowledgment

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CAPÍTULO 3: Nuptial pads of *Melanophryniscus macrogranulosus* (Anura: Bufonidae) and an unexpected dermal structure for the genus.

(Artigo nas normas de submissão do periódico *Journal of Morphology*)

Nuptial pads of *Melanophryniscus macrogranulosus* (Anura: Bufonidae) and an unexpected dermal structure for the genus

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Nuptial pads in a red-bellied-toad

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Abstract: Nuptial pads are one secondary sexual characteristics of male anuran and consist mainly of a thickened epidermis. This structure exhibits structural differences adapted to a particular courtship behavior, amplexus, or circumstances of breeding. The position of the nuptial pad is usually on the thumb, occasionally on the second and third digits, and/or on the ventral surface of the forearm, or on the feet. These structures are used in courtship and mating and it has been suggested they help the male hold onto the female and prevent it from escaping and can be used to defend himself from rival males. This structure has been reported in males of some bufonids, including the genus *Melanophryniscus,* as a sexual dimorphism, only present in males. However, only the presence of the structure is reported, usually

lacking additional details about its morphology. Our main goal is to describe the epidermal structures of the hand and foot of adult males and females *M. macrogranulosus*. We studied structures of males and females of *M. macrogranulosus* and other three species of the genus under an optical and scanning electron microscope. Males from all species have nuptial pad present, distributed at least on finger I and, frequently, on finger II and III of forelimbs. Density of elevations in males finger I (697,2 ± 103,41) and II (647,2 ± 141,1) was higher than on finger III (459,4 ± 290,9). We bring here the first record of these structures on females for the genus. Those minute structures were only found in females of *M. macrogranulosus,* they were absent in the other species studied. These protuberances are only on finger I and the density in males is twice higher than in females (319,4 ± 82,7).

Key words: red-bellied-toad, scanning electron microscope, dermal excrescence, male and females.

Introduction

Adult amphibians have various morphological specializations of the epidermis, such as adhesive toe pads, nuptial pad, spines and claws (Wells, 2007). Nuptial pads are one secondary sexual characteristics of male anuran and consist mainly of a thickened epidermis, which can have a distinctive architecture and a dermis with embedded large skin glands (Duellman and Trueb, 1986). This structure exhibits structural differences according to species and family, sometimes it can be thicker, heavily keratinized or with hypertrophied glands, adapted to a particular courtship behavior, amplexus, or circumstances of breeding (Fujikura et al., 1988; Kurabuchi, 1993). The position of the nuptial pad is usually on the thumb, occasionally on the second and third digits, and/or on the ventral surface of the forearm, or on the feet (Duellman and Trueb, 1986).

These structures are used in courtship and mating (Wells, 2007), and it has been suggested they help the male hold onto the female and prevent it from escaping (Duellman and Trueb, 1986). Another function proposed is to grasp females while defending himself from rival males (Savage, 1962). These

aggressive behaviors would be predictable in explosive breeders with extensive male-male combat and on holing mate when other males try to dislodge them (Wells, 2007). Besides that, males from many species have nuptial pads or keratinized excrescences on the forelimbs or chest, usually with modified mucus glands associated with them (Duellman and Trueb, 1986; Epstein and Blackburn, 1997). The hormonal induction of courtship gland activity coincides with the development of secondary sexual characters such as broad tail fins and nuptial pads on the hind feet of males (Singhas and Dent, 1975). Forearm musculature and nuptial pads of males typically regress outside of the breeding season in some species, however, they generally do not return to a female-like morphology (Emerson, 2000; Wells, 2007).

This structure has been reported in males of some bufonids (Coloma, 2002; Kwet et al., 2006; Recoder et al., 2010; Caramaschi, 2012) and as a sexual dimorphism in *Melanophryniscus* (Anura: Bufonidae). This genus currently has 26 recognized species (Frost, 2014) and this character is known to be present in males of *M. alipioi*, *M. cambaraensis, M. devincenzii*, *M. krauczuki*, *M. moreirae, M. pachyrhynus*, *M. rubriventris*, *M. simplex, M. stelzneri* and *M. tumifrons* (Cei, 1980; Baldo and Basso, 2004; Vaira, 2005; Langone et al., 2008; Santos et al., 2010; Baldo et al., 2012; Peloso et al., 2012). However, typically only the presence of the structure is reported, usually lacking additional details about its morphology. In most species, the pad occurs always on finger I and, frequently, also on II and III. According to observations of Peloso et al. (2012) the pad of most species is composed of many minute, keratinized spines, except for *M. setiba,* which has only a few, enlarged keratinized spines at medial

Table 1. Review of nuptial pad occurrence and structure in males of *Melanophryniscus*.

Species	Description	Reference
Melanophryniscus alipioi	Smooth, brown nuptial pads on fingers I and II	(Langone et al., 2008)
Melanophryniscus devincenzii	Pad composed by many minute, keratinized spines	(Peloso et al., 2012)
Melanophryniscus krauczuki	Smooth, brown nuptial pads on fingers I and II	(Baldo and Basso, 2004)
Melanophryniscus macrogranulosus	Conspicuous brown nuptial pad on fingers I and II	(Braun and Braun, 1979; Santos et al., 2010)
Melanophryniscus moreirae	Pad composed by many minute, keratinized spines	(Peloso et al., 2012)
Melanophryniscus pachyrhynus	Brown nuptial pads on finger I and usually also on II and III	(Baldo et al., 2012)
Melanophryniscus rubriventris	Presence of nuptial pads	(Vaira, 2005)
Melanophryniscus setiba	Enlarged, brow, keratinized spines at medial margin of finger II	(Peloso et al., 2012)
Melanophryniscus simplex	Pad composed by many minute, keratinized spines	(Peloso et al., 2012)
Melanophryniscus stelzneri	Smooth, brown nuptial pads	(Cei, 1980)
Melanophryniscus tumifrons	Pad composed by many minute, keratinized spines	(Peloso et al., 2012)

margin of finger II (Tab. 1). In *Melanophryniscus*, nuptial pads have never been observed in females and even in males this structures have not been described in detail. In this study, we described the epidermal structures of the hand and foot of adult males and females *Melanophryniscus macrogranulosus*, and report the unexpected occurrence of nuptial pads in females.

Material and Methods

Sample data

We studied individuals of *Melanophryniscus macrogranulosus* from four known localities in Rio Grande do Sul, Brazil: (1) Morro da Gruta, municipality of Dom Pedro de Alcântara (type locality); (2) Lower Garapiá, Barra do Ouro, municipality of Maquiné; (3) Floresta Nacional de São Francisco de Paula (FLONA SFP), municipality of São Francisco de Paula; (4) Serra Geral National Park, municipality of Cambará do Sul. Vouchers of all specimens used in this work are deposited in the amphibian collections at Departamento de Zoologia da Universidade Federal do Rio Grande do Sul (UFRGS 6449, UFRGS 6587, UFRGS 6588) and Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul (MCN 9791, MCN 9802, MCN 1693, MCN 13470, MCN 13477). Material collected during this study was authorized trough the Sistema de Autorização e Informação em Biodiversidade – SISBIO (IBAMA, MMA) under licenses numbers 35295-1.

We analyzed the hand of an adult male and female of four localities and the feet of one individual of each sex from Barra do Ouro, Maquiné. Furthermore, we examined adult females of other species of the genus, *M. dorsalis*, *M. sanmartini* and *M. simplex* for comparisons with *M. macrogranulosus*. Sex was determined by the presence of vocal slits for males and lack of this last character and presence of eggs for females.

Tissue preparation

All individuals, but two (from Barra do Ouro, Maquiné) were already at scientific collections. Animals collected in the field were sacrificed using an anesthetic (lidocaine) and latter we excised the

entire right hand and foot and fixed it with modified Karnovskys´ fixative (Ito and Karnovsky, 1968) at 4ºC for 48h. The rest of the exemplars were used directly after its original fixative solution (formaldehyde 10%). To prepare material for scanning electron microscope (SEM), we used the following protocol: samples were washed three times with Sorenson's Fosfate Buffer (0,2M; pH 7,2) and then dehydrated with a crescent sequence of acetone concentrations (10min in acetone 30%, 50%, 70%, 90%, followed by 20 min in acetone 90%, then 10 more min. at this concentration and 20min at 100%). Finally, they were dried with Critical Point method (using liquid CO_2) and coated with gold.

Analyses

We analyzed four individuals from scientific collection fixated in 10% formaldehyde solution and stored in 70% ethanol, one male of each species used in this work, in an optical microscope coupled to a Nikon AZ100M using gain of 2.6, 3.6 and 8x. One individual from each sex of four different populations *Melanophryniscus macrogranulosus* and one female of *M. dorsalis*, *M. sanmartini* and *M. simplex* were analyzed with a scanning electron microscope Jeol JSM-6060 at the Centro de Microscopia Eletrônica da Universidade Federal do Rio Grande do Sul. Density of protuberances was estimated on each pad of *M. macrogranulosus* males and females, by counting 3-6 squares of 200 x 200 μm^2, placed on SEM photographs of each pad using Adobe Photoshop software for male and female separately. The results are expressed by the mean ± standard deviation and range of protuberances per unit surface area (mm^2).

