INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA - INPA
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA

ASPECTOS DA ECOLOGIA QUÍMICA EM ANUROS: INTERAÇÕES ENTRE ANUROS E INVERTEBRADOS E VARIAÇÃO GEOGRÁFICA NO PERFIL DE METABÓLITOS SECUNDÁRIOS EM EXTRATOS DA PELE

Manaus, Amazonas
2021

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ANDRÉ DE LIMA BARROS

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Orientadora: Dra. Albertina Pimentel Lima

Coorientadora: Dra. Cecília Verônica Nunez

Tese apresentada ao Instituto Nacional de Pesquisas da Amazônia – INPA, como parte dos requisitos para obtenção do título de Doutor em Biologia (Ecologia).

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2021

ATA DE DEFESA

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Aos 16 dias do mês de Abril do ano de 2021, às 14h00min, por videoconferência, reuniu-se a Comissão Examinadora de Defesa Pública, composta pelos seguintes membros: o Dr. **Jorge Luis López-Lozano**, da Fundação de Medicina Tropical - FMT, Dra. **Márcia Neiva**, da Universidade Federal do Amazonas - UFAM, a Dra. **Elizabeth Franklin Chilson**, do Instituto Nacional de Pesquisas da Amazônia - INPA, a Dra. **Juliana Hipólito de Sousa**, do Instituto Nacional de Pesquisas da Amazônia - INPA, e o Dr. **Rafael de Fraga**, da Universidade Federal do Oeste do Pará - UFOPA. Tendo como suplentes o Dr. Igor Luis Kaefer, da Universidade Federal do Amazonas, e o Dr. Mario Eric Cohn-Haft, do Instituto Nacional de Pesquisas da Amazônia - INPA, sob a presidência da orientadora, a fim de proceder a argüição pública do trabalho de **TESE DE DOUTORADO** do **ANDRÉ DE LIMA BARROS**, intitulada: **"ASPECTOS DA ECOLOGIA QUÍMICA EM ANUROS: INTERAÇÕES ENTRE ANUROS E INVERTEBRADOS E VARIAÇÃO GEOGRÁFICA NO PERFIL DE METABÓLITOS SECUNDÁRIOS EM EXTRATOS DA PELE"**, orientado pela Dra. Albertina Pimentel Lima, do Instituto Nacional de Pesquisas da Amazônia – INPA e co-orientado pela Dra. Cecília Veronica Nunez.

Após a exposição, o discente foi arguido oralmente pelos membros da Comissão Examinadora, tendo recebido o conceito final:

[X] APROVADO (A) [ ] REPROVADO (A)

[X] POR UNANIMIDADE [ ] POR MAIORIA

Nada mais havendo, foi lavrada a presente ata, que, após lida e aprovada, foi assinada pelos membros da Comissão Examinadora.

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Dedico esta Tese à minha mãe, Gracilândia Farias de Lima, à meu pai, Beder Ferreira Barros e ao meu irmão Ítalo de Lima Cavalcante. O apoio de vocês me fez mais forte.

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Resumo

Anuros apresentam grande variedade de estratégias de defesa, sejam visuais, químicas ou comportamentais, como a reprodução não exclusiva em ambientes aquáticos. De maneira incomum, algumas espécies têm utilizado um mecanismo de defesa considerado raro, que é o hábito de conviver com invertebrados. De maneira geral, os aspectos ecológicos envolvidos nessas interações ainda são incompreendidos. *Lithodytes lineatus* é uma rã que apresenta distribuição conhecida para os países sul-americanos e tem sido considerada por muitos pesquisadores como sendo de baixa detectabilidade. Esta característica pode estar associada ao hábito incomum que esta espécie apresenta de viver junto a formigas saúvas (*Atta* spp.). É sugerido que a associação com formigas *Atta* seja mediada por substâncias químicas na pele da rã, porém aspectos da ecologia química de *L. lineatus* permanecem desconhecidos. Até o momento, nenhum estudo tratou de investigar padrões de composição de substâncias no tegumento e muito menos isolar e elucidar as substâncias na pele da rã, que são importantes para o entendimento das bases da associação sapo-formiga. Desta forma, o objetivo geral da presente tese foi investigar o perfil químico dos metabólitos secundários no tegumento de *L. lineatus*. No **primeiro capítulo**, investigamos o estado da arte acerca da biologia de interação entre anuros e invertebrados. Nós verificamos que, até o momento, há registros de associações anuro-escorpião, anuro-formiga e anuro-aranha e que em todas estas associações sugerem o uso de sinal químico entre a rã e o organismo parceiro, porém de todos os estudos avaliados, apenas um apresentou a descrição da substância que permite a coexistência. Observamos ainda que, para a grande maioria dos registros, a relação ecológica entre os organismos não foi avaliada ou definida. No **segundo capítulo**, avaliamos o uso de anestésicos a base de benzocaína como protocolo de morte seguro em anuros para o uso em análises de composição química ou bioensaios. Trazemos evidências robustas, utilizando técnicas espectroscópicas, espectrométricas e quimiométricas, de que não ocorre conversão da benzocaína presente no anestésico para o tegumento de *L. lineatus* após aplicação diretamente na boca, sugerindo que seu uso seja seguro e não ocasione impacto negativo em estudos que utilizem extratos da pele de animais mortos sob este protocolo. No **terceiro capítulo**, nós avaliamos diferenças entre o perfil químico de metabólitos secundários nas peles de indivíduos de *L. lineatus* em função as áreas de ocorrência. Utilizamos técnicas cromatográficas, espectroscópicas e quimiométricas e mostramos que ocorre variação geográfica no perfil de metabólitos secundários nos extratos de pele

dos indivíduos avaliados e ainda, verificamos que, além da variação geográfica, ocorre variação do perfil químico em função do tipo de ambiente, sendo mais diversos o perfil químico em indivíduos coletados em áreas de floresta primária. No **quarto capítulo**, utilizamos técnicas cromatográficas e espectroscópicas para isolar e elucidar uma substância presentes em extratos metanólicos feitos a partir das peles da rã *L. lineatus.* Nossas análises sugerem tratar-se de um esteroide, o colesterol. Sugerimos que este esteroide seja utilizado como precursor de substâncias que medeiam a associação com formigas *Atta*.

ABSTRACT

Anurans present a wide variety of defense strategies, whether visual, chemical, or behavioral, such as non-exclusive breeding in aquatic environments. Unusually, some species have used a defense mechanism considered rare, which is the habit of living with invertebrates. In general, the ecological aspects involved in these interactions are still not understood. *Lithodytes lineatus* is a frog that has a known distribution for South American countries and has been considered by many researchers to be of low detectability. This characteristic may be associated with the unusual habit that this species has of living next to leafy ants (*Atta* spp.). It is suggested that the association with *Atta* ants is mediated by chemical substances in the frog's skin, however, aspects of the chemical ecology of *L. lineatus* remain unknown. Until now, no study has attempted to investigate patterns of substance composition in the integument, let alone isolate and elucidate the substances in the frog's skin, which are important for understanding the bases of the ant-frog association. Thus, the general objective of this thesis was to investigate the chemical profile of secondary metabolites in the *L. lineatus* integument. In the **first chapter**, we investigate the state of the art about biology of interaction between frogs and invertebrates. We found that, to date, there are records of associations of anuran-scorpion, anuran-ant, and anuran-spider and that in all these associations suggest the use of a chemical signal between the frog and the partner organism, however from all the studies evaluated, only one presented the description of the substance that allows coexistence. We also observed that, for the vast majority of records, the ecological relationship between the organisms has not been assessed or defined. In **the second chapter**, we evaluated the use of benzocaine-based anesthetics as a safe death protocol in frogs for use in chemical composition analyzes or bioassays. We bring robust evidence, using spectroscopic, spectrometric, and chemometric techniques, that there is no conversion of the benzocaine present in the anesthetic to the *L. lineatus* integument after application directly in the mouth, suggesting that its use is safe and does not cause a negative impact in studies using extracts from the skin of animals killed under this protocol. In the **third chapter**, we evaluated differences between the chemical profile of secondary metabolites in the skins of individuals of *L. lineatus* according to the areas of occurrence. We used chromatographic, spectroscopic, and chemometric techniques and showed that there is a geographic variation in the profile of secondary metabolites in the skin extracts of the individuals evaluated. We also found that in addition to the geographic variation there is variation in the chemical

profile depending on the type of environment, the most diverse being the chemical profile in individuals collected in primary forest areas. In the **fourth chapter**, we use chromatographic and spectroscopic techniques to isolate and elucidate a substance present in methanolic extracts made from the skins of the frog *L. lineatus*. Our analyzes suggest that it is a steroid, cholesterol. We suggest that this steroid be used as a precursor to substances that mediate the association with *Atta* ants.

SUMÁRIO

LISTA DE TABELAS

LISTA DE FIGURAS

CAPÍTULO 2

A. galactonotus; and **LL** corresponds to extracts of *L. lineatus*. Gray rectangles describe the signals belonging to the benzocaine substance.

Figure 7. Cluster dendrogram of the spectra of methanolic and dichloromethanic extracts of *Lithodytes linetaus* and the Benzotop© product. In the image: **BEN** = corresponds to the extract of Benzotop©; **DCM** = correspond to the dichloromethanic extracts of *L. lineatus*; **MET** = correspond to the methanolic extracts of *L. lineatus*.

Figure 8. Cluster dendrogram of the spectra of methanolic extracts from the *Lithodytes linetaus* and *Adelphobates galactonotus* species and the dichloromethanic extract from the Benzotop© product. In the image: **BEN** = corresponds to the Benzotop© extract; **LL** = correspond to the *L. lineatus* extracts; **AG** = correspond to the *A. galactonotus* extracts.

Figure 9. Mass spectra of the frog *Lithodytes lineatus* skin extracts (dichloromethanic and methanolic). Gray rectangles describe the region belonging to the benzocaine substance mass (166 m/z).

CAPÍTULO 3

Figure 1. Sampling areas of *Lithodytes lineatus* individuals. CSM = Cruzeiro do Sul Municipality; CSP = Chandless State Park; RBM = Rio Branco Municipality; RJR = Right Jaci River; MNP = Mapinguari National Park. Sources: Forest types (MMA 2006); Foreste loss (INPE 2020).

Figure 2. Chemical profile of methanolic extracts from the skins of *Lithodytes lineatus* collected in Acre using various chemical developers. Values at the top of the silica gel chromatoplates 365 and 254 correspond to the frequencies of the physical UV (nm) developers. Values at the base of the chromatoplates correspond to the locations: 1 = Cruzeiro do Sul; 2 = Rio Branco; 3 = Chandless.

Figure 3. Chemical profile of methanolic extracts from the skins of *Lithodytes lineatus* from Rondônia using three chemical developers. Values at the top of the silica gel chromatoplates 365 and 254 correspond to the frequencies of the physical UV (nm)

developers. Values at the base of the chromatoplates correspond to the locations: 1 = Mapinguari - left of the Madeira River; 2 = Jaci - right of the Madeira River.

Figure 4a. Comparison of ^{1}H Nuclear Magnetic Resonance Spectra of methanolic extracts of *Lithodytes lineatus* individuals collected in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray - Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

Figure 4b. Magnification in the methyl region of the ^{1}H Nuclear Magnetic Resonance Spectra of *Lithodytes lineatus* methanolic extracts in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray - Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

Figure 4c. Magnification in the methyl region of the ^{1}H Nuclear Magnetic Resonance Spectra of *Lithodytes lineatus* methanolic extracts in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray - Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

Figure 4d. Magnification in the methyl region of the ^{1}H Nuclear Magnetic Resonance Spectra of *Lithodytes lineatus* methanolic extracts in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray - Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

Figure 4e. Magnification in the methyl region of the ^{1}H Nuclear Magnetic Resonance Spectra of *Lithodytes lineatus* methanolic extracts in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray

- Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

Figure 5. Hierarchical ordering of clusters graph of the entire spectra of the methanolic extracts of *Lithodytes linetaus*. As shown: CSP = Chandless State Park - AC; CSM = Cruzeiro do Sul Municipality - AC; RBM = Rio Branco Municipality – AC; MNP = Mapinguari National Park - AM; RJR = Right Jaci River - RO.

CAPÍTULO 4

Figura 1. Cromatoplacas de sílica gel contendo amostra da *Substância I* (fração 5-6) submetida ao processo de cromatografia em camada delgada (CCD) utilizando o reagente de Dragendorff.

Figura 2. Espectro de RMN de ^{1}H da *Substância I* ($CDCl_3$, 300 MHz).

Figura 3. Espectros bidimensionais de COSY, HSQC e HMBC, respectivamente, utilizados para realizar as correlações.

Figura 4. Espectro de RMN de ^{13}C da *Substância I* ($CDCl_3$, 75 MHz).

Figura 5. Espectros DEPT 90 e 135 da *Substância I.* Na imagem: DEPT 90, sinais positivos representam associações CH; DEPT 135, sinais positivos representam CH_3 e sinais negativos representam CH_2.

Figura 6. Estrutura da molécula de colesterol isolada de extratos metanólicos da pele de *L. lineatus*.

INTRODUÇÃO GERAL

Anuros apresentam uma gama de estratégias de defesa, que podem variar desde o desenvolvimento de habilidades como mimetismo, camuflagem, tanatose e inflar o corpo (Toledo *et al.*, 2011) até mudanças comportamentais, como exemplo, hábitos reprodutivos não totalmente restritos ao ambiente aquático (Malagoli *et al*., 2021). A utilização de modos reprodutivos diferentes tem se mostrado eficiente no estabelecimento de diversas espécies de anuros, uma vez que diminui tanto o risco de predação quanto a competição por recursos (Malagoli *et al*., 2021).

Outra linha de defesa em anuros é mediada por um sistema robusto de substâncias químicas no tegumento (Daly, 1995). De modo geral, é sabido que anuros produzem uma grande variedade de substâncias com propriedades bioativas no tegumento, como proteínas, enzimas, aminas biogênicas, peptídeos, dentre outras (Bevins e Zasloff, 1990; Daly, 1995). Uma vez que existe a produção de um grande arsenal de substâncias na pele, a utilização de moléculas para defesa não é surpresa (Daly, 1995). A maioria das espécies de anuros viventes não possuem aparelho inoculador de veneno, dessa forma, a defesa química ocorre de maneira passiva (Pough *et al*., 2008). Recentemente, Jared *et al*. (2015), mostraram pela primeira vez a presença de duas espécies de anuros, *Corythomantis greeningi* e *Aparasphenodon brunoi*, com capacidade para introduzir veneno. Essa habilidade mostra que algumas espécies estão adaptando suas linhas de defesa química da forma passiva para a ativa.

Independentemente do tipo de defesa química (ativa ou passiva), as substâncias presentes no tegumento de anuros apresentam diferentes rotas de biossíntese, podem ser produzidas diretamente no organismo do animal, através do processo de síntese "*de novo"* (Smith *et al.*, 2002) ou, como no caso dos alcaloides, sequestrada a partir da dieta (Daly *et al.*, 1994; Darst *et al*., 2005; Saporito *et al*., 2009; Saporito *et al*., 2012). No caso do sequestro de substâncias a partir da dieta o mais conhecido são os alcaloides. As famílias Dendrobatidae, Mantellidae, Myobatrachidae, Bufonidae e mais recentemente, Eleutherodactylidae, apresentam representantes com habilidade para sequestrar alcaloides a partir de artrópodes como formigas e ácaros, que por sua vez assimilam os alcaloides das plantas (Rodriguez *et al*., 2011; Saporito *et al*., 2012; Hantak *et al.*, 2013). No extremo deste tipo de adaptação as espécies de Myobatrachidae não apenas sequestram alcaloides (pumiliotoxinas), mas de modo singular, sintetizam alcaloides diretamente no seu organismo (pseudophrynaminas) e até o presente momento, são os

únicos "*poison frogs*" que sequestram e sintetizam alcaloides (Smith *et al.,* 2002). Essa "habilidade" de converter os alcaloides para proteção é conhecida como uma defesa química, uma vez que as espécies que utilizam esse tipo de estratégia tornam-se tóxicas e/ou impalatáveis para predadores potenciais e obtêm proteção contra patógenos no ambiente (Bevins e Zasloff, 1990; Daly, 1995; Williams *et al*., 2000; Rodriguez *et al*., 2011; Saporito *et al*., 2012; Hantak *et al.*, 2013).

O uso de substâncias na pele por anuros não está restrita a defesa contra predadores e patógenos no ambiente. De maneira mais incomum, algumas espécies começaram a desenvolver *parcerias* com invertebrados para proteção (Cocroft e Hambler,1989; Rödel e Braun, 1999). Esse tipo de estratégia de defesa é bem peculiar, uma vez que muitas dessas associações ocorrem entre anuros e predadores potenciais (Menin *et al*., 2005). Grande parte dos estudos sugerem o uso de registros químicos para firmar estas coexistências, porém, poucos são os trabalhos que tentaram evidenciar a identidade química desses sinais (Rödel *et al*., 2013). Apesar da quantidade limitada de estudos envolvendo ecologia química entre anuros e invertebrados é possível evidenciar que a maneira de utilizar as substâncias no tegumento têm sofrido alteração. Entender, por exemplo, como o ambiente ajuda a selecionar o perfil de substâncias na pele das espécies de anuros é fundamental para que possamos entender melhor a biologia da interação entre estes organismos. É possível evidenciar então que as substâncias no tegumento de anuros são utilizadas também para comunicação e que a disposição geográfica das espécies condiciona a variedade química.

Alguns trabalhos têm mostrado que ocorre variação geográfica no tipo, na quantidade e no número de alcaloides, não somente entre populações de anuros, mas também, variação na composição individual de espécimes dentro de uma mesma população (Clark *et al.,* 2006; Saporito *et al*., 2006; Saporito *et al*., 2007; Daly *et al*., 2008). Em *Oophaga pumilio* (Anura: Dendrobatidae) foi encontrada diferença na composição de alcaloides em relação ao sexo (Saporito *et al.,* 2009). No bufonídeo *Melanophryniscus moreirae* além da variação na produção de alcaloides entre os sexos foi encontrada variação em função do tamanho do corpo e da idade dos indivíduos (Jackel *et al*., 2015).

Com relação a compostos, que não sejam alcaloides, presentes na secreção de anuros, alguns estudos demonstram que também existe variação entre os sexos e na quantidade destes compostos produzidos. Existe variação ontogenética na composição e quantidade de toxinas nas glândulas paratoides do sapo *Rhinella marina* (Hayes *et al.,*

2009). Na perereca *Litoria splendida* foram observadas diferenças na composição de peptídeos entre machos e fêmeas (Wabnitz *et al.*, 2000) e uma grande variedade de espécies de anuros possuem odores indicando a presença de compostos voláteis na pele, utilizados para comunicação (Cummins e Bowie, 2011; Brunetti *et al*., 2015).

A comunicação química ocorre quando tanto o sinalizador quanto o receptor, utilizam especializações para informar positiva ou negativamente alguma ação (Smith *et al.*, 2004; Belanger e Corkun, 2009; Cummins e Bowie, 2011; Poth *et al*., 2012; Brunetti *et al*., 2015). Este tipo de comunicação tem um caráter importante, pois engloba uma série de traços comportamentais, como no comportamento de corte (macho-fêmea), no comportamento territorial (macho-macho), na atração direta das fêmeas e na relação predador-presa (Smith *et al.*, 2004; Belanger e Corkun, 2011; Cummins e Bowie, 2011; Maag *et al*., 2012; Poth *et al*., 2012; Brunetti *et al*., 2015).

Algumas espécies de girinos utilizam feromônios aquáticos (feromônios de alarme) para evitar a predação e/ou alertar indivíduos da mesma espécie que o predador está em atividade de forrageio. A percepção destes registros de alarme auxilia na tomada de decisões comportamentais desses indivíduos. Feromônios podem ser emitidos pelas larvas que foram atacadas e que estão com algum tipo de laceração (Fraker *et al*., 2009; Manteifel e Kiseleva, 2011; Maag *et al*., 2012). Este tipo de mecanismo de defesa (individual e/ou social) não está presente em todas as espécies de anuros (Summey e Mathis, 1998; Schoeppner e Relyea, 2005). Estudos com a perereca *Litoria splendida* mostraram que os machos utilizam um feromônio (splendiferina) para atrair as fêmeas conspecíficas e que a taxa ou a quantidade deste feromônio aumenta durante o período reprodutivo em relação à quantidade total de peptídeos na pele do animal, e que após o período reprodutivo há um decréscimo considerável em sua produção (Wabnitz *et al.*, 2000).