Results

We analyzed four populations of *Melanophryniscus macrogranulosus* (Tab.2) and other three species of the genus. Males from all species have nuptial pad present, distributed at least on finger I and, frequently, on finger II and III of forelimbs. We only found those minute structures on finger I in females of *M. macrogranulosus* (Fig.3), it was absent in the other species (Fig. 4). The excrescence on females is present merely on medium margin of finger I and absent on the others. The minute elevations from the

pad have a conical shape in both sexes, but males have much higher density than females and a larger covered area (Tab. 2).

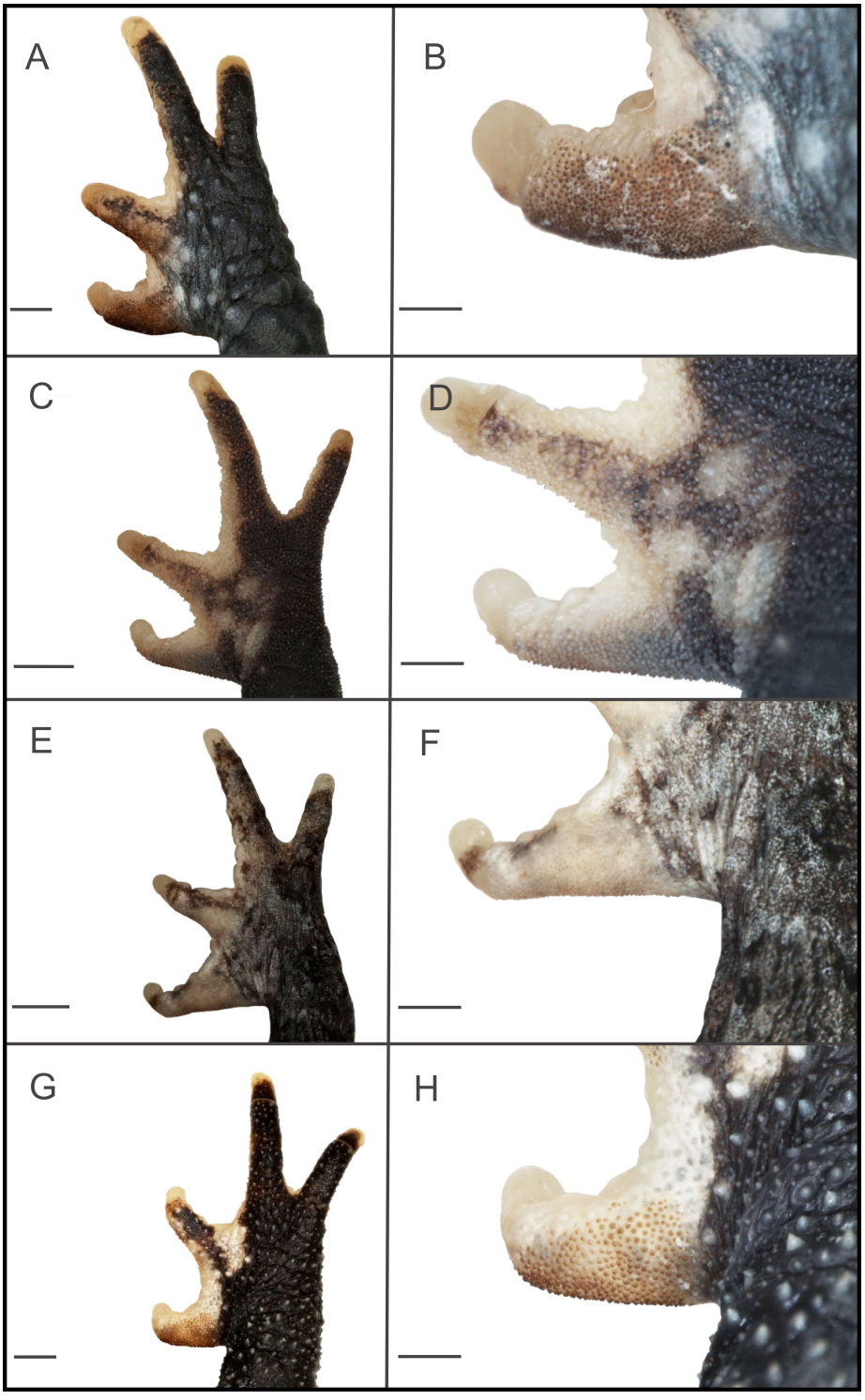

Figure 1. Optical micrograph images of the hand of a male (A) *Melanophryniscus macrogranulosus* (UFRGS 6451) (B) *M. dorsalis* (UFRGS 4199), (C) *M. sanmartini* (UFRGS 6748) and (D) *M. simplex* (UFRGS 4296). (Scale bar of images from the left= 1000µm and the right = 500µm).

Table 2. Nuptial pad data for male and female *Melanophryniscus macrogranulosus*.

Character	Male	Female
Form and occurrence	Conspicuous brown nuptial pad on fingers I and II and also on finger III (n=4)	Few minute structures only in finger I (n=4)
Estimated density - Finger I	697,2 ± 103,41 (525-875) (n=3)	319,4 ± 82,7 (225-400) (n=2)
Estimated density - Finger II	647,2 ± 141,1 (425-850) (n=3)	Lack of structures
Estimated density - Finger III	459,4 ± 290,9 (175-825) (n=3)	Lack of structures

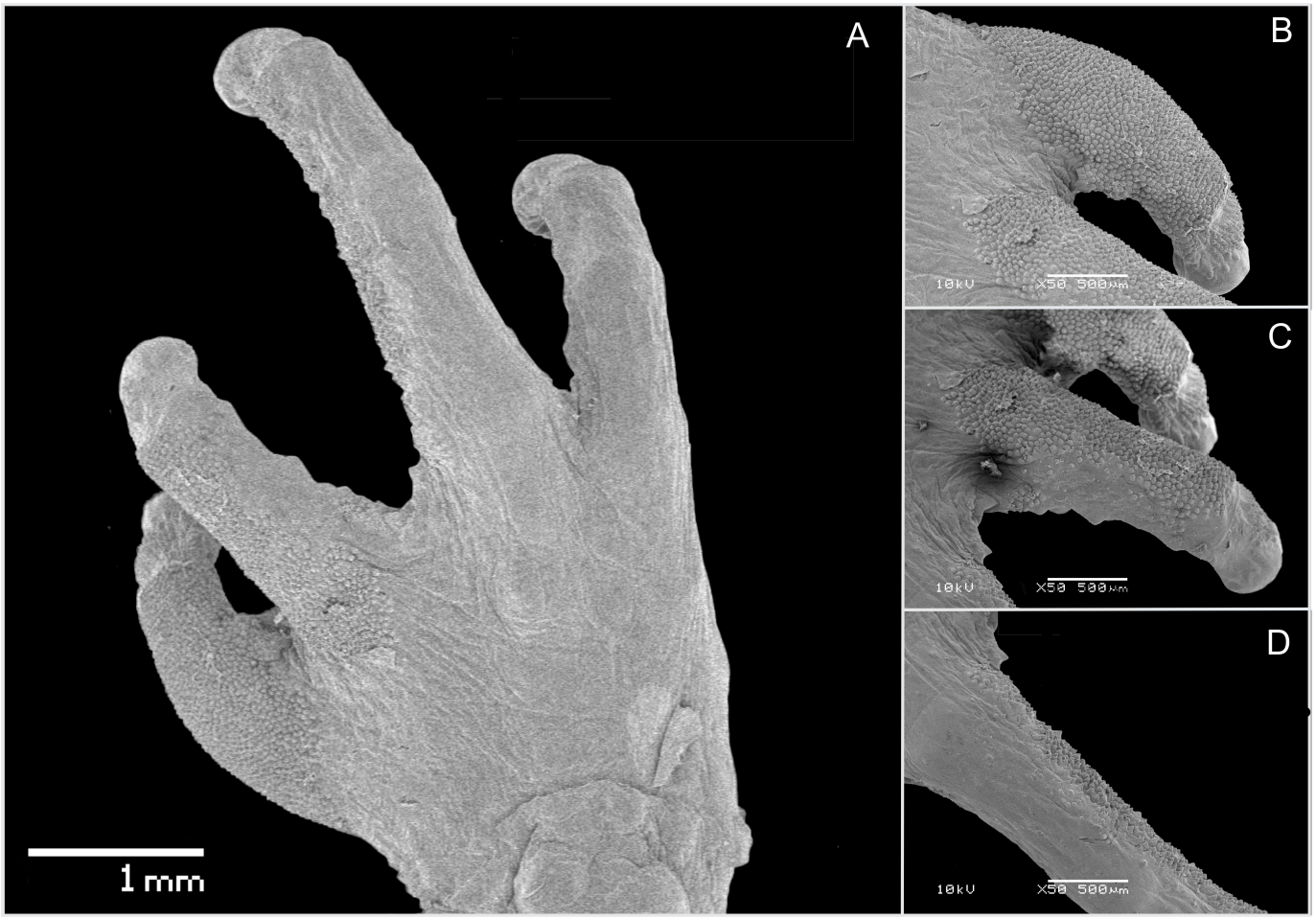

Figure 2. Scanning electron micrograph of a male *Melanophryniscus macrogranulosus* (UFRGS 6587). (A) Right hand with numerous small structures on the first three fingers and absent on the fourth. (B) Detail of nuptial pad on Finger I, (C) Finger II and (D) Finger III, (Scale bar = 500µm).

Density of elevations varies between fingers, the fist being always denser, and the following varying more between individuals. We observed a pattern of lower density at the margin of the pads. We did not find nuptial structures neither on male or female foot.

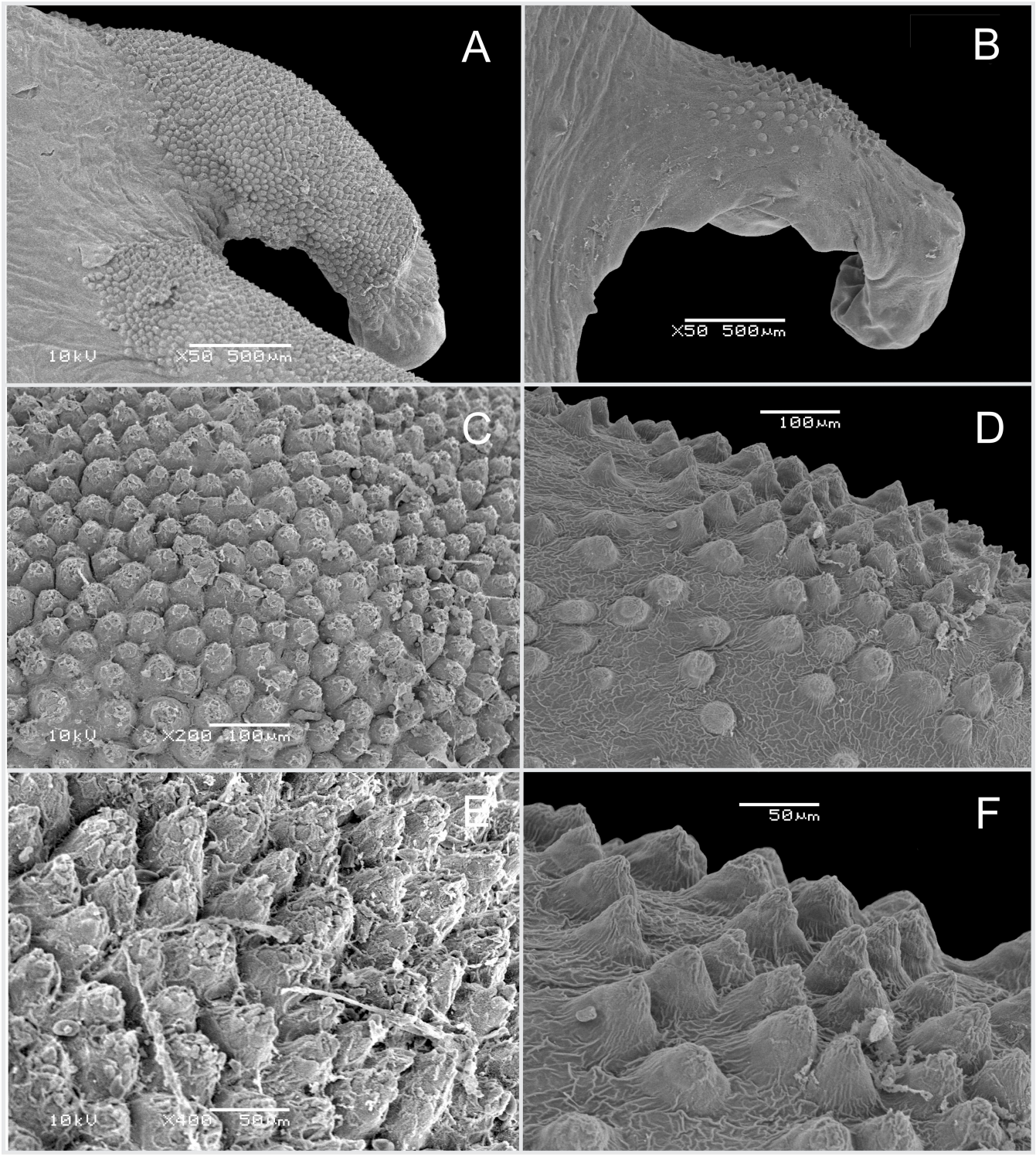

Figure 3. Scanning electron micrographs of the detail structure of the first finger of a *Melanophryniscus macrogranulosus* male (left; UFRGS 6587) and female (right; UFRGS 6588); 50x (A-B), 200x (C-D) and 400x (E-F).

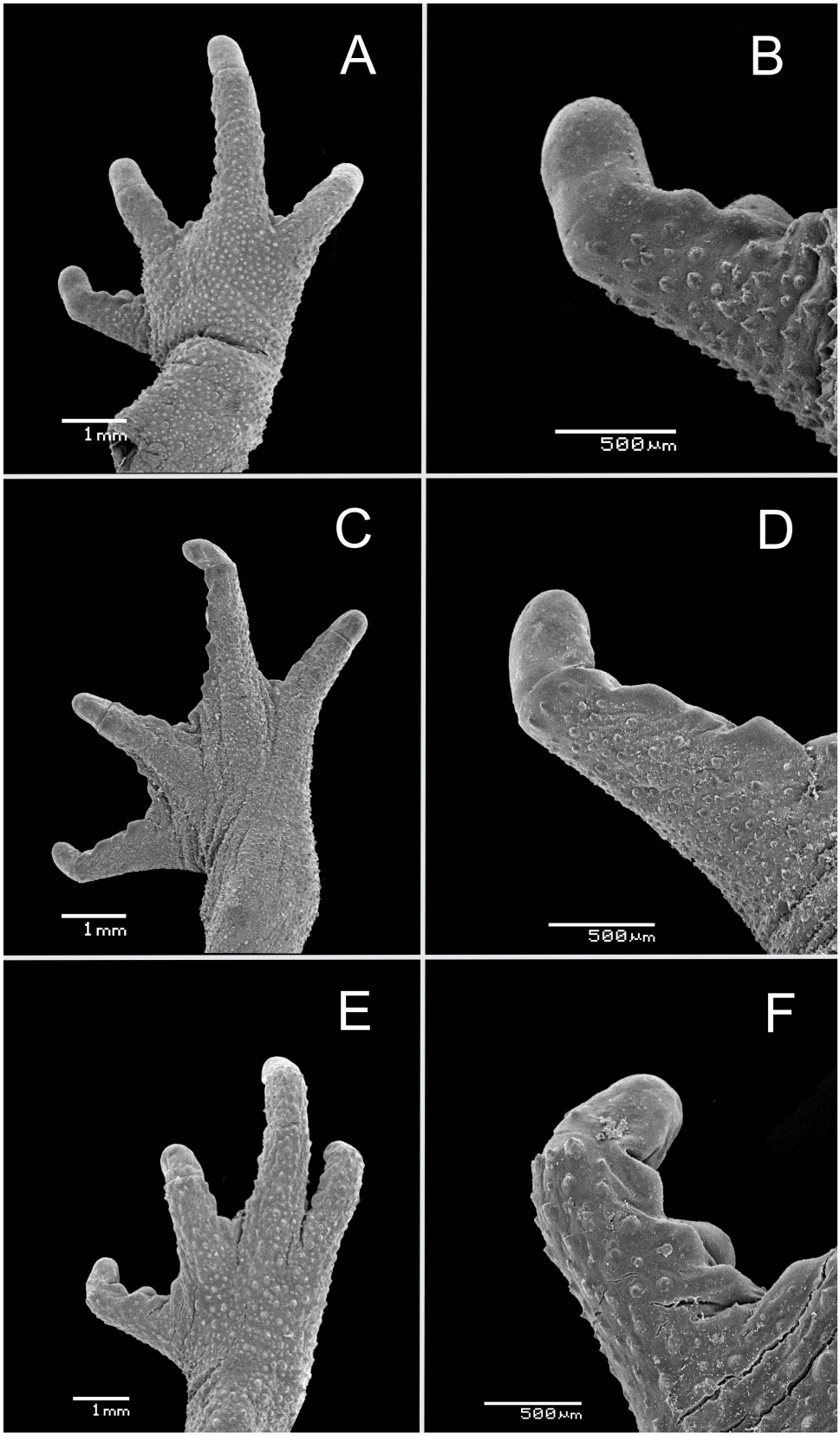

Figure 4. Scanning electron micrograph of the right hand (on the left) and finger I (on the right) of females from three species from the genus, (A) *Melanophryniscus dorsalis* - UFRGS 3256; (B) *M. sanmartini* - UFRGS 6751; (C) *M. simplex* - UFRGS 4165.

Discussion

Males of *Melanophryniscus macrogranulosus* have nuptial pads well evolved on finger I (thumb) II and III. Previous works had reported this structures only for the first two fingers (Braun and Braun, 1979; Santos et al., 2010). *Melanophryniscus macrogranulosus* species is known as an explosive breeder (Santos et al., 2010), which according to Wells (2007) could explain the function proposed for the presence of nuptial excrescence in males for grasp females or for aggressive behaviors, with male-male combat or male displacement, which we also observed Caorsi et al., (unpublished data). Other species with the same reproduction pattern and many stream-breeding frogs have very large nuptial pads, which could be an adaptation to aquatic amplexus (Wells, 2007).

We bring here the first record for the genus *Melanophryniscus* of these dermal modifications on finger I (thumb) in females. Dermal modifications in females have been reported in other few species, as *Chiasmocleis avilapiresae* (Peloso and Sturaro, 2008) and *Insuetophrynus acarpicus* (Diaz et al., 1983). In both cases, the structures are more developed in males than females. The authors reported that most cornifications of *I. acarpicus* females are on finger one (97,5%) and in some cases on finger two (40%), different from *M. macrogranulosus* females, that only have this structure present on finger one. Species of *Limnodynastes and Platyplectrum* have specialized structures during breeding season that provide a much greater surface area to the hands, which are used in paddling movements for stirring water and spawn into a foam nest (Duellman and Trueb, 1986). Other dermal modifications were reported in females, as few small spines on dorsum, and on dorsolateral surfaces of hind limbs in *Nelsonophryne aterrima* (Lehr and Trueb, 2007).