De modo geral, feromônios são utilizados na informação quimio-sensorial e na comunicação entre indivíduos pertencentes à mesma espécie. Os feromônios são substâncias que podem ser voláteis, não voláteis e solúveis em meio aquoso. Até o momento, a maioria dos feromônios conhecidos em anfíbios anuros são basicamente proteínas e peptídeos sendo esta característica esperada, uma vez que para muitas espécies de anuros têm sido descritas uma alta concentração proteica na secreção cutânea (Wabnitz *et al.*, 2000; Prates *et al*., 2012).

Desta forma, pode-se concluir que estudar aspectos químicos em anuros é de suma importância para entender mecanismos ecológicos que norteiam diversas espécies.

Biologia da interação entre a mãe do sauveiro (*Lithodytes lineatus*) e as formigas saúvas (*Atta* spp.)

Até o presente momento, *Lithodytes lineatus* é a única espécie de anuro nas Américas que tem sido reportada se associando a *Atta* (Schlüter, 1980; Schlüter e Regös, 1981; Lamar e Wild, 1995; Schlüter *et al*., 2009, de Lima Barros *et al*., 2016**a,b**). O tipo de relação ecológica entre *L. lineatus* e as formigas *Atta* ainda não é bem entendido. São claras as vantagens para a rã nessa associação, mas não são conhecidos benefícios aparentes para as formigas em tê-la como parceira de ninho. Esse fato nos leva a suspeitar que se trate de uma relação comensal, porém, não se sabe se esses indivíduos se alimentam das formigas residentes, o que o tornaria um parasita nos ninhos. Sugerimos algumas possíveis explicações para as formigas tolerarem a presença da rã, caso seja uma relação mutualística. Ninhos de formigas geralmente são utilizados por outros invertebrados, dessa forma, e como também sugerido por Schlüter e Rëgos (1981), *L. lineatus* poderia estar se alimentando destes indivíduos controlando o número de coabitantes do ninho. Outra possível explicação seria que *L. lineatus* estaria se alimentando dos inimigos naturais de formigas saúvas, como cupins, por exemplo, reduzindo a competição entre estes organismos. Estudos acerca da biologia da interação entre *L. lineatus* e as formigas *Atta* ainda não haviam sido realizados.

de Lima Barros *et al*. (2016), realizaram bioensaios em campo, para examinar como a rã *L. lineatus* permanece nos ninhos de *Atta sexdens* e *A. laevigata*, sem ser atacada. Seus resultados mostraram que nem o parentesco genético e nem a semelhança fenotípica de outras espécies de anuros (não associadas à *Atta*) influenciaram na resposta das formigas. Todas as espécies de anuros testadas foram atacadas agressivamente, porém, nenhum indivíduo de *L. lineatus* sofreu ataque. De modo similar, os indivíduos do sapo *R. major* que foram embebidos com os extratos da pele de *L. lineatus* não foram atacados pelas formigas. Esse resultado suporta a hipótese de que substâncias químicas presentes na pele da rã *L. lineatus* são responsáveis por inibir a resposta agressiva das formigas *Atta*. Porém, nesse estudo não foram descritas quais as biomoléculas encontram-se envolvidas no processo de associação.

Formigas *Atta* têm sido descritas utilizando feromônios para desempenhar diversas funções dentro das colônias (Della Lucia, 2011). Estes feromônios têm sido utilizados na identificação e diferenciação de intrusos e conspecíficos, marcação de trilhas, territorialidade e alarme contra potenciais ameaças (Hölldobler e Wilson, 1986;

Whitehouse e Jaffe, 1995; Hernández *et al*., 2002; Hernández *et al.*, 2006). É conhecido que estes feromônios são produzidos e armazenados em glândulas especializadas que estão dispersas no corpo das formigas (Hölldobler e Wilson, 1986; Whitehouse e Jaffe, 1995; Hernández *et al*., 2002; Hernández *et al.*, 2006).

A comunicação química em formigas *Atta* desempenha importante papel dentro do desenvolvimento da colônia. Essa comunicação pode ocorrer por contato, trofalaxia ou utilização de compostos químicos (Hölldobler e Wilson, 1986; Whitehouse e Jaffe, 1995; Hernández *et al*., 2002; Hernández *et al.*, 2006; Della Lúcia, 2011). Muitos dos feromônios utilizados para desencadear ações comportamentais vitais para a manutenção da colônia são formados por substâncias não voláteis, porém várias substâncias voláteis também foram descritas (Hölldobler e Wilson, 1986; Whitehouse e Jaffe, 1995; Hernández *et al*., 2002; Hernández *et al.*, 2006; Della Lúcia, 2011). Um dos feromônios de alarme bastante conhecido para *Atta* é produzido por indivíduos de *A. sexdens*. É conhecido para essa espécie que quando a cabeça destes indivíduos é esmagada, ocorre liberação de um odor semelhante a limão (por este motivo esta espécie é conhecida como saúva-limão). Deste modo, o saber a respeito da comunicação em formigas *Atta* é importante para traçar estratégias para entender quais substâncias químicas (voláteis e/ ou não voláteis) podem estar envolvidas na associação com *L. lineatus*. Do mesmo modo, o fato de já terem sido descritas as estruturas e funções de alguns feromônios utilizados por espécies de *Atta* (Della Lucia, 2011) possibilita um melhor entendimento de qual potencial sinal químico a rã está utilizando.

Schlüter *et al*. (2009) tem sugerido que *L. lineatus* possa estar utilizando algum composto químico semelhante ao utilizado por *Atta* e, por este motivo, permanece ileso dentro dos ninhos. De certo modo, *L. lineatus* apresenta forte odor quando manuseado, indicando a presença de compostos voláteis na pele (observação pessoal). Característica semelhante foi descrita por Schlüter e Rëgos (1981), que registraram odor aromático em todos os indivíduos capturados nas entradas dos ninhos de *Atta cephalotes*. Prates *et al*. (2012), descreverem o perfil molecular da secreção de *L. lineatus* e os resultados sugerem que seja altamente proteico, porém como o estudo teve como objetivo descrever o posicionamento das glândulas serosas no animal, a determinação dos compostos presentes na secreção cutânea da rã ainda é desconhecida.

Os trabalhos envolvendo a descrição de defesas químicas em sapos têm sido direcionados para evidenciar a variação (geográfica e/ou individual) na composição de alcaloides, uma vez que esta abordagem tem sido sugerida apenas para anuros

conhecidos como "*poison frogs*". Porém, assim como o sapo *Phrynomantis microps*, que utiliza peptídeos na pele como defesa para inibir a resposta agressiva das formigas na África (Rodel *et al* 2013), foi demonstrado que *L. lineatus* possivelmente também utiliza defesa química para permanecer nos ninhos de *Atta* (de Lima Barros et al., 2016), dessa forma, nesta tese utilizamos este indivíduo como objeto focal.

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OBJETIVOS

O principal objetivo desta tese foi descrever aspectos da Ecologia Química da rã *Lithodytes lineatus* e dar suporte para características relacionadas à história natural da espécie.

Capítulo 1. Determinar o estado da arte acerca das interações entre anuros e invertebrados para evidenciar potenciais lacunas no conhecimento e realizar sugestões para esta linha de pesquisa.

Capítulo 2. Avaliar se a técnica utilizada como protocolo de morte em anuros é segura para quem trabalha com extratos feitos da pele destes animais, principalmente análises de composição química e bioensaios.

Capítulo 3. Testar se a composição dos metabólitos secundários presente em extratos de pele da rã *Lithodytes lineatus* variam em função do ambiente e área geográfica.

Capitulo 4. Isolar e elucidar substâncias a partir de extratos de pele da rã *L. lineatus* que possam dar indícios de qual potencial sinal químico é utilizado para coexistir com formigas *Atta*.

CAPITULO 1

"Living in danger": a review on the interaction biology between frogs and invertebrates. Em revisão na revista **South American Journal of Herpetology.**

"LIVING IN DANGER": A REVIEW ON THE INTERACTION BIOLOGY BETWEEN FROGS AND INVERTEBRATES

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ABSTRACT

Records of anuran-invertebrate associations are uncommon regarding the global frog species diversity. Here, we performed a literature review on these associations and presented data regarding the ecological relationship involving these organisms. Our results show that the majority of known frog-invertebrate associations involve fossorial microhylid species sharing burrows with spiders. In general, it is supported that these interactions are mediated by chemical traits present in the frogs' skins that are used for recognition by the nestmates. We verified that there is a need for investments in studies aimed at detecting the chemical signal that mediates associations between frogs and invertebrates so that it is possible to determine the potential ecological relationships between these organisms.

Keywords: Anurans, Biology of interaction, Ecological relationship, Invertebrates.

INTRODUCTION

Anuran amphibians have a great diversity of predators, vertebrates (Lingnau and Di-Bernardo, 2006; Polo-Cavia et al., 2010; Garbino et al., 2020), and invertebrates (Villa et al., 1982; Vonesh, 2000; Luiz et al., 2013). A part of them is strongly dependent on water bodies for reproduction, which makes spawning and tadpoles potential targets for aquatic predators (Buxton and Sperry, 2017). Tadpoles of same species use aquatic pheromones (alarm pheromones) to prevent predation and/or alert individuals of the same species against predators' attack. The perception of these predator's alarm (or keromones) is relevant to defensive decisions. These chemical signals are pheromones emitted by the larvae that were attacked and that have some type of laceration (Fraker et al., 2009; Manteifel and Kiseleva, 2011; Maag et al., 2012). Despite being efficient, it is known that this type of defense mechanism (individual and/or social) is not present in all anuran species (Summey and Mathis, 1998; Schoeppner and Relyea, 2005), but it is an important strategy for the survival of larvae, since parental care is rare (Beck, 1998). To reduce the risk of predation in the aquatic environment, many anuran species have developed behaviors to partially oviposit the water (Magnusson and Hero, 1991).

After the post-metamorphosis process, juveniles and adults are exposed to new predators (Garbino et al., 2020), which led them to develop a wide variety of defense behaviors and conditions to prevent predation, such as inflating the body, stretching the legs, camouflage, mimicry, and thanatosis (Toledo et al., 2011). Additionally, some species have developed the ability to sequester and convert toxins to the skin from ingesting arthropods (Daly et al., 1994; Darst et al., 2005; Saporito et al., 2009; Saporito et al., 2012). Currently, five anuran families are known to have representatives which

use defenses based on the sequestration of chemical substances througth diet. Most of the species known to sequester chemical defenses (alkaloids) from diet belong to the family Dendrobatidae, but other representatives with this capacity are disposed of in the families Bufonidae, Myobatrachidae, Mantellidae, and more recently, Eleutherodactylidae (Rodriguez et al., 2011; Saporito et al., 2012; Hantak et al., 2013). Some species of the Myobatrachidae family not only sequester alkaloids (pumiliotoxins) but also synthesize alkaloids directly in their bodies (pseudophrynaminas) and, so far, they are the only "poison frogs" that sequester and synthesize alkaloids (Smith et al., 2002).

Even with all this (chemical) defense arsenal, some anuran species have rarely developed the ability to coexist with invertebrates, further reducing the risk of predation. In this review, we compare specific characteristics of these associations to observe which potential gaps in knowledge about the biology of the interaction between frogs and invertebrates.

BIBLIOGRAPHIC SURVEY

Search for articles, short communications, or any report of associations between anurans and invertebrate species was carried out. For this work, it was not considered just a locality, but any record in any geographical area. As for indicators for the study, during the searches a "mixture" was used with the following keywords: "association", "interaction", "anurans", "invertebrates", "spiders" and "ants" (the latter two to find new records more specifically). We used Google Scholar, Scielo, and Pubmed platforms as the consulted repositories.

DESCRIPTION OF RECORDS ON THE TOPIC

We found a total of 20 records involving associations between frogs and invertebrates. Among which two (9,5%) correspond to associations between frogs and scorpions, nine (42,9%) correspond to associations between frogs and ants, and 10 (47,6%) correspond to associations between frogs and spiders.

ASSOCIATIONS BETWEEN FROGS AND SCORPIONS

Until now, there are only two known associations between frogs and scorpions. The first record was published by Rao and Ramana (1925) where they described that the microhylid frog species *Uperodon montanus* (Jerdon, 1853) during periods of drought share burrows with the black scorpion *Heterometrus fulvipes* (Koch, 1837). They observed that when disturbed in the burrow, the frog walks towards the scorpion, but is not attacked. Also, they found that when the scorpion moves, the frog crouches and remains immobile, allowing the scorpion to pass. Vyas (2010) endorsed this phenomenon after detecting the presence of *U. montanus* inside the burrow of a *Heterometrus.* Even pointing out some possible explanations involving the interaction between these animals, no study has shown evidence of the potential ecological relationship between these organisms.

ASSOCIATIONS BETWEEN ANURANS AND ANTS

Frog-ant associations are uncommon, but some records have been made involving species that occur in Africa and America. Déjean and Amiet (1992) described the presence of *Kassina senegalensis* (Duméril and Bibron, 1841) (Anura, Hyperoliidae) in the nests of the ant *Megaponera foetens* (Fabricius, 1793) in Africa. Rödel and Braun detected the presence of the species *K. fusca* (Schiøtz, 1967) and

Phrynomantis microps (Peters, 1875) (Anura, Mycrohylidae) when excavating the nests of the ants *M. foetens* and *Paltothyreus tarsatus* (Fabricius, 1798). Subsequently, Rödel and Braun (1999) carried out experiments to determine which factors are responsible for inhibiting the aggressive response of ants in the presence of frogs *K. fusca* and *P. microps*. In these experiments, in addition to *K. fusca* and *P. microps*, they presented three other anuran species (*Hemisus marmoratus* (Peters, 1854), *Ptychadena maccarthyensis* (Hallowell, 1845) and *Phrynobatrachus latifrons* (Ahl, 1924)) to the *P. tarsatus* and *M. foetens* ants, both extremely aggressive. Their results showed that all species that were not known to associate with these ants were attacked aggressively by the residents, except *K. fusca* and *P. microps*. They also observed a different behavior in *P. microps* during the examination done by the ants, where they noticed that when in contact with the residents, the frog bent down, placing his head between his arms, a behavior similar to that described by Rao and Ramana (1925) for the species of frog that cohabits scorpion burrows. Thus, it was suggested that at the moment that *P. microps* performs this movement, substances present on its skin are released, inhibiting the aggressive response of the ants and favouring the permanence of these individuals in the nests.

Rödel et al. (2013) carried out experiments to better understand the ecological relationship between the anuran *P. microps* and the ants *P. tarsatus*. For the experiment, they coated termites with substances isolated from extracts from the skin of *P. microps* and coated other termites with ultrapure water (control). Subsequently, they presented the termites to the ants in both treatments. They found that termites coated only with ultrapure water were strongly attacked, while those coated with substances isolated from the frog's skin had a great decrease in attack rates. Through their results they identified that peptides present in the frog's skin inhibit the ants' attack, characterizing the frog's

126 ability as a chemical camouflage, where ants perceive the organism, but lose the ability
127 to distinguish it as an invader.
128 In the Americas, the only species of anuran known to associate with ants is
129 *Lithodytes lineatus* (Schneider, 1799) (Anura, Leptodactylidae), which specializes in
130 cohabiting nests of leaf-cutting ants of the genus *Atta* (Fabricius, 1805) (Schlüter, 1980;
131 Schlüter and Regös, 1981; Lamar and Wild, 1995; de Lima Barros et al., 2016a). Unlike
132 the association between frogs and ants in Africa, where frogs use ants' nests only as a
133 refuge, *L. lineatus* lives and reproduces inside *Atta's* nests (Schlüter et al., 2009). *Atta*
134 ants are not carnivorous and have been described using pheromones to perform various
135 functions within the colonies (Della Lucia, 1993). These pheromones have been used in
136 the identification and differentiation of intruders and conspecifics, marking trails,
137 territoriality, and alarm against potential threats (Hölldobler and Wilson, 1986;
138 Whitehouse and Jaffe, 1995; Hernández et al., 2002; Hernández et al., 2006).
139 Schlüter et al. (2009) has suggested that *L. lineatus* may be using some chemical
140 compound similar to that used by *Atta* ants and for this reason, remains unharmed
141 within the nests. Therefore, it is possible to notice that *L. lineatus* has a strong odor
142 when handled, indicating the presence of volatile compounds on the skin (personal
143 observation). A similar characteristic was described by Schlüter and Rëgos (1981), who
144 noticed an aromatic odor in all individuals captured at the entrances of *Atta cephalotes*
145 (Linnaeus, 1758) nests. The study by de Lima Barros et al. (2016**b**), investigated the
146 potential mechanisms used by the frog to coexist with the *Atta* ants without being
147 attacked. In their experiments, they used extracts made from the skins of *L. lineatus* and
148 bathed individuals of the bufonid *Rhinella major* (Müller and Hellmich, 1936). Also, as
149 a control, other individuals of *R. major* were bathed only with ultrapure water. Their
150 results showed that none of the frogs coated with extracts made from the frog's skin

suffered attacks from the ants, but all those in the control group were attacked. Also, using phenotypically similar species and phylogenetically related to *L. lineatus*, it was observed that it is a species-specific association, since all other anurans presented to ants were quickly attacked. In this case, there is still no information about the ecological relationship involving *L. lineatus* and the *Atta* ants.

ASSOCIATIONS BETWEEN FROGS AND SPIDERS

Associations between frogs and spiders have the largest number of records, and it is quite interesting since these organisms share behavioural aspects considered non-harmonious, as they are involved in the predator-prey relationship, spiders being common items in the diet composition of several species of frogs and on the other hand, several species of spiders are considered voracious predators of anurans (Menin et al., 2005). Thus, imagining that spiders and frogs could at some point coexist would be unlikely. Blair (1936) detected the presence of nine individuals of *Gastrophryne olivacea* (Hallowell, 1856) (Anura, Mycrohylidae) sharing the same burrow with the spider *Aphonopelma hentzi* (Girard, 1852*)*. However, details about their observation were inconclusive. During a general study on *G. olivacea*, Freiburg (1951) corroborated the observation made by Blair (1936) where he suggests that this species of anuran uses active burrows of spiders and vertebrates. Subsequently, Dunndee (1999) showed even more robust evidence about the association between *G. olivacea* and *A. hentzi*. He found 22 individuals from the microhylid sharing the same burrow as a female of this spider and even noted that when threatened, the frogs entered the burrows without any impediment from the resident. Dundee et al. (2012) showed that this relationship is potentially symbiotic. They presented individuals of *G. olivacea*, *G. carolinensis* (Holbrook, 1835) (another species of the same genus), and *Acris crepitans* (Baird, 1854)

to observe the response of ants. In their results, it was possible to verify that both *G. olivacea* and *G. carolinensis* did not suffer attacks from the spiders, while some individuals of *A. crepitans* were eaten. They inferred that the presence of the frogs was tolerated by chemical records present in both species of *Gastrophryne* and that the ecological relationship is symbiotic because the frog feeds on ants that could potentially feed on the eggs of the spiders, and in return, spiders provide shelter and protection for frogs.

Cocroft and Hambler (1989) detected the presence of the microhylid frog *Chiasmocleis ventrimaculata* (Andersson, 1945) cohabiting nests of the theraphosid spider *Xenesthis immanis* (Ausserer, 1875). They conducted experiments to understand the biology of the interaction between these two organisms. Initially, they monitored the spider's burrows to observe the pattern of sharing the nests with the microhylid frogs. They found that frogs emerged from the spiders' burrows and sometimes also emerged together with the spiders at certain times of the day, reinforcing the spiders' non-attack on the frogs. Subsequently, they performed experiments to observe the response of spiders to other frog species (*Rhinella marina* (Linnaeus, 1758), *Boana fasciata* (Günther, 1858), *Pithecopus palliatus* (Peters, 1873), *Pristimantis peruvianus* (Melin, 1941), *Leptodactylus wagneri* (Peters, 1862), and *Engystomops petersi* (Jiménez de la Espada, 1872)) belonging to three large families of anurans (Bufonidae, Hylidae e Leptodactylidae). Among the species evaluated, they found that only one (*E. petersi*) was not predated after being captured, with all other species being captured by the spiders and subsequently eaten. These results are important, as it supports the existence of recognition in the form of chemical records present on the skin of *C. ventrimaculata*. In this association, the authors were unable to show any potential benefit for spiders in having a frog as a nesting companion, therefore, since for the frog this association is

advantageous (using the burrow for protection) the authors considered it to be a commensal relationship. For the result observed in *E. petersi*, it was assumed that this individual has skins' substances that are unpalatable to spiders. A similar result was observed by Csakany (2003), who found that another species of theraphosid spider, *Pamphobeteus* sp. (Pocock, 1901), did not feed on *C. ventrimaculata*, also suggesting a commensal relationship and the use of chemical recognition between the frog and the spider.