The minute elevations of *Melanophryniscus macrogranulosus* pads have a conical shape in both sexes, but males have much higher density than females and a larger covered area. Architecture of pad structures varies between species. Kurabuchi (1993) reported rounded nuptial pads in *Pelophylax porosus*, cap-shaped tops in *Rugosa rugosa,* conical in *P. nigromaculatus,* and rather tall and gradually tapering in *R. ornativentris. Phyllomedusinae* can have roundish or conically shaped with spiny ends

structures on nuptial pad (Luna et al., 2012). The density of elevations on finger I in males is almost twice than in females of *M. macrogranulosus*. Our density estimations in males finger I (mean=697,2) is very similar to the results found for two species of *Litoria* (then included in genus *Nyctimystes*) and some of *Phylomedusa* (Zweifel, 1983; Luna et al., 2012). This last author also reported lower densities than found in *M. macrogranulosus* for *Agalychnis*, *Cruziohyla*, *Phrynomedusa* and *Phasmahyla*. A large variation of density estimation within a genus was reported in *Phylomedusa*, 365-923 structures/mm^2 (Luna et al., 2012), *Pelophylax,* 295-1315 structures/mm^2 and *Rugosa*, 199.5-490,7 (Kurabuchi, 1993).

The hypothesis that nuptial excrescences could have evolved due to their explosive breeding, presence of aggressive behavior or reproductive sites in streams, still very superficial. Males of most *Melanophryniscus* have nuptial pad reported, but few of them have it described. There is a lack of detailed information of these structures for most species, especially for females, in which the function remains completely unknown. More studies must be performed to better understand the relation of this excrescence and their biology and to explore the relation of structure architecture to species relation within the genus.

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CAPÍTULO 4: Advertisement calls of *Melanophryniscus macrogranulosus,* a red-bellied-toad from the Atlantic Rainforest, Brazil: Encoding variation of acoustic signals at different levels

(Artigo nas normas de submissão do periódico *Plos One*)

Advertisement calls of *Melanophryniscus macrogranulosus,* an endangered red-bellied-toad from the Atlantic Rainforest, Brazil: Encoding variation of acoustic signals at different levels

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Abstract: The bioacoustic signals of the red-bellied-toads, from the genus *Melanophryniscus*, are poorly known; only eight of the 26 species have some call described. In this work, we describe the advertisement call of *M. macrogranulosus* comparing three different populations of this species. We collected data after heavy rains in three different sample sites in Rio Grande do Sul, Brazil, between 2012 and 2013. The advertisement call of *M. macrogranulosus* is composed by two segments. It always begins with part A, lasting approximately 0,44 - 6 seconds and composed by single modulated pulses separated by long time intervals followed by part B, an extremely long train of unmodulated pulses emitted at a rate of 32,8 – 42,6 per second, with short time intervals, and lasting from 9 – 32,1 seconds. We did not find any effect of snout-vent-length or body mass in the parameters. Principal Component Analysis showed some components reflecting some temporal variation and others spectral. Multivariate Test Analysis did not indicated significant differences between populations studied. Within-individual Coefficient of Variation showed only two parameters below 5% of variation, pulse rate in call part B and peak frequency of call part B. Results from Potential for Individual Coding presented pulse interval and peak frequency of call

part A and pulse duration of call part B as the acoustic characters with lowest values, possible reflecting the species-specific call characteristics. At a species level, the same bioacoustic parameters showed the lower values of CV, but still they were higher than at individual level. Despite intra-male variation found in some parameters the analysis at group level (populations) were not strong enough to differentiate the three populations of *M. macrogranulosus*.

Key-words: Bioacoustics, Bufonidae, populations, coefficient of variation.

Introduction

The red-bellied-toads, genus *Melanophrynicus* Gallardo, 1961 comprise 26 recognized species [1] distributed in southern of South America. It is arranged in three different and weakly defined species groups [2] based on morphological characters [3]: *M. stelzneri*, *M. moreirae* and *M. tumifrons* species group. The last one is characterized by the presence of a frontal skin gland [4] and is composed of eight species including *Melanophryniscus macrogranulosus* Braun, 1973, an endemic toad from the extreme southern Atlantic Rainforest of Brazil. This endangered species has been differentiated for many years from *M. cambaraensis* Braun & Braun, 1979, basically by the color pattern of the skin. However, Caorsi *et al*. (unpublished data) morphological and molecular data for all the known populations for this species and did not find any available evidence to support the existence of the two different species, considering them all as *M. macrogranulosus*.

The structure of the acoustic signal in anurans has been considered species-specific [5] and may be used for the recognition of species with no distinguishable morphological characters [6]. Even though, it is possible to find variation of acoustic parameters within and between individuals and populations (e.g. [7,8]). Nowadays, only eight of the 26 species of the genus *Melanophryniscus* have their call structure described and only one is in the *M. tumifrons* species group. From those, the advertisement call described shows a pattern over the genus, with the call divided in two parts, call part A, generally composed by single pulses, and call part B, generally a trill. However, these two types vary between species, specially the second segment, which is continuous, but short for some species and very long in others.

In this work, we describe the advertisement call of *Melanophryniscus macrogranulosus* and compare the call of three different populations of this species.

Material and Methods

Study sites

We collected bioacoustic data in three different sample sites in Rio Grande do Sul, Brazil (Fig.1). We recorded males of *Melanophryniscus macrogranulosus* during day and night on September 18 and 19 of 2012 in Floresta Nacional de São Francisco de Paula – FLONA SFP – a conservation unit (29°25'41.3"S, 50°23'44.5"W/866 m asl); municipality of São Francisco de Paula; on September 21 and 22 of 2013 in Morro da Gruta, type locality of the species, municipality of Dom Pedro de Alcântara (29°24'21.16"S, 49°51'1.48"W/31m asl); in June 21 and September 22 of 2013 in Garapiá, Barra do Ouro (29°30'33"S, 50°14'45"W/200m asl), municipality of Maquiné. Reproductive site are temporary shallow streams formed after heavy rains. At the stream, we may only see this species. FLONA SFP and Garapiá sites are relatively quiet, there is only the stream noise and rain and wind from the storm. The opposite happens in Morro da Gruta, where besides the stream noise we also have the high way road noise pollution of cars and trucks passing every minute. All expeditions were after heavy rains (from 40-180 mm, according to the Instituto Nacional de Meteorologia of Brazil) and under temperatures that varied from 15.7-17ºC on the air and 15-16.5ºC on the water.

Data collection

Call recordings were made with a Marantz PMD 670 and a Sennheiser ME 67 directional microphone at a distance about 50 cm from the individual recorded. The recording level was adjusted manually to obtain the best signal-to-noise ratio and avoid distortion and kept constant during each session. At least five males per population were audio recorded. Sounds were recorded using a sampling rate of 48 kHz, 16-bit depth and in uncompressed .wav format. After recording, we measured air and water temperature of the specific individual calling site using a digital thermometer (accuracy 0.1ºC).

Some males were hand captured after recording and their body mass and snout-vent length (SVL) were measured using a Pesola scale to the nearest 0.1 g and caliper to the nearest 0.1 mm. All the material collected during the study was deposited in Coleção Herpetológica do Departamento de Zoologia da Universidade Federal do Rio Grande do Sul (UFRGS), RS, Brazil (UFRGS 6319-21, UFRGS 6385). Most of the sounds included in the description are deposited in Fonoteca Zoologica do Museo Natural de Ciencias Natures, Madrid, Spain (Codes FZ Sound Collection 9093-9113).