Another record of the commensal relationship between spiders and frogs was made by Siliwal and Ravichandran (2008) where they observed the microhylid frog *Uperodon taprobanicus* (Parker, 1934) sharing holes in tree trunks with the spider *Poecilotheria hanumavilasumica* (Smith, 2004). Once again, the evidence in his observations points to an association mediated by chemical recognition.

Karunarathna and Amarasinghe (2009) presented evidence of a supposed mutualistic association between *Uperodon nagaoi* (Manamendra-Arachchi and Pethiyagoda, 2001) (Anura, Microhylidae) and two species of spiders also of the genus *Poecilotheria* (*P. ornata* (Pocock, 1899) and *P. subfusca* (Chamberlin, 1917)). They found many tree holes cohabited by both species and the presence of eggs and tadpoles of *U. nagaoi* and juveniles of species of *Poecilotheria*. It's known that juveniles of *Poecilotheria* are highly predated, even by ants, and ants being one of the prey items identified for *U. nagaoi*. In this case, it is suggested that there is an exchange of benefits where the frog protects juveniles from spiders; and spiders provide shelter and protection for eggs, tadpoles, and adults. The same evidence was shown by Karunarathna et al. (2012) to evidence the association between the microhylid frog *Uperodon taprobanicus* and *Poecilotheria* spiders.

Recently, Bascoulès and Smith (2021) observed individuals of *Chiasmocleis albopunctata* (Boettger, 1885) (Mycrohylidae) in burrows of *Eupalaestrus campestratus* (Simon, 1891) (Theraphosidae) in Paraguay. It was observed that when feeling threatened, the frogs entered the burrows. Subsequently, an individual of *E. campestratus* emerged at the entrance of the burrow, demonstrating that both shared the same space. Furthermore, the authors observed that when close, the spiders have body contact with the frogs without attacking them.

FINAL CONSIDERATIONS

It is possible to observe that most of the interactions between anurans and invertebrates involve microhylid species (Table 1). Although these observations are well known, it is evident that clearer aspects regarding the ecological relationships between these organisms are still not well understood, especially for the anuran-ant and anuran-scorpion associations (Table 1). It was observed that all studies support the use of chemical records for recognition among nestmates showing the importance of research that addresses the identification of the chemical components that mediate these associations and that could give evidence of more intrinsic aspects of the life history of these species. Likewise, some of the research found does not have the central objective of understanding parameters of behavioural and chemical ecology among the species that live together, it is important to carry out future studies that address this issue in order to be able to have an evolutionary approach.

Table 1. Species of anurans and invertebrates recorded in interaction events and descriptions of the potential ecological relationship between these organisms.

Family (Anuran)	Species (Anuran)	Nestmate (Invertebrate)	Common Name (Invertebrate)	Ecological relationship
Hyperoliidae	*Kassina senegalensis*	*Megaponera foetens*	Ant	Undefined
Hyperoliidae	*Kassina fusca*	*Paltothyreus tarsatus*	Ant	Undefined
Hyperoliidae	*Kassina fusca*	*Megaponera foetens*	Ant	Undefined
Leptodactylidae	*Lithodytes lineatus*	*Atta cephalotes*	Ant	Undefined
Leptodactylidae	*Lithodytes lineatus*	*Atta laevigata*	Ant	Undefined
Leptodactylidae	*Lithodytes lineatus*	*Atta sexdens*	Ant	Undefined
Microhylidae	*Uperodon montanus*	*Heterometrus fulvipes*	Scorpion	Undefined
Microhylidae	*Uperodon montanus*	*Heterometrus* sp.	Scorpion	Undefined
Microhylidae	*Phrynomantis microps*	*Paltothyreus tarsatus*	Ant	Undefined
Microhylidae	*Phrynomantis microps*	*Megaponera foetens*	Ant	Undefined
Microhylidae	*Gastrophryne olivacea*	*Aphonopelma hentzi*	Spider	Mutualism
Microhylidae	*Chiasmocleis ventrimaculata*	*Xenesthis immanis*	Spider	Commensalism
Microhylidae	*Chiasmocleis albopunctata*	*Eupalaestrus campestratus*	Spider	Mutualism
Microhylidae	*Chiasmocleis ventrimaculata*	*Pamphobeteus* sp.	Spider	Commensalism
Microhylidae	*Uperodon taprobanicus*	*Poecilotheria hanumavilasumica*	Spider	Mutualism
Microhylidae	*Uperodon nagaoi*	*Poecilotheria ornata*	Spider	Mutualism
Microhylidae	*Uperodon nagaoi*	*Poecilotheria subfusca*	Spider	Mutualism

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CAPITULO 2

Evaluation of benzocaine-based anesthetic gel in anuran skins extracts: a case study using the frog *Lithodytes lineatus* (Anura: Leptodactylidae). Publicado na revista **PLOS ONE.**

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Full Title: Evaluation of benzocaine-based anesthetic gel in anuran skins extracts: a case study using the frog *Lithodytes lineatus* (Anura: Leptodactylidae)

Short Title: Benzocaine-based anesthetic gel and anuran skins extracts

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Credit Author Statement

Conceptualization, A.L.B and A.P.L.; Formal analysis, A.L.B. and M.T.F.; Funding acquisition, A.P.L. and C.V.N.; Investigation, A.L.B and M.T.F.; Project administration, A.L.B., A.P.L., and C.V.N.; Writing - Original Draft, A.L.B and A.P. L.; Writing - review & editing, A.L.B., C.V.N., and A.P.L.

Abstract

Extracts made from the skin of dead *Lithodytes lineatus* frog individuals with the application of the benzocaine-based anesthetic gel, introduced into the oral cavity, were analyzed by ^{1}H Nuclear Magnetic Resonance to investigate whether the application of this product (oral) can make studies that use extracts from the skins of these animals unfeasible. For comparison, we used skins of another species of anuran following the same death protocol. No trace of the benzocaine substance was found in the ^{1}H-NMR spectra of the skin extracts from any of the tested anuran species. Still, using the hierarchical clustering model, it was possible to observe the formation of well-defined groups between the skin extracts of anurans and the anesthetic used to kill these animals. Our results suggest that the lethal dose of benzocaine in gel used inside the mouth of frogs may have no influence on potential results regarding the chemical composition or even bioassays using extracts made from the skin of these animals killed under this protocol since there was no detection of this substance for the analyzed samples.

Keywords: Anurans, Anesthetics, Benzocaine, Nuclear Magnetic Resonance.

1. Introduction

Studies involving analysis of the chemical composition of the cutaneous secretion of several species of anurans have been widely carried out, because it is possible to find a large number of substances with bioactive properties in the skin of these animals that present, mainly, great antimicrobial potentials [1 – 6]. There are several methods to extract the substances present in the body of frogs. One of the most used is the preparation of extracts made from the integument [7 – 9]. The method using skin extracts requires greater care during treatment in order to avoid indirect contamination of the samples during handling.

Currently, there are several debates about the use of some types of death protocols for anurans, such as, for example, cooling and freezing [10 – 12] since the perception of pain in these animals is still not well understood [13]. Thus, the use of anesthetics as a "humane" way to kill individuals in this taxonomic group has been recommended [7, 12, 14 – 17]. Benzocaine-based anesthetics are highly effective for both, anesthesia and death in amphibians, not requiring a large amount of the product to be able to kill them [12, 18 – 21]. However, recently, Saporito and Grant [22] found traces of the benzocaine substance coming from the Orajel© (liquid) product in skin extracts made from individuals of the species *Melanophryniscus moreirae* and *Lithobates clamitans* killed with the oral application of this anesthetic and concluded that the application directly in the mouth in certain species of anurans, may invalidate potential studies on the chemical composition of the extracts of these animals, signalling false positives, such as inaccurate detection of substances and/or incorrect information about potential biological activities. The authors gave as an example the study by Amézquita et al. [23] who, through experiments, suggested that some populations of the frog *Allobates femoralis* showed higher toxicity in the extracts when used in mice and

was considered by the authors to be from alkaloids present in the integument of this species. As *A. femoralis* belongs to a frog family that has no representative known for producing or sequestering diet alkaloids [24 – 28], this result was contested by Saporito and Grant [22] who described that the possible toxicity in *A. femoralis* is due to the presence of benzocaine substance that was converted to the animals' skin, detecting the presence of this substance experimentally using mass spectrometry (MS).

Studies showing the detection of substances used to kill anurans present in skin extracts of these animals are still scarce, but they are of great value since many experiments are conducted using different death protocols [12] and also, using anesthetics [7, 13 – 16].

In this study, we test whether the benzocaine used to kill individuals of the frog *Lithodytes lineatus* is transferred to the skin extracts of these animals.

2. Material and methods

2.1. Obtaining of the *Lithodytes lineatus* skin extracts and of the Benzotop© product

Individuals of *L. lineatus* collected in three locations in the Brazilian Amazon (Cruzeiro do Sul [n = 3], Chandless State Park [n = 5] and Rio Branco [n = 3], all in the state of Acre), were killed using 40 mg of benzocaine-based anesthetic gel (Benzotop© gel, DFL Indústria e Comércio S.A.) with direct application in the mouth to avoid contamination of the skins. Immediately after the deaths were confirmed the animals' skins were removed through the inguinal incision from one end of the body to the other and sampled, by location, in microtubes containing methanol (100%) and prepared for extraction. Two substance extraction systems (10 mL / g of skin) were used based on differences in polarities between the solvents: 1) dichloromethane + methanol at a concentration 9:1 (v/v), and 2) methanol (100%). The use of different extracting

101 solvents was made to be able to access different substances, potentially including, the
102 benzocaine substance. Subsequently, in each extractor system, the skins were taken to
103 an ultrasound bath (Unique©, model USC - 1800, frequency 40Hz) for 20 minutes, and
104 subsequently filtered on filter paper. This process was repeated three times, and the
105 concentrates for each extraction, by location, packed in a single bottle. The extracts
106 obtained were taken to the fume hood for total evaporation of the solvents.

107 Still, for comparison, we used skins of another species of anuran, *Adelphobates*
108 *galactonotus* (n = 3), killed with the oral anesthetic application (based on benzocaine),
109 however, disregarding the standardized amount (higher or lower) of the product used in
110 *L. lineatus* individuals. The *A. galactonotus* skins used in this study were stored in the
111 tissue bank of the Ecology Laboratory of the National Institute of Amazonian Research
112 (INPA) and were used separately to assess whether there is an individual effect and / or
113 the conservation time on the results. For analyzes that included this species of anuran,
114 only methanolic extracts were used.

115 A sample containing 10 mg of the Benzotop© gel product flavoured Pina Colada
116 was solubilized using dichloromethane (DCM) and left to dry in a fume hood for total
117 solvent evaporation. Subsequently, 10 mg of the methanolic extracts from the skins of
118 individuals of *L. lineatus* and *A. galactonotus,* as well as the diluted sample of
119 Benzotop©, were solubilized in deuterated chloroform ($CDCl_3$) containing
120 tetramethylsilane (TMS) as a reference solvent to be analyzed by the method Hydrogen
121 Nuclear Magnetic Resonance spectroscopic (^{1}H-NMR / NMR: Bruker, model Fourier
122 300, magnet 300 SB UltraShieldTM, 7.05T, 300 MHz).

123 The search for the ^{1}H-NMR spectrum of the benzocaine substance (standard)
124 was performed in the *SciFinder* repository database (Figure 1). As benzocaine is a well-
125 known substance, there was no difficulty in finding an available spectrum.

Subsequently, the spectra referring to the tested samples were compared to the standard. All samples were solubilized in chloroform for ^{1}H-NMR analysis and tetramethylsilane (TMS) was used as the reference solvent.

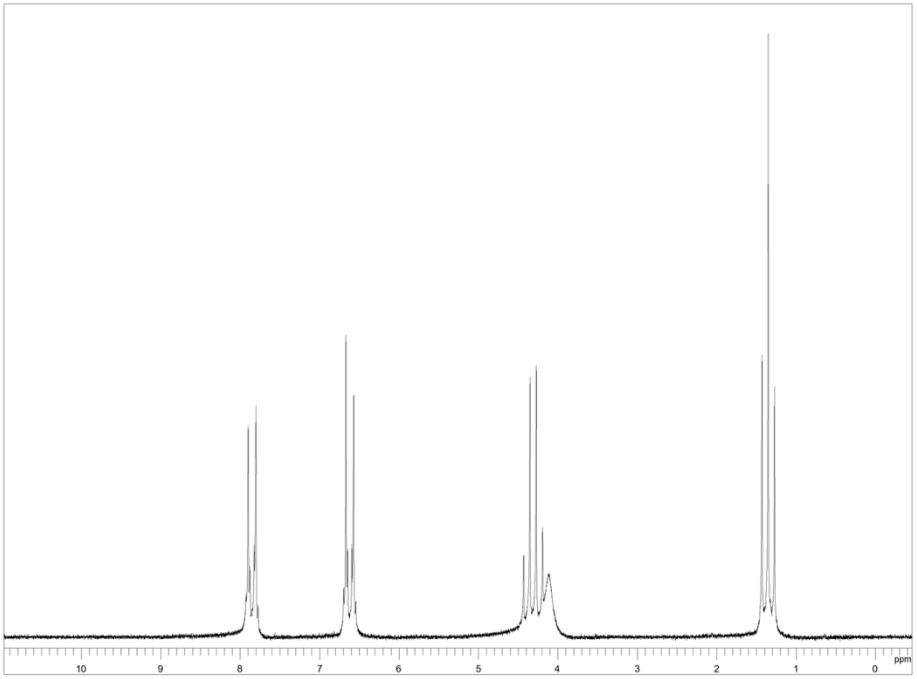


Figure 1. ^{1}H-NMR spectrum of the substance benzocaine solubilized in $CDCl_3$. Withdrawal from and available at: https://scifinder.cas.org.

Mass spectrometry analysis – MS/MS, ESI+, 50 to 300 m/z amplification, positive mode (M+H) – was used to test the presence or absence of benzocaine substance in *L. lineatus* skin samples. The presence of benzocaine in the analysed samples is confirmed by detecting mass fragments of 166 m/z (benzocaine mass at positive mode) according to *SciFinder* repository database.

2.2. Chemometric analysis

The ^{1}H-NMR spectra of extracts from frog' skins and the Benzotop© product had both phase distortions and baselines adjusted in the TopSpin program (version

142 4.0.7, Bruker Biospin) where they were automatically converted to CSV files (Comma-
143 separated values). Subsequently, the CSV files were analysed using the R Studio
144 program (R Studio, version 3.3 [29]). We evaluated potential differences or similarities
145 between the samples tested, selecting specific regions in the ^{1}H-NMR spectra to be
146 excluded from the analyses, to perform a comparison based on the chemical shifts of
147 interest, and eliminate potential noise from the sample (Table 1). In this analysis, only
148 the regions corresponding to the chemical shifts of benzocaine were maintained. In
149 total, five regions were chosen for exclusion.
150

151 **Table 1**. Regions of chemical shifts excluded from the chemometric analysis.

Number of cuts	Exclusion region (in ppm)
1	0.01 – 1.15
2	1.40 – 4.27
3	4.36 – 6.61
4	6.68 – 7.82
5	7.89 – 9.9

152

153 To highlight potential similarities or divergences between the different samples
154 analysed, we used a Hierarchical Cluster Analysis (HCA) to assess whether there is the
155 formation of groups between samples from Euclidean distances, the results of which are
156 illustrated in a dendrogram. The analyzes were performed using the software R Studio.
157

158 2.3. Ethical approval

159 The National Institute of Amazonian Research approved the experiments with
160 the frog species used in this study and the permissions for the use of animals were
161 granted by the Ethics Committee for Research in the Use of Animals (CEUA) (INPA /

CEUA, Protocol: 030 / 2014). The permissions for the animal collection were granted by the Chico Mendes Institute for Biodiversity Conservation (ICMBio / license number 57123-1). All experiments were carried out following the relevant guidelines and regulations.

3. Results

The signs referring to the benzocaine substance were detected in the sample made with the Benzotop© anesthetic and are formed by regions with signs between I) 1.33 and 1.37 ppm; II) 4.27 and 4.37 ppm; III) 6.61 and 6.66 ppm, and IV) 7.83 and 7.87 ppm (Figure 2). The other signals detected in the spectrum come from other substances that are part of the general composition of the product, besides the solvent signal.

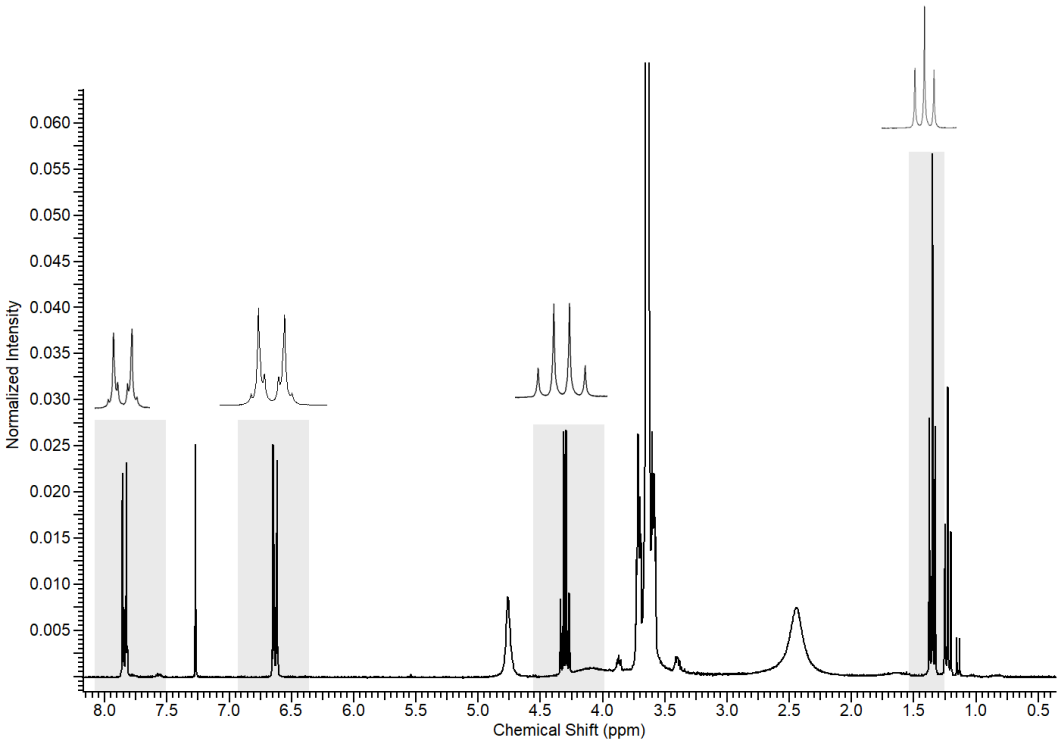


Figure 2. ^{1}H-NMR spectrum of Benzotop© dichloromethanic extract. Gray rectangles describe the signals belonging to the substance benzocaine. Above, enlargements of the signals from the highlighted regions.

3.1. Comparative analysis between the ^{1}H-NMR spectra of individuals of *Lithodytes lineatus* and the Benzotop© product

The methanolic (**MET**) and dichloromethanic (**DCM**) extracts made with the skins of individuals of *Lithodytes lineatus* were compared with the dichloromethanic extract of the Benzotop© (**BEN**) product to verify the presence or absence of characteristic signs of the benzocaine substance (Figure 3).

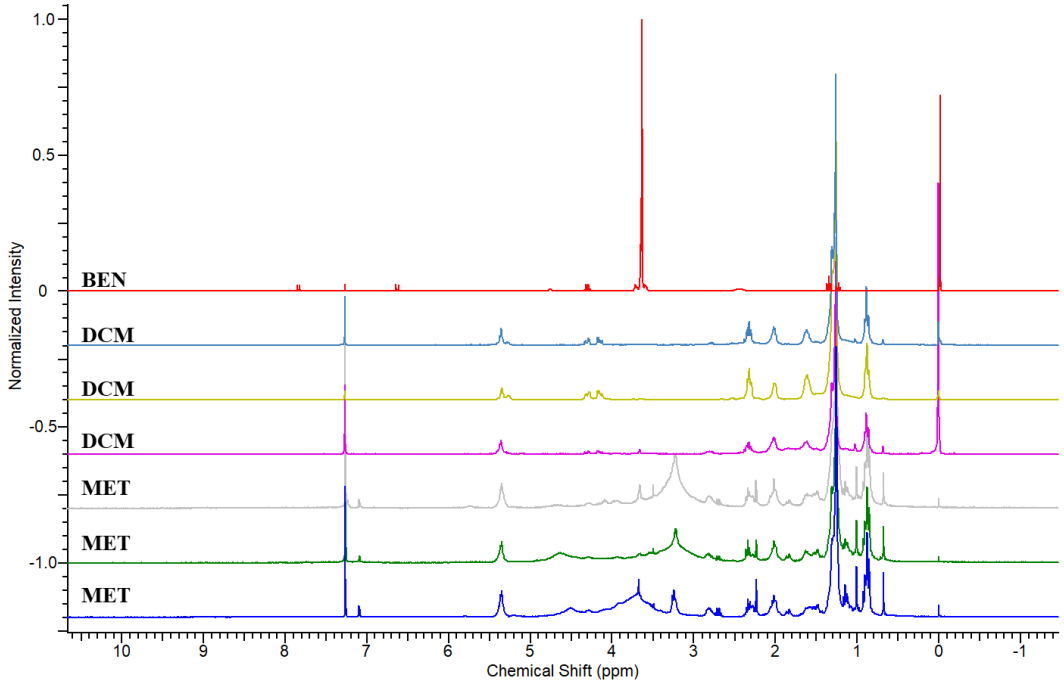


Figure 3. Overlapping ^{1}H-NMR spectra of methanolic and dichloromethanic extracts from the skins of individuals of *Lithodytes lineatus* and the Benzotop© product. In the spectrum: **BEN** corresponds to the extract of the Benzotop© product; **DCM** corresponds

to the dichloromethanic extracts of *L. lineatus*; **MET** corresponds to the methanolic extracts of *L. lineatus*.

Through the analysis of the ^{1}H-NMR spectra, it was possible to observe that **MET** presents a greater chemical complexity concerning **DCM**, clear by the superior amount of signals present in the spectra. Also, it was found that the benzocaine substance was not incorporated into the general composition of the skin extracts of *L. lineatus* individuals (Figure 4) killed with **BEN**, in neither of the two extractions, since the signs and consequently the characteristic chemical shifts of the substance in question were not detected.

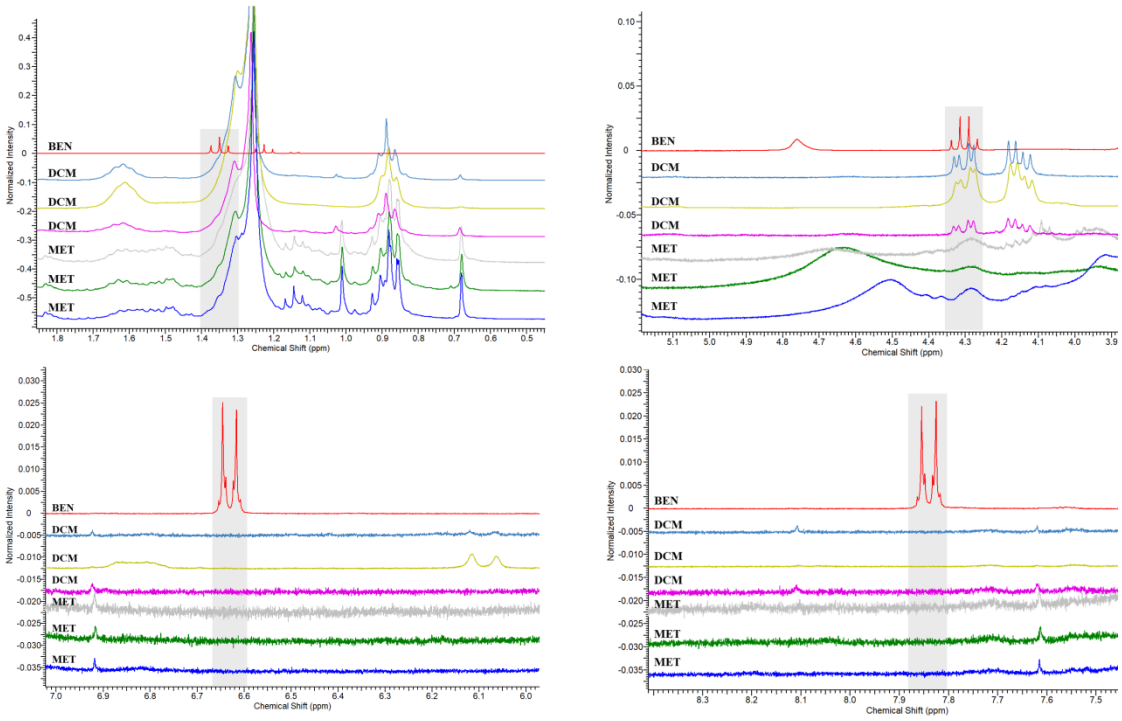


Figure 4. Magnification of the regions in the ^{1}H-NMR spectra of methanolic and dichloromethanic extracts from the skins of *Lithodytes lineatus* individuals and the Benzotop© product. In the spectrum: **BEN** corresponds to the extract of the Benzotop© product; **DCM** corresponds to the dichloromethanic extracts of *L. lineatus*; **MET**

corresponds to the methanolic extracts of *L. lineatus*. Gray rectangles describe the signals belonging to the benzocaine substance.

To validate the experiment, a comparison was made between BEN with extracts from the skin of another frog, *Adelphobates galactonotus* (**AG**), and the methanolic extracts from *L. lineatus* (**LL**) (Figure 5). The use of only *L. lineatus* methanolic extracts was due to the complexity of the samples compared to the dichloromethanic extracts, previously observed in the ^{1}H-NMR spectra (Figure 4).

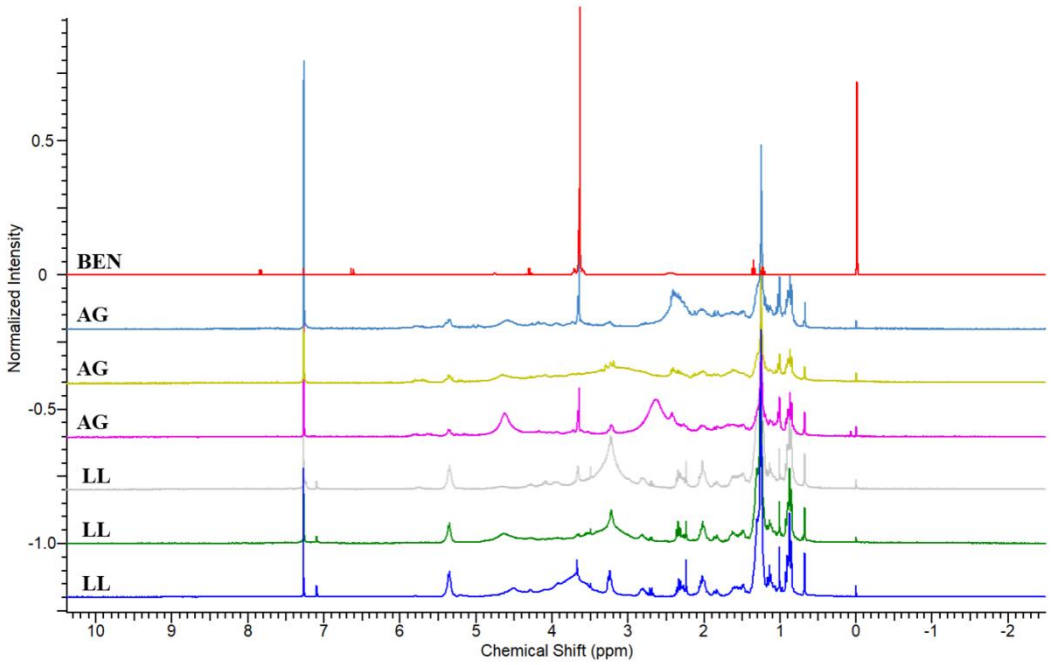


Figure 5. Overlapping ^{1}H-NMR spectra of methanolic extracts from the skins of *Lithodytes lineatus* and *Adelphobates galactonotus* individuals and the Benzotop© product. In the spectrum: **BEN** corresponds to the extract of the product Benzotop©; **LL** corresponds to *L. lineatus* extracts; **AG** corresponds to the extracts of *Adelphobates galactonotus*.

There were also no signs of chemical shifts characteristic of the substance benzocaine in the ^{1}H-NMR spectra of **AG** skin extracts. Thus, we found that there was no conversion of benzocaine to the skin extracts of either of the two tested species (Figure 6).

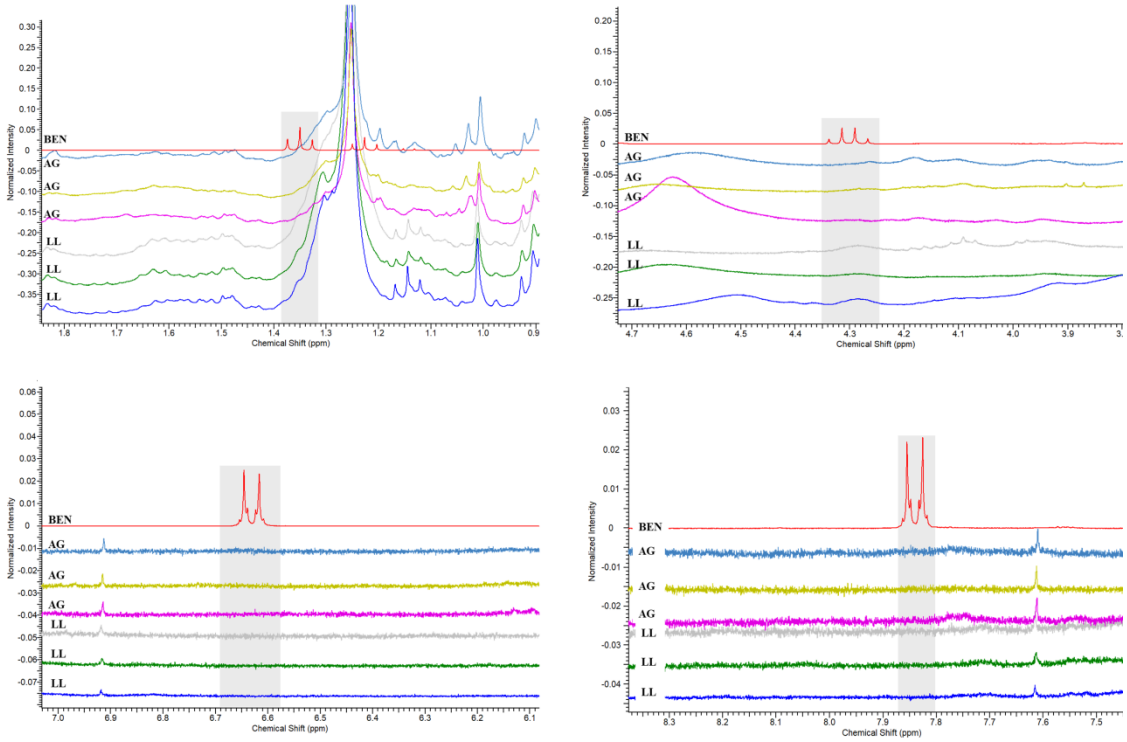
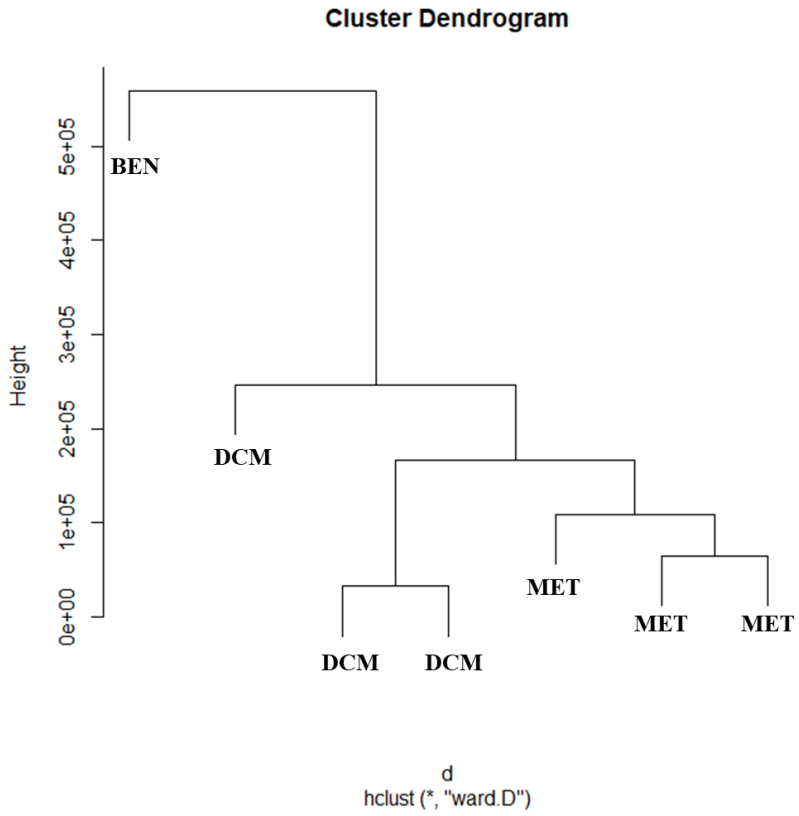


Figure 6. Magnification of the regions in the ^{1}H-NMR spectra of the methanolic extracts of the skins of *Lithodytes lineatus* and *Adelphobates galactonotus* individuals and the dichloromethanic extract of the Benzotop© product. In the spectrum: **BEN** corresponds to the extract of the Benzotop© product; **AG** corresponds to the extracts of *A. galactonotus*; and **LL** corresponds to extracts of *L. lineatus*. Gray rectangles describe the signals belonging to the benzocaine substance.

234 3.2. Hierarchical Cluster Analysis (HCA)

235 The clusters analysis associated the samples by the similarity between the
236 regions of interest (characteristic signs of the benzocaine substance). The formation of
237 two well-defined clusters between the samples was evidenced, one formed by the skin
238 extracts of *Lithodytes lineatus* (**LL**) individuals, in both extractions, and the other
239 formed by Benzotop© (**BEN**) (Figure 7).



240

241 **Figure 7**. Cluster dendrogram of the spectra of methanolic and dichloromethanic
242 extracts of *Lithodytes linetaus* and the Benzotop© product. In the image: **BEN** =
243 corresponds to the extract of Benzotop©; **DCM** = correspond to the dichloromethanic
244 extracts of *L. lineatus*; **MET** = correspond to the methanolic extracts of *L. lineatus*.

245

246 Still, another analysis of clusters was made by adding **AG** and again showed that
247 **BEN** formed a separate group in comparison to both samples, **AG** and **LL** (Figure 8).

These results reinforce that one found in the analysis of the ^{1}H-NMR spectra, where the presence of the benzocaine substance was not detected in any of the evaluated samples.

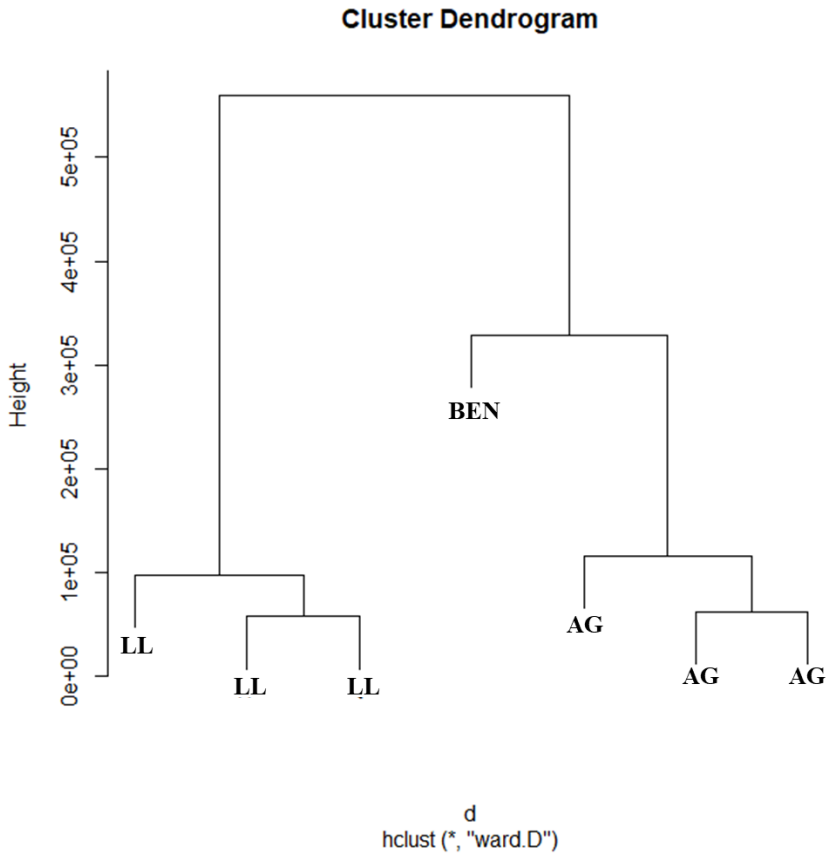


Figure 8. Cluster dendrogram of the spectra of methanolic extracts from the *Lithodytes linetaus* and *Adelphobates galactonotus* species and the dichloromethanic extract from the Benzotop© product. In the image: **BEN** = corresponds to the Benzotop© extract; **LL** = correspond to the *L. lineatus* extracts; **AG** = correspond to the *A. galactonotus* extracts.

As well as verified by ^{1}H NMR and Cluster Dendrogram analysis, benzocaine was not detected in *L. lineatus* skin extract samples by using mass spectroscopy analysis (**Figure 9**).

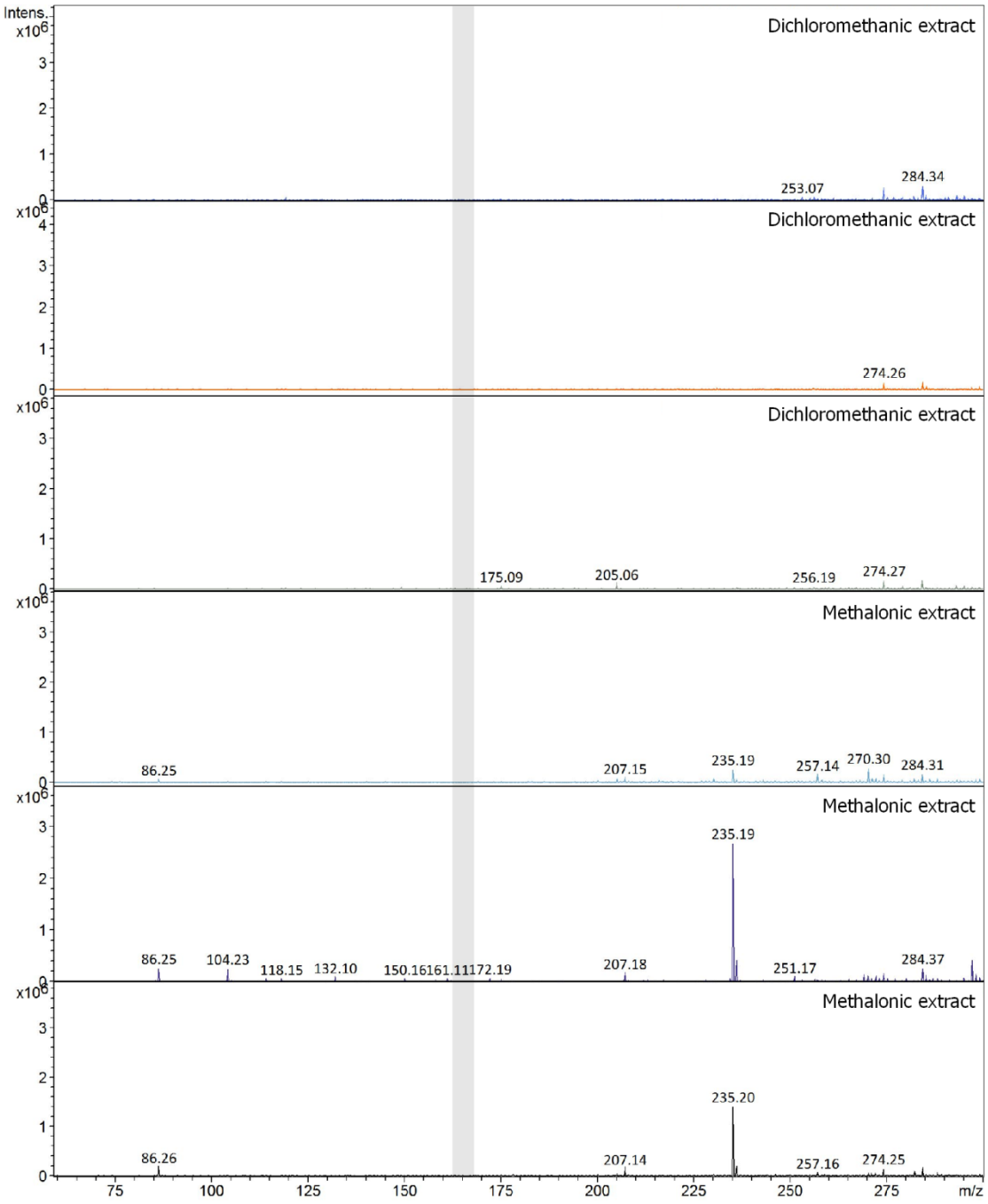


Figure 9. Mass spectra of the frog *Lithodytes lineatus* skin extracts (dichloromethanic and methanolic). Gray rectangles describe the region belonging to the benzocaine substance mass (166 m/z).