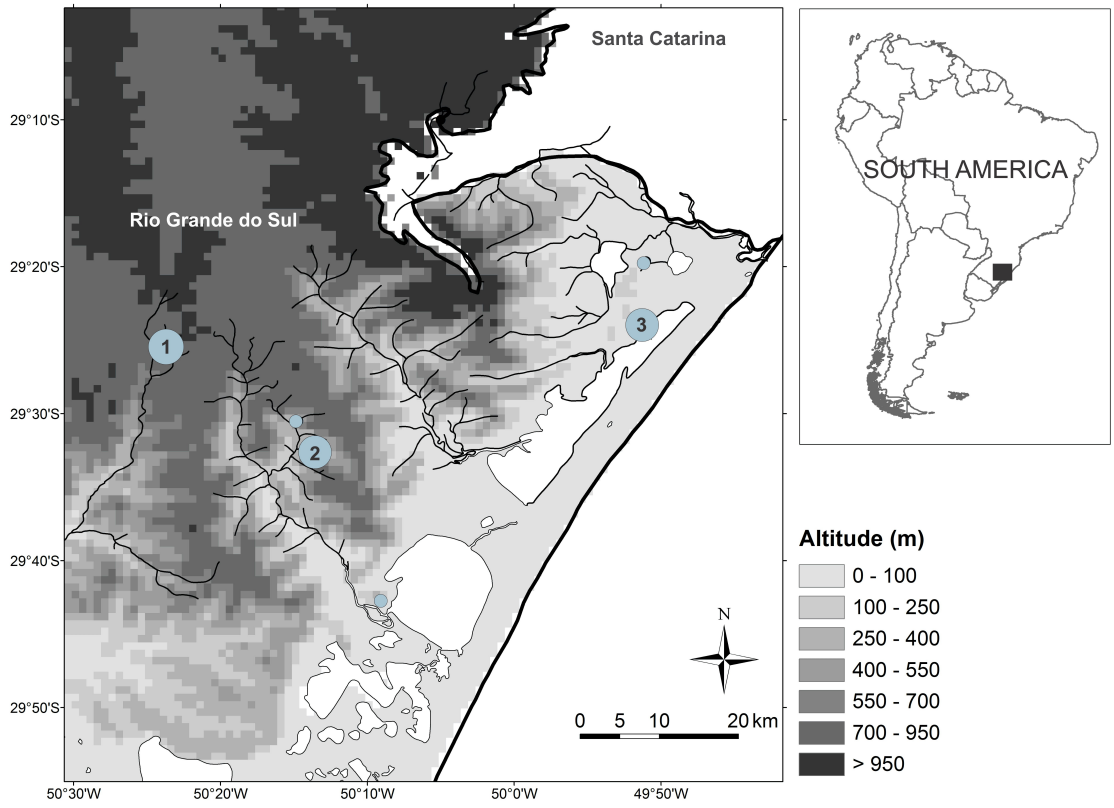

Figure 1. Distribution of *Melanophryniscus macrogranulosus* (blue dots)*;* FLONA SFP (1), Garapiá (2), Morro da Gruta (3).

Bioacoustic Analyses

Calls were analyzed with an Apple MacBook Pro using Software RAVEN Pro. 1.5: Interactive Sound Analysis (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY, USA). For the sound analyses, we constructed energy detectors to help capturing all pulses of the call. Energy

Detector supplied in Raven is a time-frequency energy detector. It estimates the background noise of a signal and uses this to find sections of signal that exceed a user-specified signal-to-noise ratio threshold in a specific frequency band, during a specific time (Raven Pro 1.4 User's Manual, Revision 11 10 December 2010). Spectral properties were assessed with a Hann type window and Fast Fourier Transform of 256 point length, window overlap.

We analyzed from one to three calls of the same individual, a total of 30 calls: 21 calls from nine individuals that were measured after recording and nine calls from individual that were not captured. The nomenclature used for call variables follows [9] and vocal repertories and behaviors follows [10]. The advertisement call of *Melanophryniscus macrogranulosus* is composed by two segment: call part A, single pulses, and call part B, a continuous trill. The following measurements were taken: Call duration (s), Call part A duration (s), Nr of pulses per call part A, Pulse duration of call part A (s), Pulse interval of call part A (s), Peak Frequency of call part A (Hz), Interval between call part A and call part B (s), Call part B duration, Nr of pulses per call part B, Pulse rate in call part B (pulses/sec), Pulse duration of call part B (s), Pulse interval of call part B (s), Peak Frequency of call part B (Hz).

To understand and evaluate call structure variation within and between individuals and populations we completed some analyses: We analyze if there was some difference between the parameter "interval of the pulses of call part B" using ten of the best recorded individuals. We divided the trill (call part B) into three sections: the first ten pulses (FP10), pulses in between (PB), and the last ten pulses (LP10), and analyzed them separately. We tested the effect of body mass and body size on some acoustic parameters of all populations separate and together. The correlation was not calculated with "duration of pulses of call part B" due to their extremely short duration and high relative measurement error. A Principal Component Analysis (PCA) including all males recorded from all populations was performed to observe whether population groups were separated along some acoustic characteristic. Factors with eigenvalues ≥ 1 were analyzed to extract the bioacoustic parameters that reflect the variance in the group. After the PCA, assuming having transformed the variables into non-correlated components,

we used all characters but two, pulse duration of call A and pulse interval of call B (eigenvalues ≤ 1), to perform a Multivariate Analysis of Variance (MANOVA). Using only individuals recorded more than once, we calculated the Coefficient of Variation (CV) (*sensu* [11]), [CV = standard deviation/mean x 100], for all acoustic properties within individuals for each population. We then classified those with CV <5% as static, CV> 12% as dynamic following [11]. Within a call session, the static parameters remain considerably constant in a population, and presumably better reflect the species-specific call characteristics. The Potential for Individual Coding (PIC) [12] for each parameter was calculated as the ratio [CVb/mean CVi] (CVb being the Coefficient of variarion between all individuals together and CVi the mean of CV intra-individual of all males recorded. PIC values indicate the relation of the between-individual and within-individual variation. High values, greater than one, suggest a better use of the parameter for individual recognition and those with low values for species recognition (see also [13]). Nr of pulses per call part B was recorded to make comparisons to other species of the genus, but we did not include it in the analyses due to its correlation with the parameters Call part B duration and Pulse rate in call part B (pulses/sec). Finally, using previously published data of *Melanophryniscus* acoustic communication, we plot values of pulse rate for each species of the genus and then the relation of two parameters: call duration (s) and pulse rate of the call part B (pulse/sec.), using the mean value showed in the articles. All statistical analyses were performed using the Software Statistica 6.0 and Sigma Plot 10.0.

Results

We observed many males calling at the three reproductive sites, after heavy rains. Calls could be heard at a distance of more than five meters, but not much further than that. Most of them called from inside the water of a shallow temporary stream where we also saw some pairs in amplexus. In the field, we watched some males walking around the site and jumping on females and other males that passed by and jumped on calling males. When that happened males often emitted a call, different from advertisement call, and tried to evict the intruder by grabbing him with the hind limbs or pushing with their legs and feet. We also observed amplexus pairs being attacked by other males, in struggles that

reached up to five individuals including the amplectant pair. Figure 2 shows a male in the water stream in Flona SFP (A), calling male Garapiá (B), an amplexus pair (C) and struggle (D) in Dom Pedro de Alcântara.

Figure 2. Reproductive activity of *Melanophrynicus macrogranulosus* in different samples sites.

The advertisement call of *Melanophryniscus macrogranulosus* is composed by two segments, call part A and call part B (Fig.3). It always begins with part A, composed by single modulated pulses separated by long time intervals and lasting approximately 0.44 - 6 seconds. Call part A is always followed by part B, an extremely long train of unmodulated pulses emitted at a rate of 32.8 – 42.6 per second, with short time intervals, and lasting from 9 – 32.1 seconds (Tab. 1). The call presents weak harmonic frequency bands.

Effects of SVL and body mass in spectral and temporal parameters with all individuals together were not significant: peak frequency of call part A and SVL $R^2 = 0.0752$; $p > 0.005$; peak frequency of

call part A and body mass R^2 = 0.0526; p > 0.005; peak frequency call part B and SVL R^2 =0.125; p > 0.005; peak frequency of call part B and body mass R^2 =0.109; p > 0,005. When testing them separately by population, we also did not find a significant relation (Power of all the tests was below 0,8).

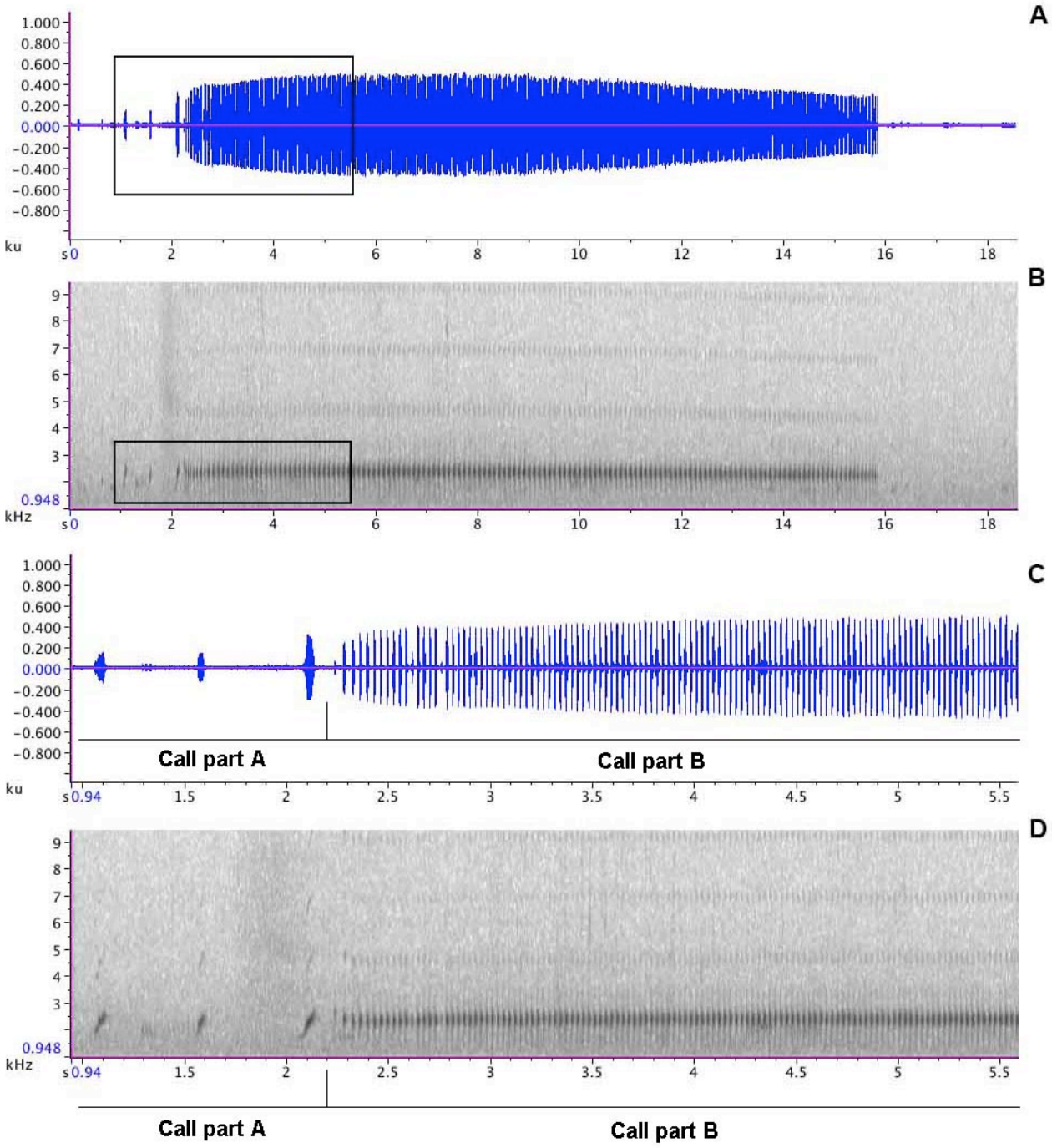

Figure 3. Oscilogram (A) and spectrogram (B) of *Melanophryniscus macrogranulosus* call from Flona SFP; enlarged section (box) from 4,5s (C, D).