4. Discussion

The use of benzocaine-based anesthetics is considered a common practice and can be used for several purposes within herpetology. Kaiser and Green [30] observed that very low amounts of Orajel© and Anbesol© anesthetics applied to the heads of several anuran species cause temporary anesthesia, drastically reducing movement and can be used as a non-lethal method to facilitate "photographic tests" of these animals in field and laboratory. The use as an anuran death protocol is highly effective concerning lethality even when compared to other anesthetics [20, 21, 31, 32] besides to being considered a low-cost product and without restrictions on the purchase, facilitating access [12, 18]. On the other hand, the use of anesthetics to kill frogs that will subsequently have their skins removed for the preparation of extracts used in biological tests or investigation of the chemical composition present in the integument can be a problematic factor due to the potential contamination [22].

Some authors have used other anuran death protocols to avoid bias that can be caused by the use of anesthetics, such as, for example, cooling and freezing [9 – 11]. Despite eliminating the risk of skin contamination either by contact with the product or by the potential conversion of the anesthetic to the integument, the "cool and freeze" death protocol has generated many controversies in the scientific community, being considered by the American Association of Veterinary Medicine [12], as not humane and unacceptable.

The result of our study was contrary to that described by Saporito and Grant [22], in which large amounts of the benzocaine substance, of the product Orajel© liquid, were found in the skins extracts of the *Melanophrynsicus moreirae* and *Lithobates clamitans* species. We found that in none of the extracts analyzed by ^{1}H-NMR, of both species of anurans, the presence of the benzocaine substance from the anesthetic

Benzotop© in gel was detected, including in the skins (*Adelphobates galactonotus*) that were stored in tissue banks, whose lethal dosage used was indiscriminate. This allows us to infer that the use of Benzotop© in gel introduced inside the mouth (lethal dose) for anurans does not prejudice possible studies regarding the chemical composition and/or biological activity of the extracts since the conversion to the skin was not evidenced.

We also suggest, based on the study by Saporito and Grant [22], that the use of benzocaine in liquid form should be avoided for studies involving the use of anuran skins extracts in bioassays since there is a possibility of signalling false positives; besides allowing inaccuracies as to the general chemical composition present in the extracts (mainly in the case of the use of crude extracts and not of isolated substances). We reinforce that other substances detection techniques (such as NMR and chemometrics) are important tools for better robustness and understanding of the results in these types of studies and that there is a need to investigate other parameters involving the interaction between the use of anesthetics and anurans, for example, whether sex, size, and age of these animals can influence the speed of conversion of these products to the integument.

5. Conclusion

Our results showed that the lethal dose of benzocaine in gel (Benzotop© product) used inside the frogs' mouth was not converted to the integument. The benzocaine substance was not found in the extracts of both species of anurans evaluated, as evidenced by the ^{1}H-NMR spectra. Also, in the Hierarchical Analysis of Clusters (HCA) it was possible to confirm the absence of benzocaine in the samples of the frogs' skin extracts.

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Support Information

AG1_FID_CSV File: CSV file containing chemical shifts of the skin extract (DCM + MeOH) of *Adelphobates galactonotus* (specimen 01).

AG2_FID_CSV File: CSV file containing chemical shifts of the skin extract (DCM + MeOH) of *Adelphobates galactonotus* (specimen 02).

AG3_FID_CSV File: CSV file containing chemical shifts of the skin extract (DCM + MeOH) of *Adelphobates galactonotus* (specimen 03).

LL1_DCM+MeOH_FID_CH_CSV File: CSV file containing chemical shifts of the dichloromethanic skin extracts from the frog *Lithodytes lineatus*. In the caption, CH corresponds to individuals collected at Chandless State Park.

LL2_DCM+MeOH_FID_CR_CSV File: CSV file containing chemical shifts of the dichloromethanic skin extracts from the frog *Lithodytes lineatus*. In the caption, CR corresponds to individuals collected in Cruzeiro do Sul municipality.

LL3_DCM+MeOH_FID_UFAC_CSV File: CSV file containing chemical shifts of the dichloromethanic skin extracts from the frog *Lithodytes lineatus*. In the caption, UFAC corresponds to individuals collected at the Federal University of Acre.

LL1_MeOH_FID_CH_CSV File: CSV file containing chemical shifts of the methanolic skin extracts from the frog *Lithodytes lineatus*. In the caption, CH corresponds to individuals collected at Chandless State Park.

LL2_MeOH_FID_CR_CSV File: CSV file containing chemical shifts of the methanolic skin extracts from the frog *Lithodytes lineatus*. In the caption, CR corresponds to individuals collected in Cruzeiro do Sul municipality.

LL3_MeOH_FID_UFAC_CSV File: CSV file containing chemical shifts of the methanolic skin extracts from the frog *Lithodytes lineatus*. In the caption, UFAC corresponds to individuals collected at the Federal University of Acre.

CAPITULO 3

Forest type and urbanization influence the geographic variation of the chemical profile of secondary metabolites of *Lithodytes lineatus* (Anura: Leptodactylidae) skin extracts
Em Revisão na revista **Toxicon.**

Forest type and urbanization influence the geographic variation of the chemical profile of secondary metabolites of *Lithodytes lineatus* (Anura: Leptodactylidae) skin extracts

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Abstract

Variations in the secondary metabolite's profiles in the skins of different species and among anuran populations from different geographic areas have been widely described, however, the greatest emphasis has been given to the detection of alkaloids. In this study, we analysed whether the chemical profile of secondary metabolites present in methanolic extracts made from the skins of different populations of the frog *Lithodytes lineatus* is influenced by geographic distance and the type of environment. We use comparative thin-layer chromatography (TLC) with different developers to highlight the potential types of substances by chemical classes. The samples were also submitted to a hydrogen nuclear magnetic resonance spectroscopic process (^{1}H-NMR) for observation and validation of chemical complexity. A hierarchical ordering model of clusters was created to highlight the similarities and/or dissimilarities between the samples of the different populations tested. We showed that the methanolic extracts from the *L. lineatus* skins displayed differences between types and amounts of secondary metabolites in relation to the geographical area. Distance was the main factor influencing these differences, since populations separated by the Madeira River did not show significant differences. However, environmental disturbance has an effect on concentration and patterns of chemical differentiation. We revealed that individuals collected in more preserved areas had a higher amount of metabolites in the skin, within the same chemical class, compared to those collected in an urban environment. Our results demonstrate that geographical distance and environmental disturbance influence the chemical diversity in the skin of these animals.

Keywords: methanolic extracts; skin; chemical variation; Anura.

1. Introduction

Vertebrates in general have protective layers on the skin such as hair, scales or feathers (Pough 2008; Williams et al. 2000) while anuran amphibians have a true chemical arsenal in their coat, this strategy is important in the action against predators and pathogens in the environment, such as fungi and bacteria (Bevins and Zasloff 1990; Daly 1995; Williams et al. 2000; Rodriguez et al. 2011; Saporito et al. 2012; Hantak et al. 2013; Mina et al. 2015). Currently, it is known that it is possible to find a wide variety of substances in the skin of anurans, such as biogenic amines, peptides, proteins, alkaloids, among others (Bevins and Zasloff 1990; Daly et al. 1994; Daly 1995; Clarke 1996 ; Williams et al. 2000; Darst et al. 2005; Saporito et al. 2009; Saporito et al. 2012; Jeckel et al. 2019). Many of these substances come from the *de novo* synthesis process, characterized by the production of a certain metabolite directly in the organism of the animal itself or by the ability to sequester substances from the dietary, as is the case with some species of dendrobatid anurans specialized in alkaloids sequester from the ingestion of ants, beetles and/or mites (Daly et al. 1994; Darst et al. 2005; Saporito et al. 2009; Saporito et al. 2012; Jackel et al. 2015). Although it is already known that there is a relationship between the alkaloids profiles and the distance or environment between populations, the answers vary between species, for *Dendrobates pumilio* the alkaloids profiles are related to the geographical distance, with the closest populations being more similar than those more distant (Saporito et al. 2012), for *Adelphobates galactonotus* the alkaloids profiles did not differ between colors and even with distance even having an aquatic barrier between populations (Jeckel et al. 2019), while the bufonids of the genus *Melanophryniscus* had alkaloids profiles related more to the type of environment than to distance (Daly et al. 2008).

Lithodytes lineatus belongs to a monophyletic genus and is a leptodactylide known to associate with *Atta* ants during the reproductive period (Schluter 1980; Schluter and Rëgos 1981; Schluter et al. 2009). Other than that, associations like this, between ants and anurans, had only been registered for the African continent between anurans *Phrynomantis microps* and *Kassina fusca* with ant species *Paltothyreus tarsatus* and *Megaponera foetens* (Rödel and Braun 1999). Coexistence is possible due to the action of two peptides present on the skin of *P. microps* that act by inhibiting the ant's aggressive response (Rödel et al. 2013).

Data on the biology of the interaction between *L. lineatus* and *Atta* ants are scarce. In a study performed by de Lima Barros et al. (2016), it was demonstrated that the frog's presence in the nests of these ants is possible due to substances present in the anuran's skin. Anurans of the Leptodactylidae family are not known to have skin alkaloids (De Sá et al. 2014; Frost 2019) and studies on the chemical composition of other types of secondary metabolites in *L. lineatus* have not yet been carried out and therefore we do not know whether the chemical profile of skin secretion varies according to geographical distance or depending on the environment.

In this study we will describe differences in the chemical composition of the methanolic skin extracts of *L. lineatus* from different locations in the Brazilian Amazon and assess whether there is a relationship with distance, geographical barrier and/or environment.

2. Material and Methods

2.1. Sample areas

Searches for *L. lineatus* individuals were carried out in two campaigns and in areas whose occurrence and abundances were previously described in the literature (de

Lima Barros et al. 2016). *L. lineatus* individuals were collected from five locations in the Amazon (Figure 1). The first sampling was carried out in one area in the state of Rondônia, Brazil: in Jaci River, right bank of the Madeira River (**RJR**) (n = 8) and in the Mapinguari National Park, left bank of the Madeira River (**MNP**), Amazonas, Brazil (n = 5). The second sampling was carried out in three areas in the state of Acre, Brazil: Cruzeiro do Sul municipality (**CSM**) (n = 3); campus of the Federal University of Acre in Rio Branco (**RBM**) (n = 3); and Chandless State Park (**CSP**) (n = 5).

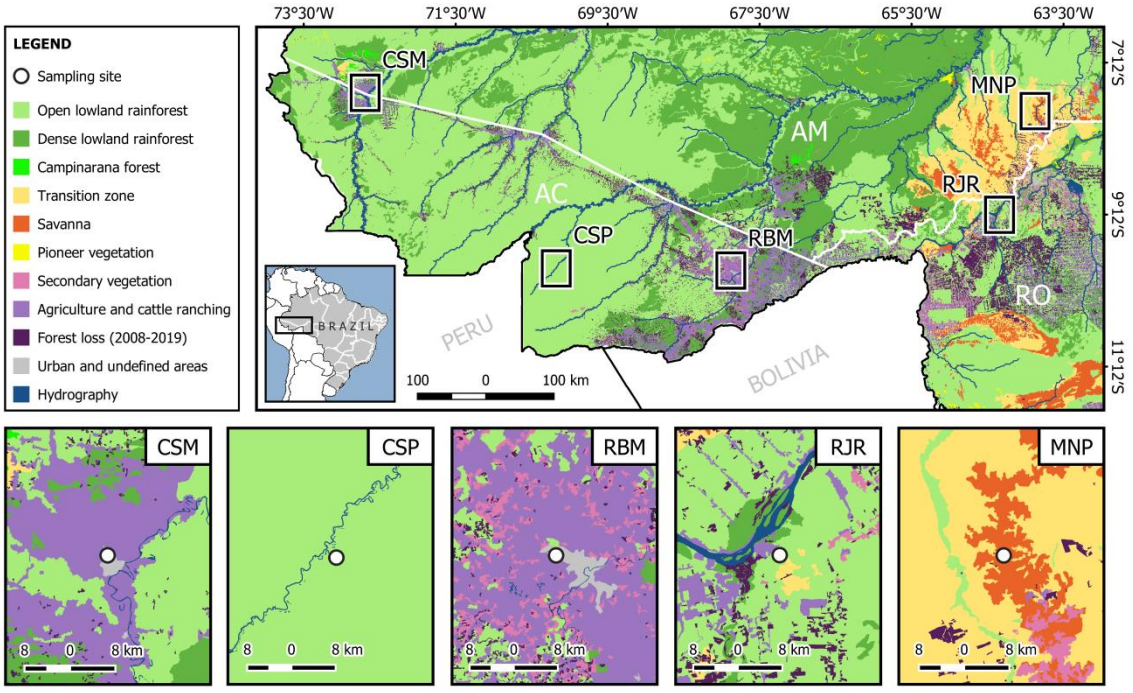

Figure 1. Sampling areas of *Lithodytes lineatus* individuals. CSM = Cruzeiro do Sul Municipality; CSP = Chandless State Park; RBM = Rio Branco Municipality; RJR = Right Jaci River; MNP = Mapinguari National Park. Sources: Forest types (MMA 2006); Foreste loss (INPE 2020).

2.2. Obtaining *L. lineatus* skin extracts

Individuals of *L. lineatus* were killed using an anesthetic gel based on benzocaine (2%). The application of the gel was carried out directly inside the animal's

mouth to avoid contamination of the skins. The skins were removed immediately after the deaths to avoid potential conversion of the anesthetic to the animal's skin (de Lima Barros et al. 2020), making an inguinal incision from one end of the animal to the other. The skins were submitted to the substance extraction process using methanol (100%) in a concentration of 1 g skin/10 mL of solvent. For the extraction, the skins were taken to the ultrasound bath for 20 minutes and then filtered. This process was repeated three times and the concentrated solution was stored in a single bottle. The solvent was evaporated from the samples using a rotary evaporator at 30 ºC at 55 rotations per minute.

2.2.1. Analysis of the chemical profile of *L. lineatus* skin extracts

We analysed the chemical profile of methanolic extracts by Comparative Thin-Layer Chromatography (TLC), using aluminium microplates containing silica gel. To elute the substances in the extracts, a system containing dichloromethane (DCM) and methanol (MeOH) in a 9: 1 (v/v) ratio was used. We use physical (254 nm and 365 nm) and chemical developers (Anisaldehyde, Ferric Chloride, Ceric Sulphate and Dragendorff reagent) to have indications of the potential chemical classes present in the samples. Extracts were also analysed by Hydrogen Nuclear Magnetic Resonance (^{1}H-NMR) (Bruker, model Fourier 300, magnet 300 SB UltraShieldTM, 7.05T, 300 MHz), where 10 mg of each methanolic extracts were solubilized in deuterated chloroform ($CDCl_3$) containing tetramethylsilane (TMS) as a reference standard. Spectra processing was performed using TopSpin program (version 4.0.7, Bruker Biospin).

140 2.3. Chemometric analysis

141 All ^{1}H-NMR spectra had both phase distortions and baselines adjusted in the
142 TopSpin program (version 4.0.7, Bruker Biospin) where they were automatically
143 converted to CSV files (Comma-separated values). Subsequently, we evaluated
144 potential differences or similarities between the samples tested, selecting specific
145 regions in the ^{1}H-NMR spectra to be excluded from the analyses, in order to perform a
146 comparison based on the chemical shifts of interest and eliminate potential noise from
147 the sample (Table 1). We selected the spectrum that presented the greatest complexity
148 (number of peaks) to serve as a comparison standard. Therefore, the cutting regions
149 were based on the ^{1}H-NMR spectrum of the extract made from the skins of individuals
150 collected at Chandless State Park, in Acre. In all, 10 regions were chosen for exclusion.

151

152 **Table 1**. Regions of chemical shifts excluded from the chemometric analysis.

Number of cuts	Exclusion region (in ppm)
1	0.1 – 0.66
2	0.68 – 0.84
3	1.35 – 1.41
4	1.63 – 1.80
5	2.12 – 2.21
6	2.36 – 2.65
7	2.84 – 3.22
8	3.71 – 5.33
9	5.37 – 6.99
10	7.2 – 8.99

153

154

155 To highlight potential similarities and/or divergences between the different
156 samples analysed, we used a hierarchical cluster analysis (HCA) to assess whether there
157 is formation of groups between samples from Euclidean distances, the results of which
158 are illustrated in a dendrogram. The analyses were performed using the software R
159 Studio, version 3.3 (R Core Team 2019).

160

161 2.4. Ethical approval

162 The National Institute of Amazonian Research approved the experiments with
163 the frog species used in this study and the permissions for the use of animals were
164 granted by the Ethics Committee for Research in the Use of Animals (CEUA) (INPA /
165 CEUA, Protocol: 030 / 2014). The permissions for the animal collection were granted
166 by the Chico Mendes Institute for Biodiversity Conservation (ICMBio / license number
167 57123-1). All experiments were carried out following the relevant guidelines and
168 regulations.

169

170 3. Results

171 3.1. Comparison of the chemical profile of *Lithodytes lineatus* methanolic skin extracts

172 Through comparative thin-layer chromatography analysis it was possible to
173 detect the presence of terpenes, since it showed reactivity with the anisaldehyde reagent
174 (lilac color; Figure 2a) in all samples of methanolic extracts from individuals collected
175 in Acre. The presence of terpenes was confirmed by the reactivity of the samples with
176 the ceric sulphate reagent (lilac to brown color; Figure 2b). There was no reactivity with
177 the ferric chloride reagent (Figure 2c) indicating the absence of phenolic substances.
178 There was reactivity with Dragendorff's reagent (orange color; Figure 2d) indicating the
179 presence of nitrogenous substances and in addition, according to the substance's
180 retention profile in the plates, it is possible to suggest that these metabolites are
181 different.

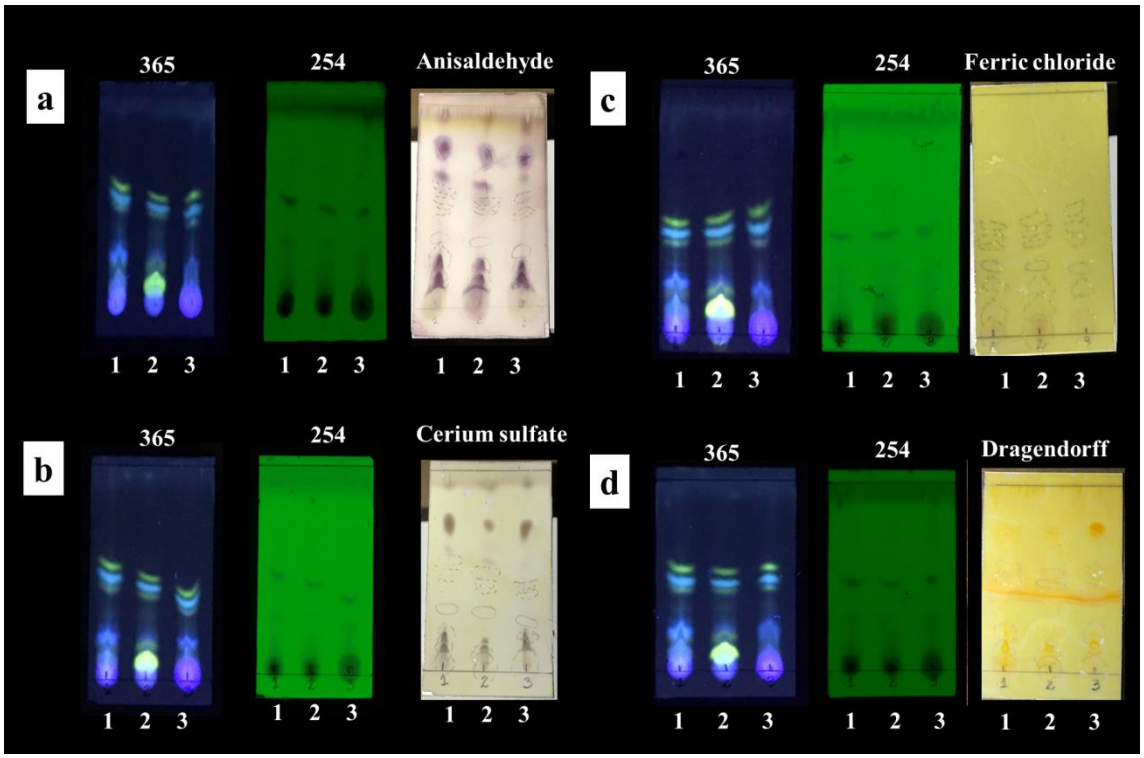

Figure 2. Chemical profile of methanolic extracts from the skins of *Lithodytes lineatus* collected in Acre using various chemical developers. Values at the top of the silica gel chromatoplates 365 and 254 correspond to the frequencies of the physical UV (nm) developers. Values at the base of the chromatoplates correspond to the locations: 1 = Cruzeiro do Sul; 2 = Rio Branco; 3 = Chandless.

For both populations **RJR** and **MNP**, the samples showed reactivity with the anisaldehyde reagent (lilac color; Figure 3a) indicating the presence of terpenes, and confirmed by the reactivity with the ceric sulphate reagent (lilac to brown color; Figure 3b). Likewise, there was reactivity with Dragendorff's reagent (orange color; Figure 3c) which indicates the presence of nitrogenous substances in extracts from both locations. Through the retention of the substances in the chromatographic plates it was possible to observe that the potential substances indicated in the chemical developers are also different between the two locations, despite belonging to the same chemical class.

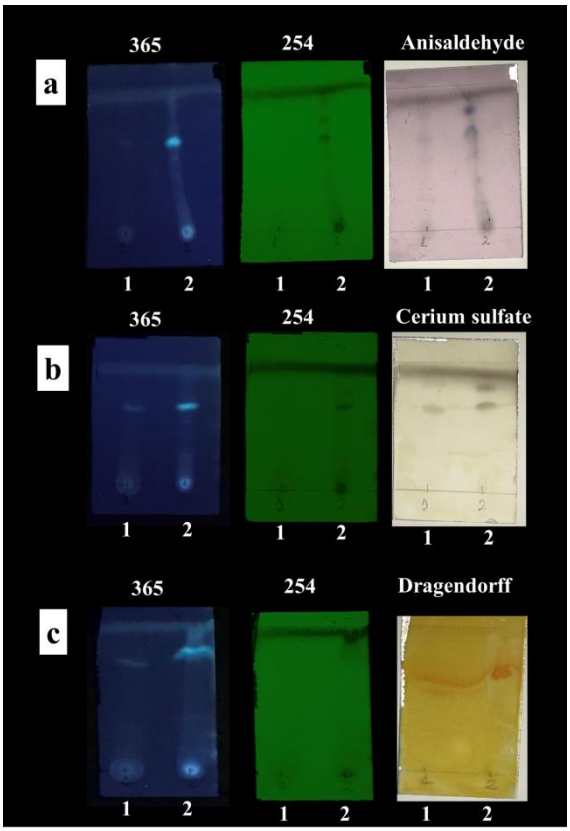

Figure 3. Chemical profile of methanolic extracts from the skins of *Lithodytes lineatus* from Rondônia using three chemical developers. Values at the top of the silica gel chromatoplates 365 and 254 correspond to the frequencies of the physical UV (nm) developers. Values at the base of the chromatoplates correspond to the locations: 1 = Mapinguari - left of the Madeira River; 2 = Jaci - right of the Madeira River.

The quantity between the types of substances, by chemical class, of the populations of **RJR** and **MNP** is lower than that of the populations of **CSM**, **CSP** and **RBM**, since there was a clear decrease in the number of spots revealed in all solvents to which the samples were submitted.

3.2. Analysis of ^{1}H nuclear magnetic resonance spectra of methanol extracts from *L. lineatus*

The methanolic extracts from the *L. lineatus*' skins analysed by ^{1}H-NMR showed differences between the evaluated areas, visible by the low amount of chemical

shifts signs present in the samples of individuals from **RJR** and **MNP** compared to samples extracted from **CSM**, **CSP** and **RBM** individuals (Figure 4a).

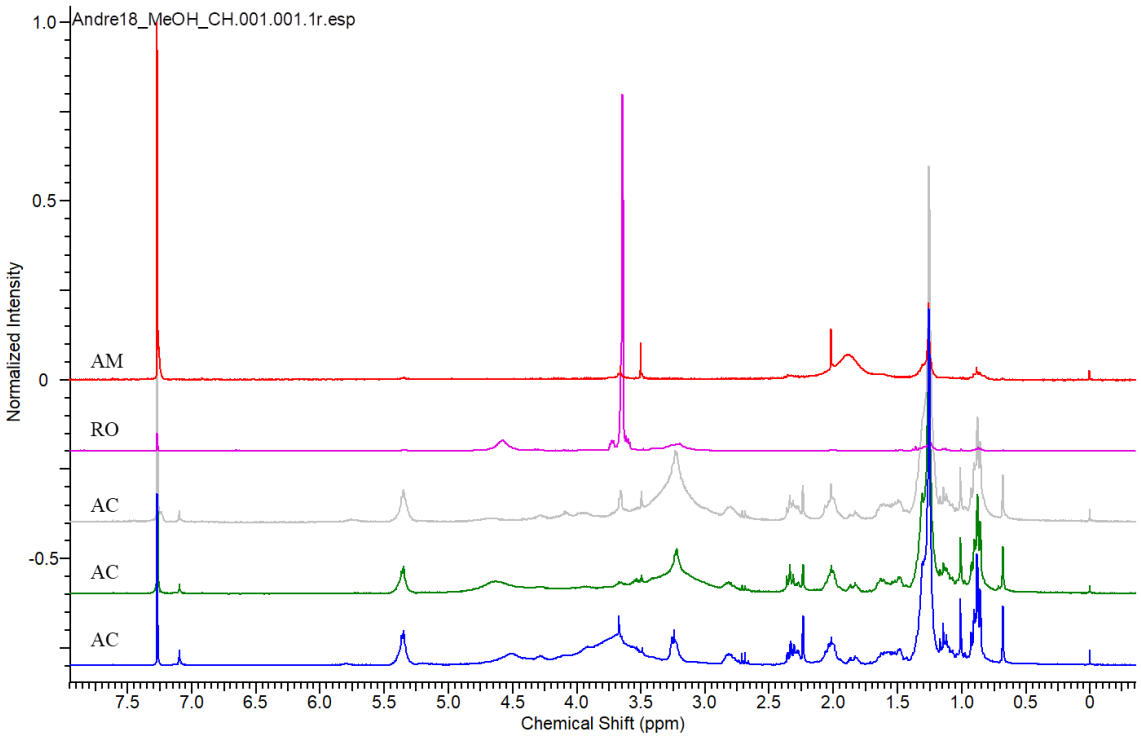

Figure 4a. Comparison of ^{1}H Nuclear Magnetic Resonance Spectra of methanolic extracts of *Lithodytes lineatus* individuals collected in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray - Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

The spectra of samples from **CSM, CSP** and **RBM** showed signs of chemical shifts in the methyl region (0.8 to 1.5 ppm), characteristic of terpenes and steroids (Fig. 4a). On the other hand, samples from individuals in **RJR** and **MNP** showed lower signal intensity in the same region of the spectrum (Figure 4b).

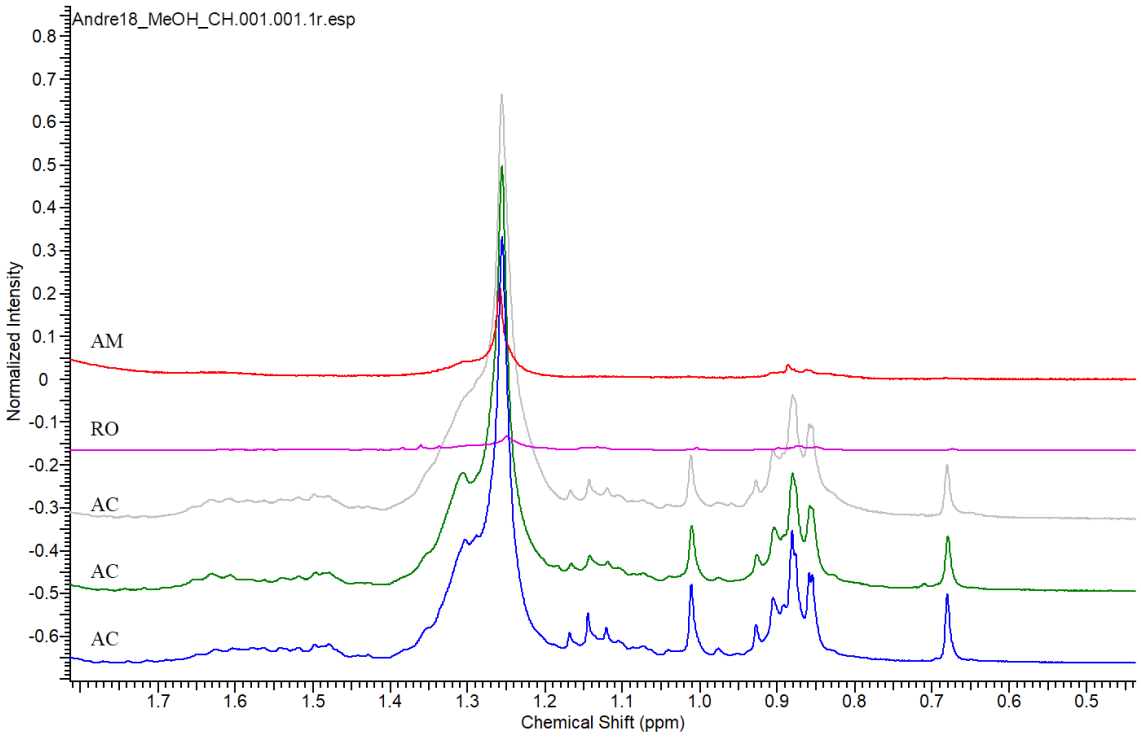

Figure 4b. Magnification in the methyl region of the ^{1}H Nuclear Magnetic Resonance Spectra of *Lithodytes lineatus* methanolic extracts in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray - Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

Also, signs of chemical shifts in the region of methylenic (1.8 to 2.5 ppm) and methinic (2.5 to 3.5 ppm) hydrogens were detected only in the spectra of methanolic extracts from individuals in Acre (Figure 4c).

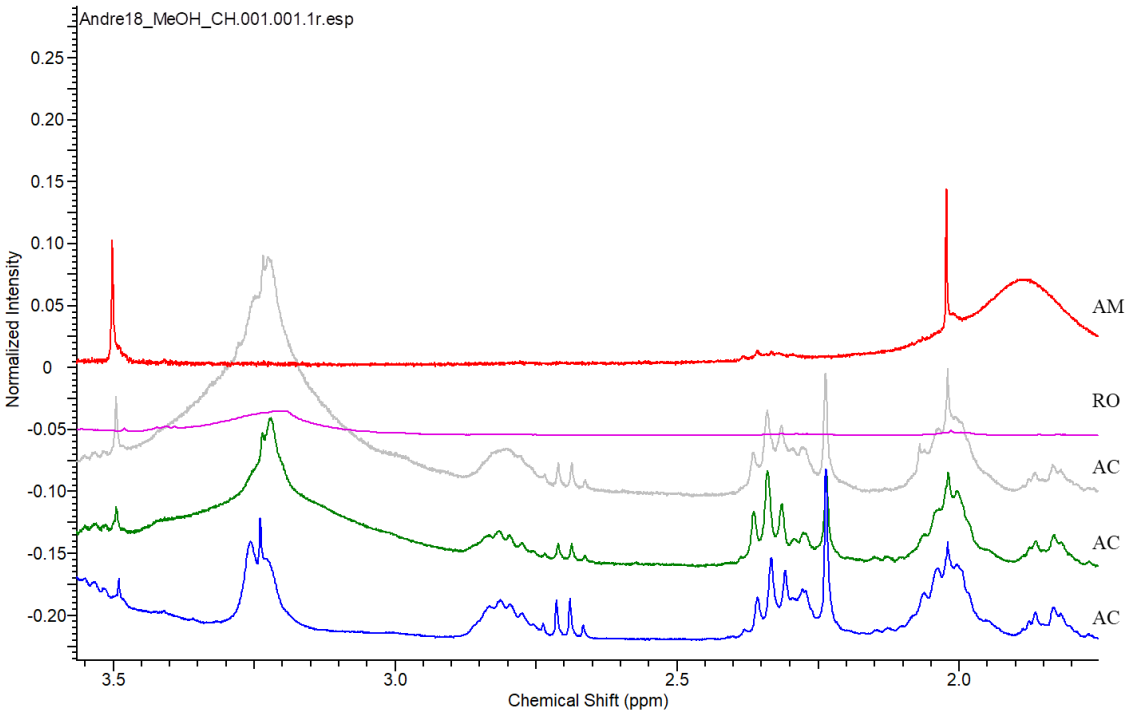

Figure 4c. Magnification in the methyl region of the ^{1}H Nuclear Magnetic Resonance Spectra of *Lithodytes lineatus* methanolic extracts in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray - Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

It was possible to detect the presence of signs of carbinolic hydrogens (3.66 ppm) in all spectra obtained from individuals from **MNP, RJR, CSM** and **RBM**. There were no signs of hydrogens of this type only in the spectra of the samples of the **CSP** individuals (Figure 4d).

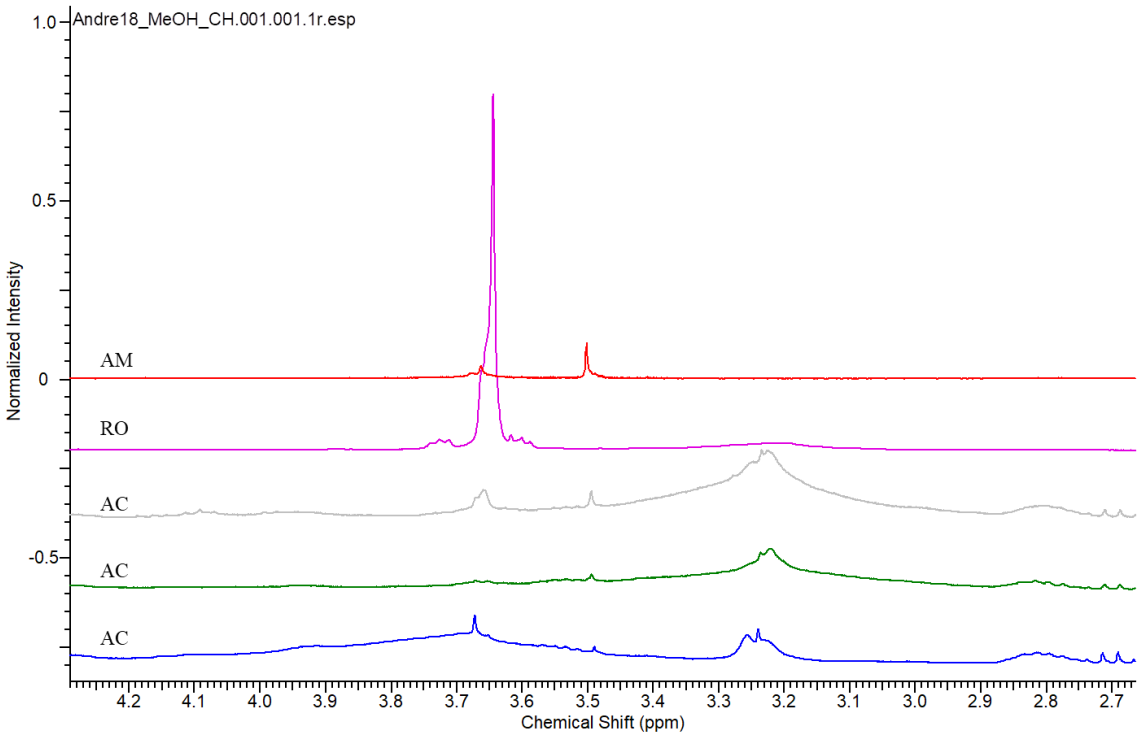

Figure 4d. Magnification in the methyl region of the ^{1}H Nuclear Magnetic Resonance Spectra of *Lithodytes lineatus* methanolic extracts in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray - Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

Signs of olefinic hydrogens (5.35 ppm) were detected only in extracts from Acre individuals (Figure 4e). Signs in this chemical shift are commonly characteristic of steroids. Some few hydrogens characteristic of aromatic rings were detected in all extracts from **CSM, CSP** and **RBM**, with the exception of extracts from the skins of individuals collected in **MNP**, where there was an absence of hydrogens of this type. Likewise, the skin extract of individuals from **MNP** was the only one that did not show signs of aromatic ring hydrogens (Figure 4e). In addition, the extract of individuals from **RJR** showed two doublets in positions 6.7 and 7.9 ppm, respectively.

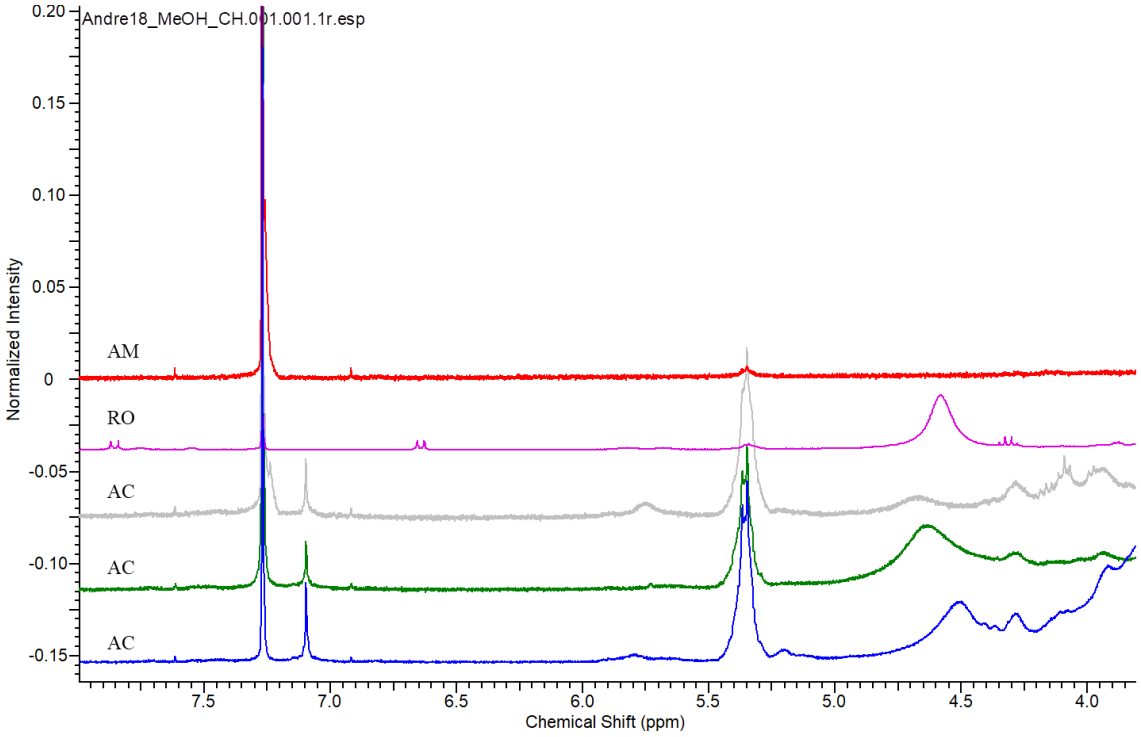

Figure 4e. Magnification in the methyl region of the ^{1}H Nuclear Magnetic Resonance Spectra of *Lithodytes lineatus* methanolic extracts in the five sampled areas. The colors in the spectra correspond, respectively: red - Mapinguari (MNP); lilac - Jaci (RJR); gray - Rio Branco (RBM); green – Chandless (CSP); and blue - Cruzeiro do Sul (CSM). The acronyms on the left indicate the States: AM = Amazonas; AC = Acre and RO = Rondônia.