Table 1. Call characteristics of *Melanophryniscus macrogranulosus*. Values in the table are Average ± standard deviation (range).

	Flona SFP (ncalls=13, nind.=8)	Garapiá (ncalls=10, nind.=6)	Morro do Gruta (ncalls=9, nind.=5)	Total (ncalls=32, nind.=19)
Call duration (s)	16.6 ± 6.51 (12.6 - 33.4)	21.3 ± 5.8 (11.9 - 29.1)	17.4 ± 1.9 (14 - 18.4)	19 ± 5.5 (11.9 - 33.4)
Call part A duration(s)	1.61 ± 1.32 (0.852 - 4.5)	2.56 ± 1.91 (1.17 - 6)	1.67 ± 0.73 (0.44 - 2.1)	2.1 ± 1.5 (0.44 - 5.99)
Nr of pulses in call part A (s)	4.5 ± 3.23 (3 - 11)	6.5 ± 4.23 (5 - 14.3)	4 ± 1.5 (2 - 6)	6 ± 3.5 (2 - 14.3)
Pulse duration in call part A (s)	0.02 ± 0.004 (0.013 - 0.027)	0.015 ± 0.04 (0.01 - 0.12)	0.02 ± 0.01 (0.17- 0.04)	0.026 ± 0.024 (0.01 - 0.12)
Pulse interval in call part A (s)	0.39 ± 0.052 (0.28 - 0.46)	0.34 ± 0.08 (0.31 - 0.53)	0.35 ± 0.09 (0.28 - 0.53)	0.376 ± 0.07 (0.28 - 0.53)
Peak Frequency in call part A (Hz)	2109 ± 162 (1968 - 2437)	2413.5 ± 277.7 (2250 - 3000)	2374 ± 159.3 (2062 - 2437)	2298 ± 231.7 (1968 - 3000)
Interval between call parts A and B (s)	0.22 ± 0.155 (0.043 - 0.533)	0.26 ± 0.16 (0.06 - 0.46)	0.27 ± 0.39 (0.12 - 0.9)	0.298 ± 0.24 (0.043 - 0.93)
Call part B duration	15.0 ± 6.8 (9 - 32.2)	18.6 ± 4.9 (10.3 - 23.5)	14.82 ± 1.13 (13.3 - 16.4)	16.6 ± 5.18 (8.9 - 32.2)
Nr of pulses in call part B (s)	576.5 ± 156 (314 - 948)	658.6 ± 99.3 (563 - 811)	523.4 ± 55.5 (497 - 599)	583.2 ± 132.1 (314 - 948)
Pulse rate in call part B (pulses/s)	35.4 ± 3.1 (32.8 - 42.7)	35.3 ± 1.05 (34.2 - 36.9)	34.9 ± 1.63 (33.4 - 36.7)	35.5 ± 2.2 (32.8 - 42.6)
Pulse duration of call B (s)	0.008 ± 0.0007 (0.006 - 0.008)	0.006 ± 0.0011 (0.01 - 0.01)	0.006 ± 0.0013 (0.004 - 0.01)	0.007 ± 0.001 (0.004 - 0.01)
Pulse interval of call B (s)	0.020 ± 0.0019 (0.016 - 0.022)	0.021 ± 0.001 (0.02 - 0.02)	0.022 ± 0.0084 (0.02 - 0.04)	0.022 ± 0.005 (0.016 - 0.04)
Peak Frequency of call B (Hz)	2250 ± 166 (1968 - 2500)	2413.5 ± 311 (2119 - 3000)	2302 ± 155.5 (2062 - 2437)	2318.8 ± 235 (1968 - 3000)

When analyzing the interval between pulses of call part B, we found that the beginning and end of the trill pulse intervals were larger than in between, where a stable maximum pulse rate is attained (Fig. 4). For all individuals (n=10) the mean of parameters interval of the “first 10 pulses” was 0.027s, “pulses in between” was 0.021s and the “last 10 pulses” was 0.026s (Tab. 2). Effects of SVL and body mass in

these parameters were also not significant: FP10 and SVL (R^2 = 0.0121, p > 0.005); PB and SVL (R^2 = 0.0861, p > 0.005); LP10 and SVL (R^2 = 0.00927, p > 0,005) FP10 and body mass (R^2 = 0.238, p > 0.005); PB and body mass (R^2 = 0.00185, p > 0.005); LP10 and body mass (R^2 = 0.175, p > 0.005) (Power of the tests were all below 0.8, very low values).

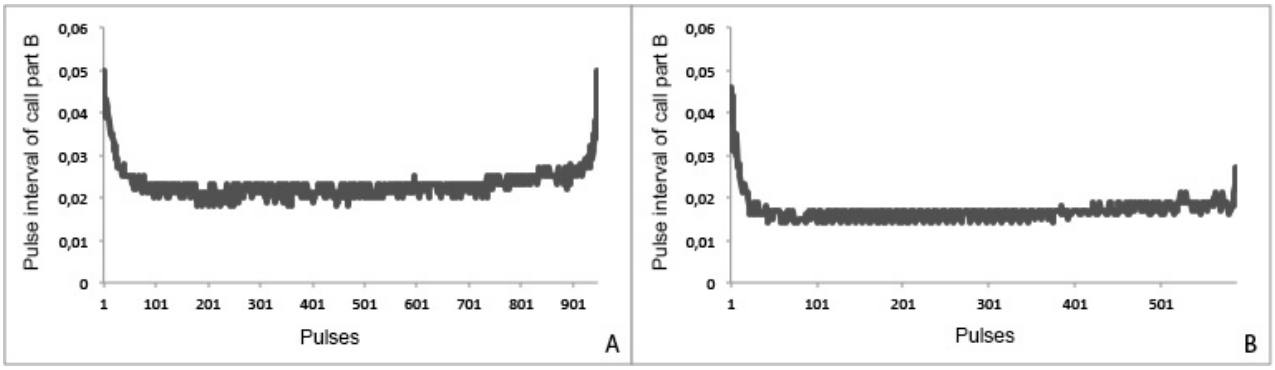

Figure 4. Interval between pulses of call B from two males of FLONA SFP; UFRGS 6320-21, SVL 32.5 and 30.8 mm respectively.

Table 2. Mean Interval between pulses (s) in call part B per individual and sample site.

	First 10 pulses		Pulses in between		Last 10 pulses	
Flona SFP (n=6)	**0.029**	**0.005**	**0.020**	**0.005**	**0.025**	**0.003**
Garapiá (n=2)	**0.029**	**0.009**	**0.021**	**0.003**	**0.026**	**0.003**
Morro da Gruta	**0.021**	**0.004**	**0.021**	**0.005**	**0.026**	**0.001**
	0.027	0.005	0.021	0.005	0.026	0.003

The PCA using twelve acoustic characteristics explained 83.2% of the variance, reflected in five components (n total for the analysis=17 individuals; n=5 from Morro da Gruta, n=6 from Garapiá and n=8 form FLONA SFP). The Principal Components 1, 2, 4 and 5 reflected temporal variables, call duration of part A and part B, Nr. of pulses of call part A, interval between parts A and B, pulse duration of call part B and pulse interval of call part A. Principal Component 3 reflected spectral variables as peak frequency of call part A and call part B (Tab.3). We only used the variables appointed by PCA, non-correlated, for the Multivariate Test Analysis. MANOVA test did not indicate significant differences

between bioacoustic parameters of the three different sample sites analyzed (Wilks lambda = 0.18454; F = 1.3279; P > 0.05). Despite some differences observed between mean values of parameters between samples sites, they show large variation and extremes overlap (Fig.5).

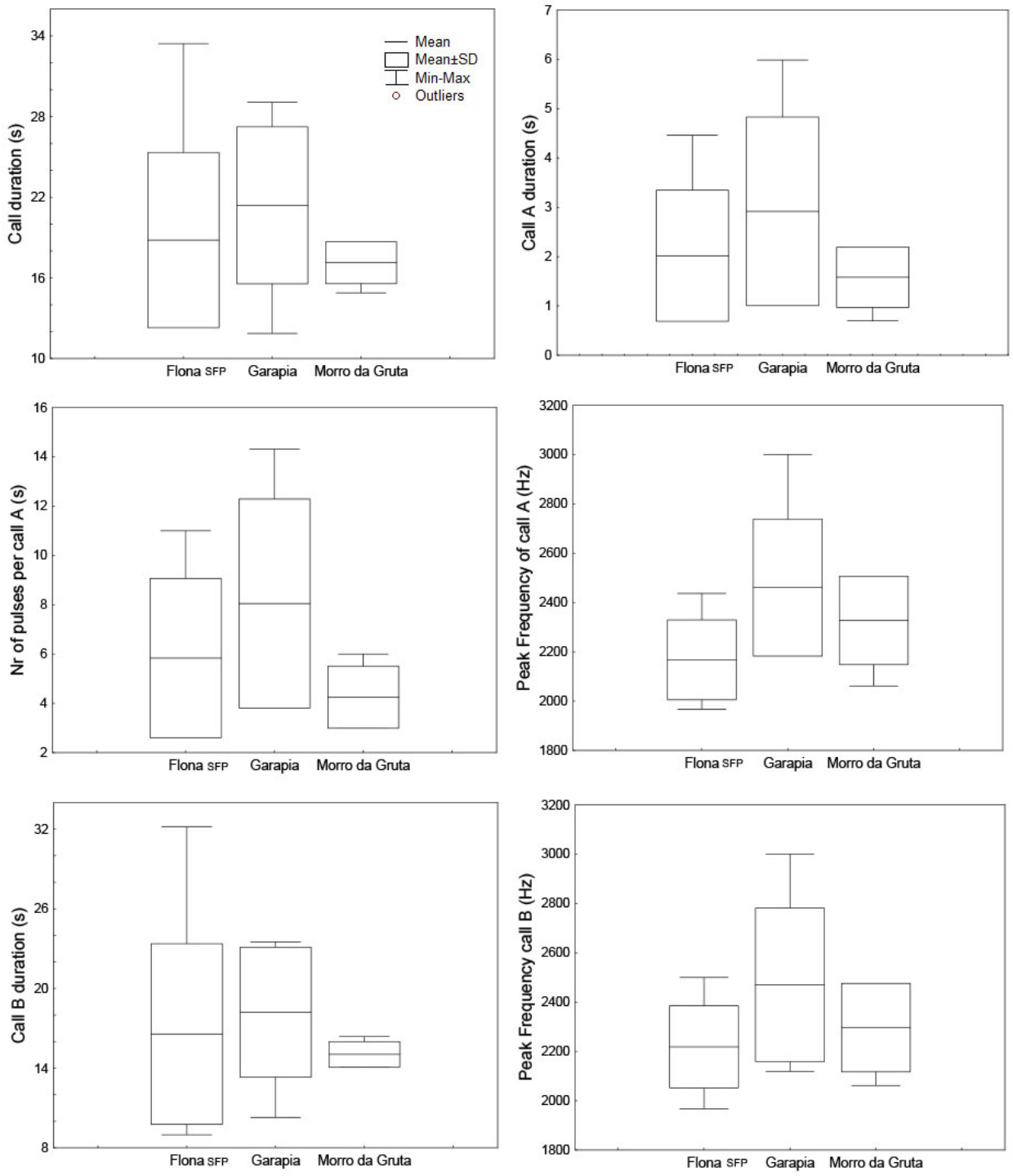

Figure 5. Comparison between populations of some parameters selected by PCA (box plot: mean, SD and extremes).

Table 3. PCA of acoustic parameters *Melanophryniscus macrogranulosus* call. Numbers in bold are those with more weight

	CP1	CP2	CP3	CP4	CP5
Call duration (s)	-0.253	**-0.893**	0.006	0.191	0.125
Call part A duration(s)	**-0.766**	-0.283	-0.013	-0.314	-0.441
Nr of pulses in call part A (s)	**-0.835**	-0.235	0.083	-0.387	-0.247
Pulse duration in call part A (s)	-0.425	0.008	-0.143	-0.453	0.286
Pulse interval in call part A (s)	-0.032	-0.061	-0.095	0.468	**-0.756**
Peak Frequency in call part A (Hz)	0.332	-0.308	**0.840**	-0.142	-0.065
Interval between call parts A and B (s)	**0.622**	-0.035	-0.049	-0.224	-0.455
Call part B duration	-0.082	**-0.859**	0.013	0.300	0.276
Pulse rate in call part B (pulses/s)	-0.421	0.533	0.578	-0.068	0.200
Pulse duration in call part B (s)	-0.418	0.114	-0.071	**0.807**	0.108
Pulse interval in call part B (s)	0.501	-0.513	-0.434	-0.367	0.105
Peak Frequency in call part B (Hz)	0.205	-0.270	**0.883**	0.054	0.188
% Variance	22.3	20	17.1	13.8	10%

Table 4. Individual Coefficient of Variation (%) in each sample site and the total of individuals together.

Call parameters	Flona SFP (ncalls=8, nind.=3)		Garapiá (ncalls=7, nind.=3)		Morro da Gruta (ncalls=6, nind.=2)		Total		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Classification
Call duration (s)	9.9	2.9	24.1	37.8	15.1	13.2	16.5	21.9	Dynamic
Call part A duration(s)	64.1	33.9	11.1	8.4	79	79.6	48	47.2	Dynamic
Nr of pulses in call part A (s)	49.6	24.3	28.4	12	68.4	3.2	46.4	22.2	Dynamic
Pulse duration in call part A (s)	24.6	8.3	17.1	17.5	44.4	38.2	26.7	21.1	Dynamic
Pulse interval in call part A (s)	5.3	4.9	21.2	18.2	_	_	22.8	28.6	Dynamic
Peak Frequency in call part A (Hz)	5.7	5.5	4.6	5.7	8.2	5.1	5.9	4.9	Dynamic
Interval between call part A and B (s)	60.4	50.7	60.5	22	39.6	13.1	55.2	31.5	Dynamic
Call part B duration	10.9	3.2	39.5	64.5	13.3	6.9	22.2	37.5	Dynamic
Pulse rate in call part B (pulses/s)	**0.8**	**0.5**	**1.5**	**1.3**	**2.8**	**0.8**	**1.6**	**1.2**	Static
Pulse duration in call part B (s)	20.2	15.1	23.9	9.1	21.5	13	21.9	10.8	Dynamic
Pulse interval in call part B (s)	6.9	3.6	6.2	7.8	65.9	80.4	21.4	41.2	Dynamic
Peak Frequency in call part B (Hz)	**3.1**	**2.7**	**2.8**	**2.8**	**2.6**	**3.6**	**2.9**	**2.5**	Static

The Coefficient of Variation varied among call parameters. Variation within-individual of different samples sites, showed only two parameters with values below 5% (Tab.4), pulse rate in call part B (pulses/sec.) and peak frequency of call part B (Hz), and the peak frequency of call part A (Hz) was

almost under 5%. When observing the results from PIC, pulse interval and peak frequency of call part A (s) and pulse duration of call part B (s) were the acoustic characters with lowest values (PIC≤1) (Tab.5).

Table 5: CV between individual; Mean CV within individuals and PIC (%): CVb/mean CVi.

	CVb	Mean Cvi	PIC
Call duration (s)	37.3	16.5	2.3
Call part A duration(s)	80.7	48.0	1.7
Nr of pulses in call part A(s)	68.2	46.4	1.5
Pulse duration in call part A(s)	43.4	26.7	1.6
Pulse interval in call part A (s)	20.3	22.8	**0.9**
Peak Frequency in call part A (Hz)	6.0	5.9	**1.0**
Interval between call part A and B (s)	73.7	55.2	1.3
Call part B duration	39.9	22.2	1.8
Pulse rate in call part B (pulses/s)	8.7	1.6	5.6
Pulse duration in call part B (s)	19.8	21.9	**0.9**
Pulse interval in call part B (s)	30.7	21.4	1.4
Peak Frequency in call part B (Hz)	7.6	2.9	2.6

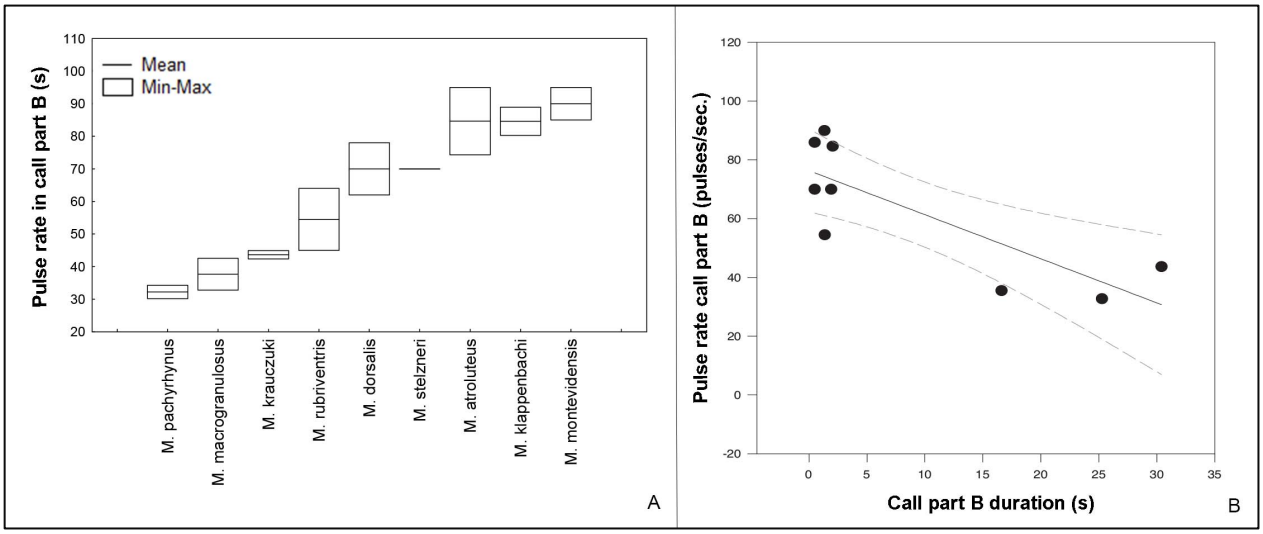

Figure 6. Pulse rate (A) and relation of call B duration and pulse rate (B) in *Melanophryniscus*.