3.3. Hierarchical clusters Analysis (HCA)

A Clusters analysis associated the extracts of individuals by region, with the two areas in **RJR** and **MNP** being closer to each other and more distant from the three areas of the Acre region (Figure 5). In addition, there was a greater similarity between the chemical constituents present in the extracts of individuals collected in the city of **RBM** and **CSM**, while the **CSP** extract was more different in relation to the types of metabolites (Figure 5).

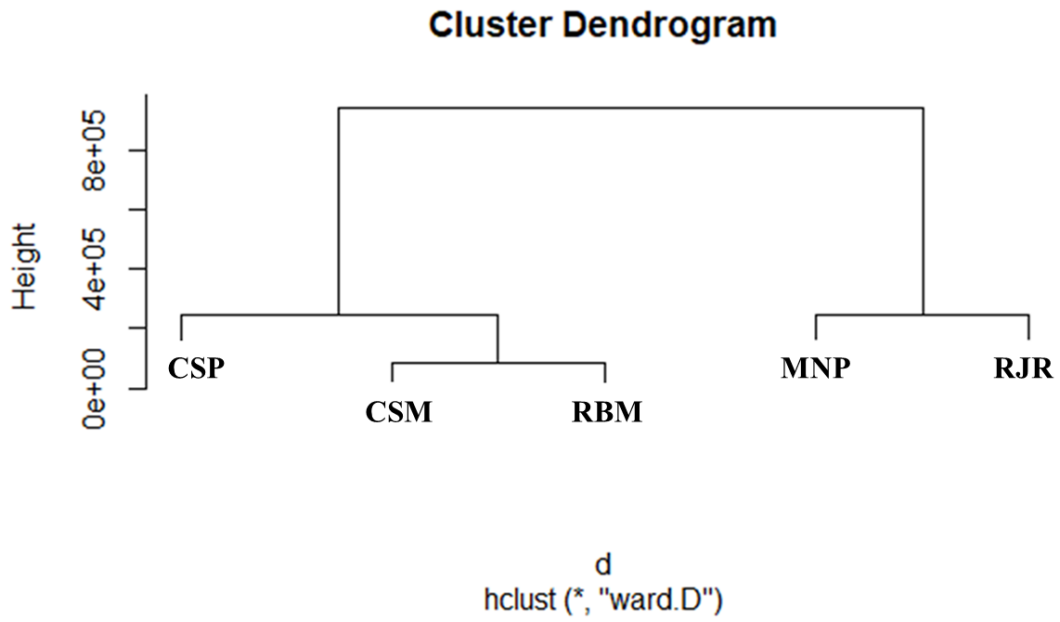

Figure 5. Hierarchical ordering of clusters graph of the entire spectra of the methanolic extracts of *Lithodytes linetaus*. As shown: CSP = Chandless State Park - AC; CSM = Cruzeiro do Sul Municipality - AC; RBM = Rio Branco Municipality – AC; MNP = Mapinguari National Park - AM; RJR = Right Jaci River - RO.

This information, together with the analysis of the spectra and thin-layer chromatography, reinforces the evidence in the formation of well-defined patterns among the evaluated samples.

4. Discussion

With these results, we have evidenced geographical and environmental influence, but not the river as being a barrier, in the profile of secondary metabolites in the methanolic skin extracts of *Lithodytes lineatus*. So far, Prates and collaborators (2012) have analysed the primary metabolites of *L. lineatus* and their results showed that there is a large amount of peptides and some high molecular weight proteins. Most studies involving geographic variation in anuran poisons commonly investigate only

316 one class of substances, mostly alkaloids, as these metabolites are associated, in many
317 cases, with defence strategies (Saporito et al. 2010; Grant et al. 2012). Regarding
318 compounds that are not alkaloids and that are present in the secretion of anurans, studies
319 have shown that there is variation between the sexes and ontogeny in the number of
320 compounds produced. For example, for the *Rhinella marina* frog, ontogenetic variation
321 occurs in the composition and amount of toxins (bufodienolides) present in its parotoid
322 glands (Hayes et al. 2009). Differences in peptide composition between males and
323 females were demonstrated for the *Litoria splendida* tree frog (Wabnitz et al. 2000).
324 The presence of terpenes in the skins extracts of all *L. lineatus* populations
325 studied is considered advantageous, since some known terpenes have antifungal,
326 antibacterial, insect repellent properties, among others (Cakir et al. 2004; Gillij et al.
327 2008; Lima et al. 2011; Montanari et al. 2011), with the sesquiterpene, β-caryophyllene
328 terpene and the monoterpenes limonene, eucalyptol and ocimene isolated from the
329 secretion of *Litoria caerulea* (Smith et al. 2004). Likewise, the presence of steroids in
330 the chemical composition of the extracts is important and is directly involved in defence
331 mechanisms (Hayes et al. 2009; Hantak et al. 2016). Furthermore, we observed that the
332 samples showed reactivity with Dragendorff's reagent, indicating the possibility of the
333 presence of alkaloids. However, *L. lineatus* is found within a family of frogs
334 (Leptodactylidae) that has no representative known for kidnapping or producing
335 alkaloids (Daly et al. 1994; Darst et al. 2005; Saporito et al. 2009; Saporito et al. 2012).
336 In this way, we isolate the substance and are in the process of structural elucidation to
337 characterize the type of metabolite. On the other hand, according to the ^{1}H-NMR
338 spectrum pattern obtained from the isolated substance, it was possible to rule out the
339 possibility of dealing with the benzocaine used to kill the individuals used in this study
340 (de Lima Barros et al., 2020). For leptodactylids, most studies describe the presence of

biogenic amines, enzymes and in large quantities, peptides with an antimicrobial character (Cei et al. 1967; Anastasi et al. 1970; Nascimento et al. 2004; Conlon 2008; Conlon et al. 2009; Colon 2011; Libério et al. 2014; Barbosa et al. 2015; Castro et al. 2017).

The variations in the profile of secondary metabolites present in the skin extracts analysed in this study demonstrated that chemical differentiation occurs between geographically distant populations, but not between populations isolated by the Madeira River. The distance effect is expected due to some characteristics such as, for example, the physical-chemical composition of the soil and the availability of trophic resources, which can be quite different between the most distant areas (Saporito et al. 2012), but the river as a barrier having no effect was not expected, however the pattern found for *L. lineatus* is similar to that of *A. galactonotus*, where the river also did not act as a barrier in chemical variation (Jeckel et al. 2019), indicating that the arthropod community changes little with distance, even though it has a geographical barrier.

In addition to the geographical influence on the profile of secondary metabolites, we found that environmental variation also occurs. Through the ordering techniques used in this study, we demonstrated that there was a greater similarity between the profile of secondary metabolites in the skin extracts of urban individuals in the cities of Rio Branco and Cruzeiro do Sul, compared to Chandless, despite all being from the state of Acre. We believe that the gap between the chemical profile of individuals in urban and preserved areas is an indication of change due to the effects of anthropization and isolation of populations in fragments. This results leads us to believe that possibly the availability of trophic resources for individuals who live in Chandless is greater compared to those who live in an urban environment, this being the determining characteristic to explain the differentiated amount of secondary metabolites in the skin

of these individuals, a since anurans usually convert certain types of substances to their integument from the ingestion of certain types of prey.

5. Conclusion

In this study we showed that the chemical composition of the methanolic extracts of the skins of *Lithodytes lineatus* varies with the distance and the place of occurrence but does not suffer the effect of the river as a barrier. The chemical profile of individuals' skin extracts and the quantity of substances are more similar to shorter distances and less similar to longer distances. Our data showed that in the Acre region, extracts from individuals collected in an urban environment are more similar chemically than those collected in a primary forest environment, regardless of distance.

6. Conflicts of interest

There are no conflicts of interest.

7. Acknowledgments

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8. Ethical Statement

The species used in this study (*Lithodytes lineatus*) is not endangered or protected in Brazil and the collection of frogs complied with the current laws in their country of origin.

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CAPITULO 4

Isolamento de componentes lipídicos no extrato da pele da rã *Lithodytes lineatus* (Anura, Leptodactylidae): Colesterol. Manuscrito em preparação para a revista **Química Nova**.

Isolamento de componentes lipídicos no extrato da pele da rã *Lithodytes lineatus* (Anura, Leptodactylidae): Colesterol

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Resumo

Lithodytes lineatus é uma espécie de rã que se especializou em coexistir com formigas *Atta* e a permanência nas colônias destas formigas é creditada à registros químicos presentes na pele da rã. Neste estudo, utilizando técnicas cromatográficas (Cromatografia em Camada Delgada Comparativa) e espectroscópicas (Ressonância Magnética Nuclear de ^{1}H, ^{13}C e bidimensionais) foi caracterizado a estrutura de um esteroide (colesterol) isolado do extrato metanólico feito a partir das peles da rã *L. lineatus*. Este é o primeiro estudo a isolar e descrever uma molécula presente na pele desta rã, além do primeiro registro de colesterol isolado a partir do tegumento de uma espécie da família Leptodactylidae.

Palavras-Chave: Extratos da pele, Anura, *Lithodytes lineatus*, Esteroides, Colesterol.

Introdução

Anuros apresentam uma linha de defesa química bastante robusta, sendo possível encontrar, por exemplo, proteínas, peptídeos, alcaloides, esteroides e outros (Bevins e Zasloff, 1990; Daly, 1995; Saporito *et al*., 2009). Dentre os esteroides detectados na secreção de anuros os mais conhecidos são os bufodienolídeos, mas também há registros de isolamento de β-sitosterol, ergosterol e colesterol (Croce e Bolognani, 1975; Medeiros *et al*., 2019; de Souza et al., 2020).

Representantes da família Leptodactylidae apresentam uma grande quantidade de substâncias de origem proteica no tegumento, sendo bastante estudados acerca das propriedades bioativas de peptídeos com potencial antimicrobiano (Nascimento *et al*., 2004; King *et al*., 2005; Dourado *et al*., 2007; Sousa *et al*., 2009). A rã *Lithodytes lineatus* é uma espécie de leptodactylideo conhecido por viver no interior de galerias de formigas cortadeiras do gênero *Atta* (Schlüter, 1980; Schlüter e Regös, 1981; Lamar e Wild, 1995; Schlüter *et al*., 2009, de Lima Barros *et al*., 2016**a,b**). A convivência harmônica entre *L. lineatus* e as formigas *Atta* têm sido creditada à presença de substâncias no tegumento da rã (de Lima Barros *et al*., 2016**b**), porém o sinal químico que medeia a associação ainda não foi descrito, assim como substâncias presentes na secreção cutânea. A única referência indicando que a secreção cutânea de *L. lineatus* é altamente proteica foi o estudo de Prates *et al*. (2012) através de técnicas de separação de substâncias como Cromatografia Líquida de Alta Eficiência (CLAE) e Eletroforese SDS-PAGE, porém, não foi realizada a identificação de nenhuma proteína ou peptídeo.

Com o intuito de ter indícios do sinal químico utilizado pela rã para conviver com as formigas *Atta* neste estudo apresentamos pela primeira vez a descrição de uma substância isolada de extratos feitos a partir de peles de indivíduos de *L. lineatus*. Esse é o primeiro passo para poder obter substâncias que possam ser testadas em experimentos

em laboratório ou no campo que permitirá uma maior compreensão de como se dá essa relação.

Material e Métodos

Comitê de ética

O Instituto Nacional de Pesquisas da Amazônia aprovou os experimentos com a espécie de rã utilizada neste estudo e as permissões para o uso dos animais foram disponibilizadas pelo Comitê de Ética para Uso de Animais (CEUA) (INPA / CEUA, Protocolo: 030 / 2014). As permissões para a coleta dos animais foram dadas pelo Instituto Chico Mendes para Conservação da Biodiversidade (ICMBio / número de licença 57123-1). Todos os experimentos foram conduzidos seguindo as normas e protocolos dos órgãos consultados. Ainda, foi realizado o cadastro no Conselho de Gestão do Patrimônio Genético (CGEN, número do cadastro: AE53FCB) para uso de componentes oriundos da biodiversidade.

Obtenção do extrato das peles de *Lithodytes lineatus*

Buscas por indivíduos de *L. lineatus* foram realizadas durante o mês de janeiro de 2018 no Parque Estadual Chandless, estado do Acre, Brasil. Ao todo, foram coletados cinco indivíduos (todos machos). Os espécimes foram mortos utilizando gel anestésico à base de benzocaína (2%) com aplicação direta na boca para evitar a contaminação das peles (de Lima Barros *et al*., 2020). Imediatamente após a comprovação das mortes, as peles dos animais foram retiradas através da incisão inguinal de uma extremidade a outra do corpo e reunidas em um microtubo contendo 4 mL de metanol (100%) e preparadas para extração. O sistema de extração de

substâncias (10 mL/g de pele) utilizado foi MeOH (100%). Posteriormente, as peles foram levadas ao banho de ultrassom (Unique©, modelo USC – 1800, frequência 40Hz) por 20 minutos, sendo filtradas em papel de filtro. Este processo foi repetido por três vezes e os concentrados de cada extração acondicionados em um único frasco. O concentrado foi levado à capela para evaporação total dos solventes e obtenção do extrato.

Fracionamento do extrato metanólico de *Lithodytes lineatus*

Para o isolamento de substâncias presentes no extrato metanólico das peles de *L. lineatus*, 20mg da amostra foi submetida ao fracionamento em Cromatografia em Coluna Aberta (CCA; h x d = 16 x 1.0 cm) utilizando sílica gel (Sigma-Aldrich 230-400 mesh) como fase estacionária e eluida com misturas dos solventes diclorometano (DCM) e metanol (MeOH), utilizando um volume de 30 ml para cada mistura do gradiente (Tabela 1).

Tabela 1. Sistemas de eluição e frações obtidas do extrato metanólico das peles de *Lithodytes lineatus*.

Sistema de eluição	Frações coletadas
DCM/MeOH 90:10	1-2
DCM/MeOH 85:15	3-4
DCM/MeOH 80:20	5-6
DCM/MeOH 70:30	7-9
DCM/MeOH 60:40	10-11
DCM/MeOH 50:50	12-13
MeOH 100%	14

Análise do perfil químico do extrato metanólico das peles de *L. lineatus*

Nós analisamos o perfil químico do extrato metanólico das peles da rã por Cromatografia em Camada Delgada Comparativa (CCDC), empregando cromatofolhas

de alumínio com sílica gel impregnado com indicador de fluorescência UV_{254} (Alugram SIL G/UV_{254}). Para eluição das amostras foi utilizado um sistema contendo diclorometano (DCM) e metanol (MeOH) na proporção 90:10 (v/v). Utilizamos reveladores físicos (luz UV 254 e 365) e químicos (Anisaldeído sulfúrico e reagente de Dragendorff) para termos indícios das classes químicas presentes nas amostras.

Análise espectroscópica por Ressonância Magnética Nuclear

Após o fracionamento por CCA as frações de interesse foram reunidas por características semelhantes, observadas quando analisadas por CCDC. As frações consideradas como em elevado grau de pureza foram submetidas ao método espectroscópico de Ressonância Magnética Nuclear de ^{1}H (RMN de ^{1}H / RMN: Bruker, modelo Fourier 300, magneto 300 SB UltraShieldTM, 7.05T, 300 MHz) uni e bidimensionais (COSY, HSQC e HMBC) e RMN de ^{13}C (75 MHz). O experimento foi realizado utilizando o tetrametilsilano (TMS) como padrão interno. Ainda utilizamos o DEPT 90 e 135 para o processo de elucidação estrutural da molécula.

Resultados

Análise do perfil químico do extrato metanólico das peles de *L. lineatus*

Através da análise de CCDC foi possível observar a presença de manchas laranjas quando utilizado o reagente de Dragendorff e manchas de cor lilás reveladas quando utilizado o anisaldeído sulfúrico. Essas manchas (laranjas e lilás) foram observadas simultaneamente nas amostras das frações 5 e 6 e apresentaram os mesmos tempos de retenção nas cromatoplacas.

As amostras das frações 5 e 6 foram então reunidas e analisadas por conta da reatividade com o reagente de Dragendorff e similaridade nos tempos de retenção (**Figura 1**). Nossas análises deram indícios que a fração 5-6 se tratava de uma substância isolada, dessa forma, a identificamos como *Substância I*.

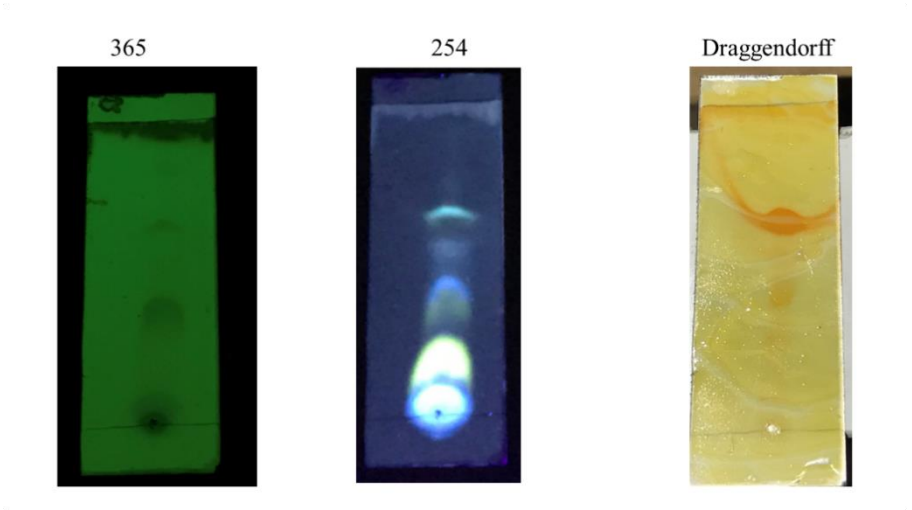


Figura 1. Cromatoplacas de sílica gel contendo amostra da *Substância I* (fração 5-6) submetida ao processo de cromatografia em camada delgada (CCD) utilizando o reagente de Dragendorff.

A *Substância I* apresentava aspecto oleoso e quando analisada por CCD (**Figura 1**) foi possível observar um Rf de 0,66 (Rf = da / ds; onde *da* = distância da amostra e *ds* = distância do solvente) após ser eluida utilizando o sistema DCM/MeOH (90:10 v/v).

Análises por Ressonância Magnética Nuclear (RMN)

Através da análise do espectro de RMN de ^{1}H (**Figura 2**) da *Substância I* apareceu sinais com deslocamentos químicos na região entre δ_H 0,67 e 2,50. Esses sinais são característicos de hidrogênios metilênicos e metílicos presentes em substâncias químicas que apresentam um núcleo esteroidal.

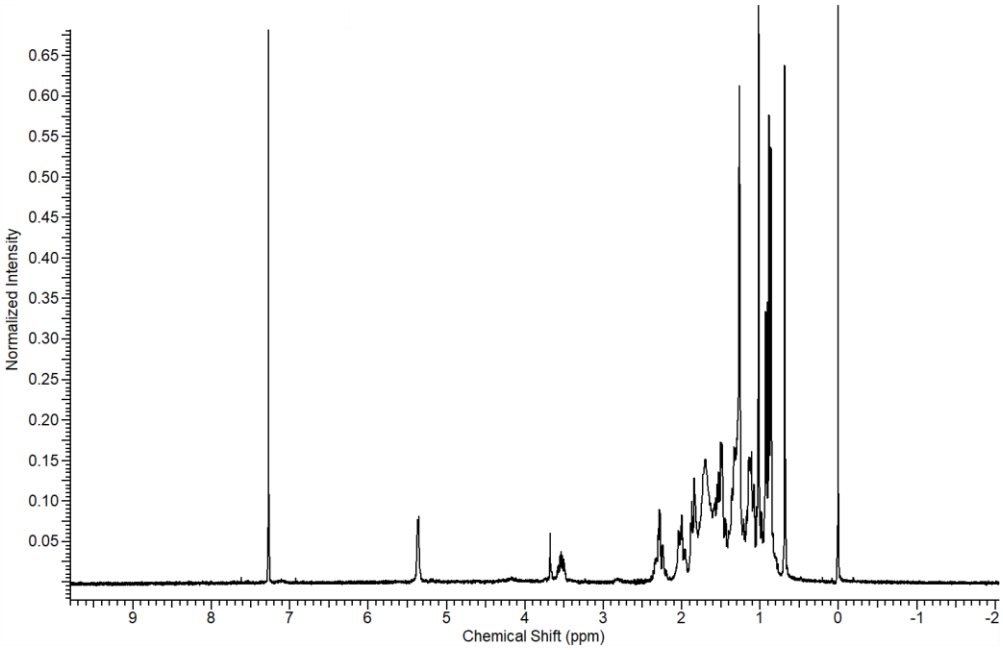


Figura 2. Espectro de RMN de ^{1}H da *Substância I* ($CDCl_3$, 300 MHz).