Using data from previous published studies of *Melanophryniscus* bioacoustics [14–18] and results from our study, we plot values of pulse rate for each species (Fig. 6A) and then the relation of two

parameters (Fig. 6B), duration and pulse rate of the call part B. The results show some differences in pulse rate between species of the genus and a trend ($R^2 = 0.652$; $p < 0.005$) that species with long call part B have lower pulse rate per second.

Discussion

The genus *Melanophryniscus* is an explosive breeder (*sensu* [19]), characterized by many individuals migrating to a temporary streams with breeding activity for a few days [20–22]. *Melanophryniscus macrogranulosus* is an aseasonal explosive breeder [23] and the behaviors observed during our field trips, as active search or male competition, were also reported for other species of the genus [18,24,25] and bufonids [26–28]. According to [10] these behaviors can be influenced by male density in the reproductive events and the same can happen with some bioacoustic parameters as variation in call duration.

Melanophryniscus macrogranulosus as other species of the genus have the advertisement call structure divided in two parts: the first with single pulses and the second with a trill. Between species, they differ in number of single notes in call part A and call part B duration. This trill is commonly found in other Bufonids, including the studied genus [14,29–31]. *Melanophryniscus macrogranulosus* has a very long trill duration (ranging from 11.9 – 33.4 seconds) as *M. pachyrhynus* (up to 45.7 sec. [18]) and *M. krauczuki* (up to 36.6 sec. [14]), this last one belonging to a species group different from the one in our study. The trill from species of *M. stelzneri* group is shorter, *M. atroluteus* (up to 4.3 sec.), *M. dorsalis* (up to 2.2 sec.), *M. klappenbachi* (up to 0.652), *M. montevidensis* (up to 2.0 sec.), *M. rubriventris* (up to 2.5 sec.) [14–17]. This same pattern of exception inside the *M. stelzneri* group was reported by [32]. The authors described that even though *M. krauczuki* is assigned to that group, its larval morphology resembles that of the *M. tumifrons* group and the same happens with the type of reproductive site inhabited, that is a temporary stream instead of a temporary pond, like other species of its group.

Pulse rate of call part B in *M. macrogranulosus* (32.8 – 42.7 pulses/sec.) is lower than in most species of the genus studied, where this parameter varied at all from 32.8-95 pulses/sec.

Melanophryniscus macrogranulosus, *M. krauczuki* and *M. pachyrhynus*, the species with very long call part B, are the ones with the lowest pulse rate per seconds and the rest, with shorter calls, have higher pulse rate per second. The results from comparison of pulse rate between species of the genus *Melanophryniscus* and the relation between call duration and pulse rate o part B show some differences between species of the genus and a trend ($R^2 = 0.652$; $p < 0.005$) that species with long calls have lower pulse rate per second. We detected that differences in pulse rate between species with long and short calls is influenced by interval between pulses. Species with long call duration have higher interval between pulses (*M. pachyrhynus* – 0.014s; *M. macrogranulosus* - 0.022s) than those with short calls (*M. montevidensis* – 0.0052s; *M. dorsalis* – 0.0069s). Peak Frequency of call part B (1968 – 3000Hz) also has a high range in all other species studied, varying more than 1000Hz within the species.

The relevance of body size and mass effects in call variation was studied for some species (e.g. [33,34]) including in *M. rubriventris*, where the authors reported a significant effect of body size in dominant frequency of call part A and call part B [16]. Although for some species that relation was relevant, it does not explain all the variation within individuals of a species (e.g [35]), and even when a positive correlation among body size and larynx exists, these morphological characters demonstrate a significant degree of independence among populations in their development [36]. Our results do not support the hypothesis that SVL and body mass have an effect in spectral and temporal parameters, however, the power of the performed tests were below the desired power of 0.800, indicating that we were likely not to detect it if the effect actually existed.

The parameter of Interval between pulses (s), divided in three categories, showed an interesting pattern, the beginning and end of the trill of call part B, have larger intervals between pulses (mean = 0.027 and 0.026 sec. respectively) than the middle (0.021), where a stable maximum call rate is attained. This aspect has never being reported for any species of the genus *Melanophryniscus* and may be related to interesting morphological characteristics (see [37]).

Principal Component Analysis indicated some call characteristics are more variable within our data, but the Multivariate Test Analysis for comparison between different sample sites did not show any significant difference. Although individuals from different samples sites seemed to show some variation in call properties, they have large variation and their extremes overlap. These agree with results found on a population study of *M. rubriventris* [16], where some spectral and temporal parameters appear reflecting the variability of some axis, but comparing between populations, this difference was not significant.

Analyses of *Melanophryniscus macrogranulosus* call at individual level considered two parameters as static (CV <5%), pulse rate in call part B and Peak Frequency of call part B, as pointed out by [16] for *M. rubriventris*. We also find peak frequency of call part A as a stereotyped propriety (5.9%). This variation within individuals indicates relatively small changes from call to call within males. This pattern of low variability within individual in spectral parameters might be due to its strong morphological constrains and weak relation with environmental variables [38]. It was registered for other anurans (e.g. [7,11,39]) and may be a general pattern across anurans [40]. Temporal properties as call duration, Nr. of pulses per call part A and interval between call part A and part B with very high CV values within individuals were considered dynamic. [38] found that call duration and intercall duration were always more variable than pulse rate and fundamental frequency in all different levels of analyses in *Bufo viridis*. Following [11] this last category includes highly variable characters, often changing by more than 100% in a matter of seconds, especially if there is some interaction with another male or a female. Despite intra-male variation found in some parameters, the analyses (PCA, MANOVA) at group level (populations) were not strong enough to differentiate the groups. At a species level, the same bioacoustic parameters showed the lower values of CV, but still they were higher than at individual level. This pattern of individual variation was also reported for other different species [7,39,41].

According to [40] low variability of call parameters within males does not necessarily mean that this pattern is the same observed between males. Categorizing properties as static or dynamic depends on

the focal object of comparison. The data obtained for variation at different levels (Tab. 5) indicate which parameters are probably more important in sexual selection and inter-individual recognition or those more likely involved in species recognition. The latter ones normally present low variation at all levels, between (CVb) and within (CVi) individuals. In *Melanophryniscus macrogranulosus* advertisement call, peak frequency of call A and B and pulse rate of call B presented the lower absolute variation for both levels, indicating some potential for species recognition, the same found by [13] for some anurans in Bali. However, the intra-individual variation was higher than inter-individual variation, so PIC value for peak frequency of call B, was higher than for the others characteristics. All other parameters had high values for inter and intra-individual variation and Potential for Individual Coding, except for pulse interval of call part A and pulse duration of call part B, that had high absolute values of CV, but low values of PIC. According to [38] these properties that vary more within individuals have a lower rate of evolution and might be more relevant to intraspecific mate choice information.

The convergence of the results of [16] with our work and other studies of genus *Melanophryniscus* allow us to interpreted the character "pulse rate of call part B (s)" as a stereotyped parameter, classified as static and maybe useful for species recognition and for further studies on taxonomy and bioacoustic for the genus. As we find some different features in interval between pulses of call B, we suggest this parameter should be more carefully analyzed and compared in future studies of *Melanophryniscus*. Other parameters such as the long duration or the extremely high number of pulses in part B may be advantageous for redundancy in a noisy environment or may be important for sexual selection, or both.

Finally, we call on the relevance of the acoustic communication for taxonomic studies for they potential mechanism of anuran isolation. Even cryptic or morphologically similar species of anura can be recognized by differences in their call and many works have shown that event sympatric species do not have identical advertisement calls, being distinguished at least by one spectral or temporal feature [10]. Tests of male and female responses to changes in these parameters should verify the actual role of the potential for information that the different call characteristics actually have.

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CONCLUSÃO

Os aspectos morfológicos, genéticos e bioacústicos estudados nas populações conhecidas de *Melanophryniscus macrogranulosus* e *M. cambaraensis* não demonstraram evidência para a separação de grupos bem definidos. Estes caracteres, portanto, não corroboraram o status taxonômico atual das duas espécies, reconhecidas atualmente como válidas. Portanto, *M. cambaraensis* Braun e Braun, 1979 foi considerado um sinônimo Junior de *M. macrogranulosus.* Seguindo os critérios da lista vermelha da IUCN (IUCN 2013), os fatores de ameaça da espécie, associados com sua reduzida extensão de distribuição (B1 <5000 km^2) justificam a categorização de *M. macrogranulosus* como Em Perigo (EN) – B1ab (i, ii, iii).

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