Análises bidimensionais e RMN de ^{13}C

Através das análises dos espectros bidimensionais, foi possível realizar potenciais correlações entre os átomos de C e H presentes na molécula. Abaixo é possível observar os espectros de COSY, HSQC e HMBC utilizados neste estudo para as análises de correlações (**Figura 3**).

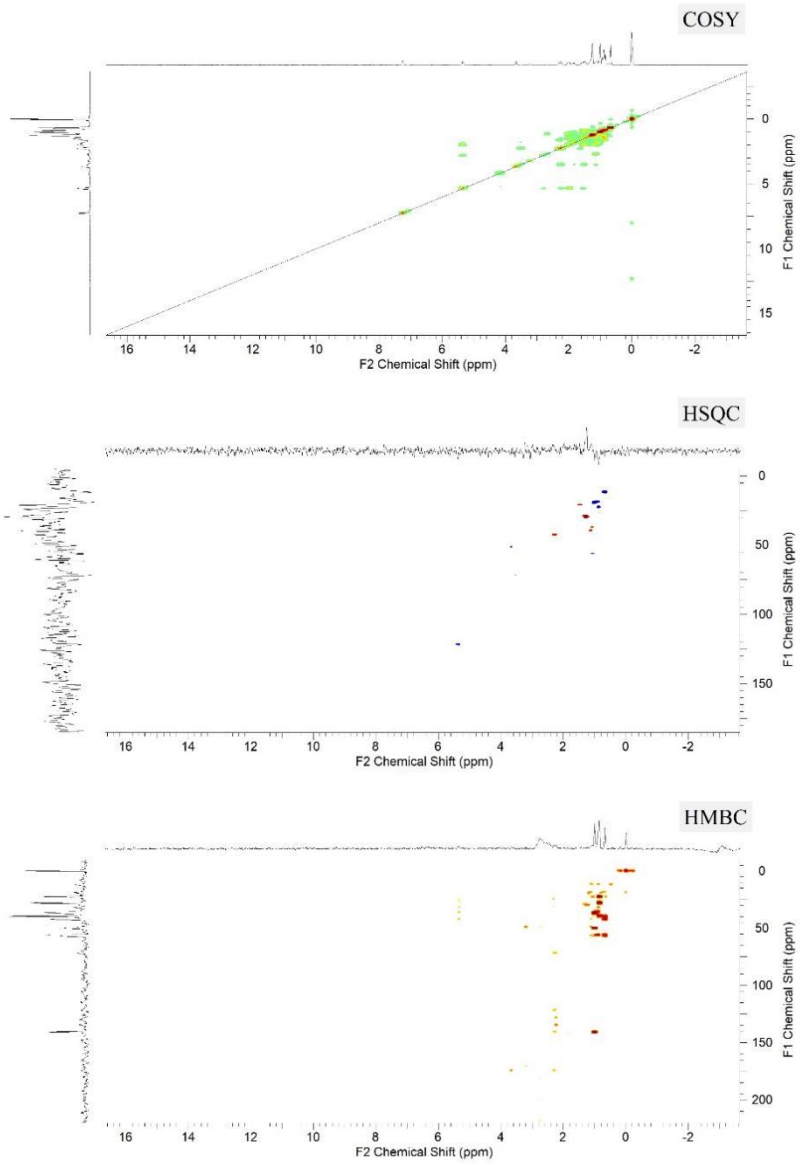


Figura 3. Espectros bidimensionais de COSY, HSQC e HMBC, respectivamente, utilizados para realizar as correlações.

Ainda, foi analisado o espectro de RMN de ^{13}C, onde foi possível detctar a presença de aproximadamente 27 sinais bem definidos (**Figura 4**).

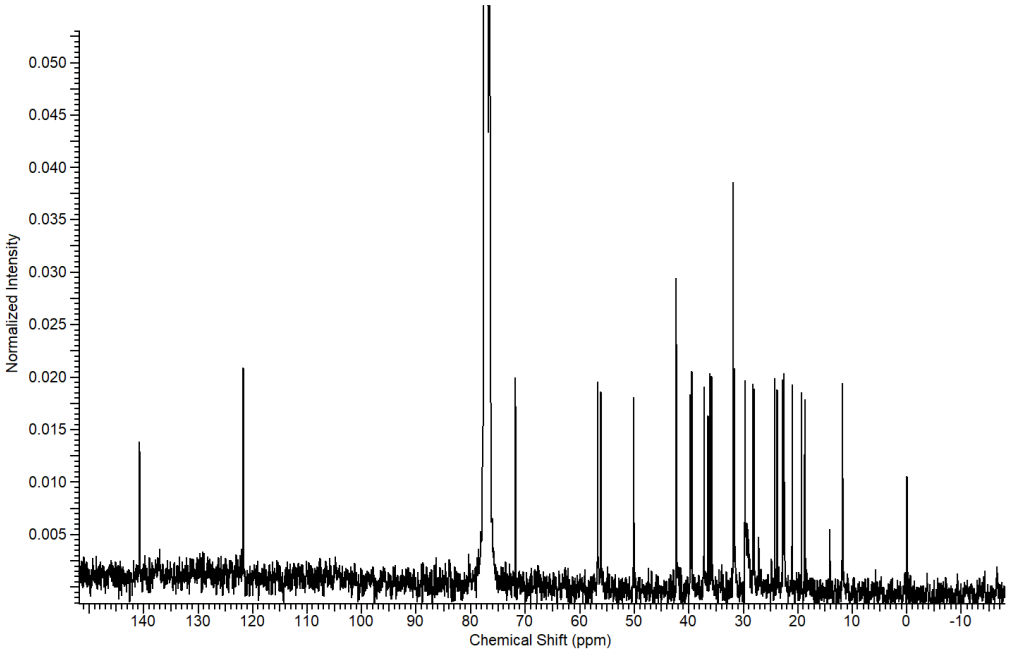
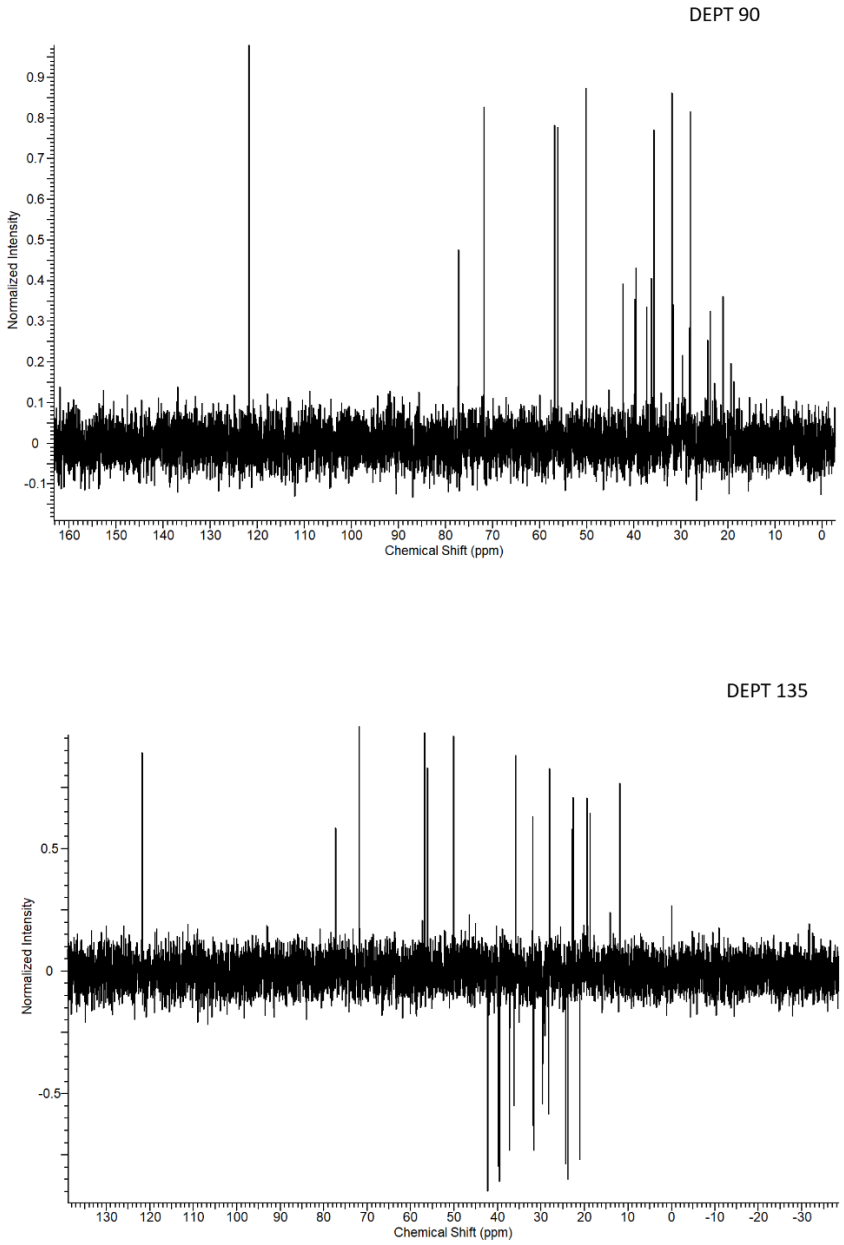


Figura 4. Espectro de RMN de ^{13}C da *Substância I* ($CDCl_3$, 75 MHz).

Através da análise dos espectros de DEPT 90 e 135 (**Figura 5**) foi possível estimar a quantidade de hidrogênios associados (CH, CH_2 ou CH_3) a cada um dos carbonos observados no espectro de RMN de ^{13}C.



176

177 **Figura 5**. Espectros DEPT 90 e 135 da *Substância I*. Na imagem: DEPT 90, sinais
178 positivos representam associações CH; DEPT 135, sinais positivos representam CH_3 e
179 sinais negativos representam CH_2.

180

181 A partir das análises foi possível confirmar a presença de hidrogênios metílicos
182 identificados pela detecção de dois singletos em δ_H 0,67 e 1,00 correspondentes aos

hidrogênios ligados aos carbonos C-18 (δ_C 11,86) e C-19 (δ_C 19,40), e de dupletos em δ_H 0,91 e 0,87 referentes aos hidrogênios ligados aos carbonos C-21 (δ_C 18,71) e C-26 e 27 (δ_C 22,56 e 22,83) respectivamente. Também foram observados multipletos com deslocamento em δ_H 5,3 sugerindo a presença de hidrogênios olefínicos (C-6) e em δ_H 3,5 que foi atribuído ao hidrogênio carbinólico (H-3).

Após as análises dos espectros e a realização das correlações foi possível estimar que a *Substância I* trata-se de um esteroide, o colesterol. Abaixo apresentamos a molécula proposta (**Figura 6**), bem como os deslocamentos observados no espectro de ^{13}C neste estudo e os dados dos deslocamentos de ^{13}C acerca da molécula proposta presente na literatura (**Tabela 2**).

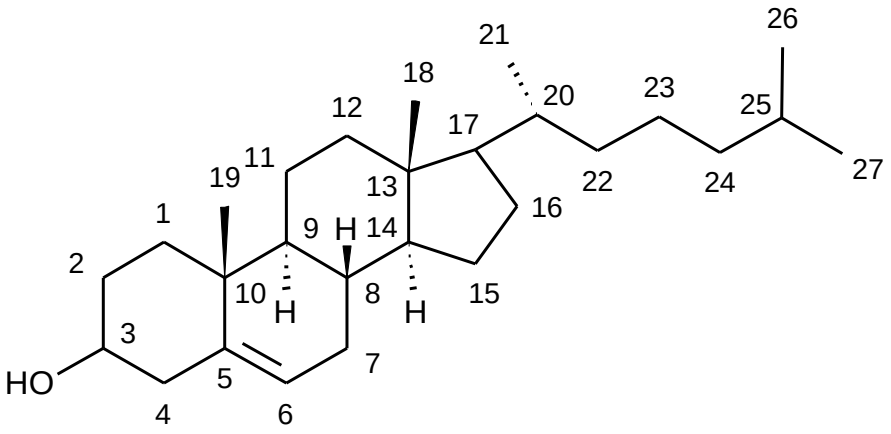


Figura 6. Estrutura da molécula de colesterol isolada de extratos metanólicos da pele de *L. lineatus*.

Tabela 2. Dados de RMN de ^{13}C da *Substância I* identificada como colesterol.

Posição	Dados observados[a]	Dados da literatura[b]
	^{13}C (75 MHz)	^{13}C
1- CH_2	37,23	37,5
2- CH_2	31,65	31,6
3- CH	71,81	71,3
4- CH_2	42,29	42,4
5- C	140,75	141,2
6- CH	121,74	121,3
7- CH_2	31,91	32,0
8-CH	31,89	32,3
9-CH	50,11	50,5
10- C	36,49	36,5
11- CH_2	21,07	21,2
12- CH_2	39,76	28,3
13- C	42,29	42,4
14- CH	56,75	56,9
15- CH_2	21,29	24,3
16- CH_2	28,23	40,0
17- CH	56,12	56,5
18- CH_3	11,86	12,0
19- CH_3	19,40	19,4
20- CH	35,78	35,8
21- CH_3	18,71	18,8
22- CH_2	36,17	36,4
23- CH_2	23,81	24,1
24- CH_2	39,51	39,6
25- CH	28,02	28,0
26- CH_3	22,56	22,5
27- CH_3	22,83	22,8

[a] = deslocamentos químicos do espectro de ^{13}C da molécula colesterol encontrada neste estudo;

[b] = deslocamentos químicos do espectro de ^{13}C da molécula colesterol descrita na literatura (Silverstein *et al*., 2019).

Discussão

Nossas análises mostraram pela primeira vez a existência de um esteroide que identificamos como colesterol na pele de *Lithodytes lineatus* da família Leptodactylidae. A presença de colesterol na secreção cutânea de anuros era conhecida apenas nas famílias Pipidae, Bufonidae, Ranidae e Bombinatoridae e para poucas espécies (Croce e Bolognani, 1975).

Essa descoberta iniciou com o fato das amostras de pele de *Lithodytes lineatus* apresentarem reatividade com Dragendorff na fração 5-6. É conhecido que este reagente apresenta alta sensibilidade à presença de alcaloides, assim como a qualquer substância nitrogenada (Simões *et al*., 1999). A rã *Lithodytes lineatus* pertence à família Leptodactylidae que, até o momento, não possui nenhuma espécie conhecida por sintetizar ou sequestrar alcaloides (Daly, 1995; Saporito *et al*., 2012), esse fato nos levou a pesquisar mais a fundo e então descobrimos que a reação era relacionada a presença de um esteroide.

A presença de colesterol na pele de anuros é considerada relativamente rara, tendo poucos estudos que detectaram a presença deste metabólito (Croce e Bolognani, 1975). É sabido que o colesterol é precursor de alguns compostos como hormônios, vitaminas, alcaloides e esteroides com atividade cardíaca (Nelson e Cox, 2014; Medeiros *et al*., 2019; de Souza et al., 2020). Um dos esteroides cardíacos mais conhecidos sem dúvidas são os bufadienolídeos, encontrados em algumas espécies de anuros não leptodactylídeos, como a espécie *Rhinella marina* (Bufonidae), na qual se sabe que ocorre variação ontogenética na produção de bufadienolídeos (Hayes *et al*., 2009).

Neste estudo, não foi possível testar potenciais atividades biológicas da molécula isolada (colesterol), mas alguns estudos evidenciaram uso de outras moléculas não

esteroides e nem alcaloides para mediar comportamentos de comunicação química. Na perereca *Litoria splendida* é conhecido que ocorre liberação de peptídeos específicos (splendiferina) pelos machos durante todo ano, porém foi determinado que durante o período reprodutivo ocorre aumento na produção e liberação desses componentes, funcionando como um feromônio, uma vez que é utilizado para atração das fêmeas (Wabnitz et al., 2000). Na fase inicial do desenvolvimento de anuros também é possível verificar que algumas espécies utilizam de sinais químicos para alertar ataques aos conspecíficos. Esses sinais são emitidos pelos girinos que estão feridos e são liberados diretamente no ambienta aquático (Fraker *et al*., 2009; Manteifel e Kiseleva, 2011; Maag *et al*., 2012).

É possível verificar que ocorre diferentes usos nos sinais químicos oriundos da grande diversidade de substâncias presentes no tegumento desses animais. Sugerimos que a presença de colesterol nos extratos de pele de *L. lineatus* não seja de fato o componente que inibe a resposta agressiva das formigas, mas o metabólito que funciona como precursor potencial da substância que medeia a associação. Reforçamos que estudos sobre atividade biológica do colesterol isolado dos extratos de pele de *L. lineatus* será testado em estudos futuros, bem como isolamento de outras substâncias presentes no tegumento da rã.

Conclusão

Foi detectada a presença da molécula de colesterol em extratos metanólicos de peles da rã *Lithodytes lineatus*. Este é o primeiro estudo a isolar e elucidar uma substância presente no tegumento desta rã. Dados como o disponibilizado neste estudo são fundamentais para traçar métricas para o melhor entendimento da diversidade química presente no tegumento dessa espécie de rã e acerca da biologia da interação

entre *L. lineatus* e as formigas *Atta,* que atualmente é creditada a presença de substâncias na pele.

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Conflitos de interesse

Não existem conflitos de interesse.

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SÍNTESE

No primeiro capítulo desta tese mostramos que o conhecimento acerca da biologia da interação entre anuros e invertebrados encontra-se muito aquém do que se espera para que seja possível o entendimento dos mecanismos utilizados por estes animais para evitar ou inibir ataque dos residentes de ninhos e tocas a qual ocorre compartilhamento. Ficou evidente que algumas associações têm sido mais bem estudadas (como no caso da associação anuro-aranha), porém, existe um número baixo de estudos que investigaram não apenas aspectos ecológicos como também aspectos químicos que medeiam essas interações, sendo importante que estudos futuros venham a ser conduzidos nesta temática.

No segundo capítulo trazemos evidências de que o uso do gel anestésico a base de benzocaína, comumente utilizado como protocolo de morte em anuros, não interfere em estudos que envolvam a análise da composição química ou potenciais estudos com ensaios biológicos que utilizem extratos da pele de sapos, uma vez que mostramos que a aplicação do produto diretamente na boca impede a contaminação da pele e não é convertida para o tegumento.

No terceiro capítulo, mostramos que ocorre variação geográfica no perfil dos metabólitos secundários presentes em extratos da pele da rã *Lithodytes lineatus*, onde além da distância o tipo de ambiente também influência no perfil químico destes metabólitos, tendo visto que indivíduos coletados em áreas mais preservadas apresentaram perfil químico de metabólitos secundários mais diversos do que os coletados em ambiente alterado ou antropizado.

No quarto capítulo, descrevemos pela primeira vez uma substância isolada a partir de extratos feitos de peles da rã *L. lineatus*. A substância isolada trata-se de um esteroide, o colesterol. Apesar de não realizarmos análises de atividades biológicas, sugerimos que a presença do colesterol pode ser como precursor de potenciais substâncias (feromômonios) que medeiam a associação com formigas *Atta*.

Os resultados apresentados nesta tese representam avanços importantes, não somente no conhecimento acerca da ecologia química da rã *L. lineatus*, mas também para estudos com a composição química da pele de anuros em geral, uma vez que o protocolo de morte utilizado e validado em nosso estudo mostra-se como um meio mais humanizado para acessar substâncias ou potencial biotecnológico presente no tegumento destes animais. Esta tese apresenta grande potencial de replicabilidade para avaliar aspectos da ecologia química em outras espécies de anuros, uma vez que a

região amazônica possui uma alta diversidade de representantes deste grupo taxonômico e para muitos não se conhece absolutamente nada a respeito da diversidade química presente na pele destes animais.