Adriana Moriguchi Jeckel

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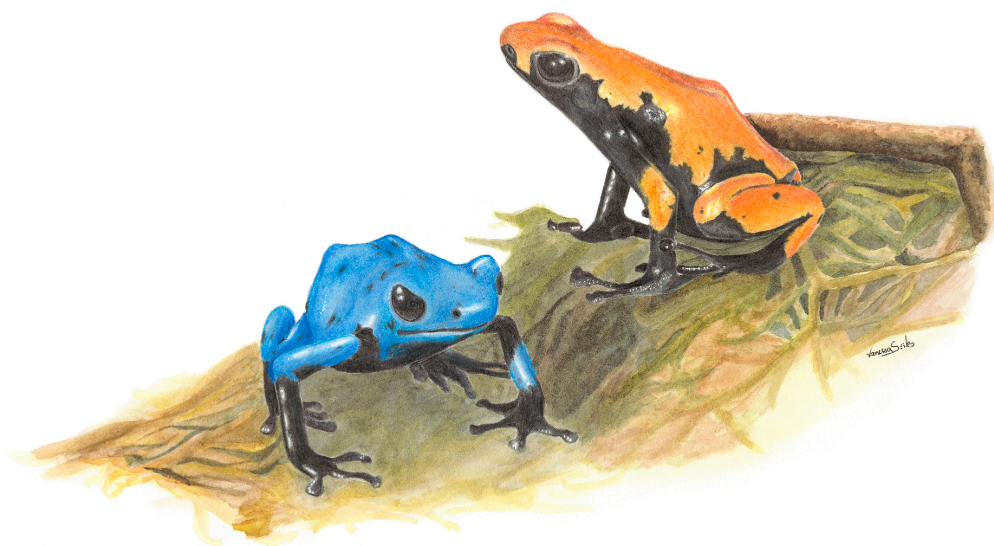
Sequestration efficiency and alkaloid composition in poison frogs of the family Dendrobatidae



São Paulo

2020

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Zoologia.

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Dedicatória

Dedico esta tese aos meus pais,
Emilio e Cristina

Epígrafe

Penso que só há um caminho para a ciência
ou para a filosofia:
Encontrar um problema, ver a sua beleza
e apaixonar-se por ele; casar e viver feliz com ele até que a
morte vos separe — a não ser que encontrem
um outro problema ainda mais fascinante, ou, evidentemente,
a não ser que obtenham uma solução.
Mas, mesmo que obtenham uma solução, poderão então
descobrir, para vosso deleite,
a existência de toda uma família de problemas-filhos,
encantadores ainda que talvez difíceis, para cujo bem-estar
poderão trabalhar, com um sentido,
até o fim dos vossos dias.

Karl Popper

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Introdução Geral

A defesa química ocorre numa interação interespecífica que funciona através de compostos químicos que influenciam negativamente alvos moleculares de predadores ou patógenos, protegendo os organismos portadores dessa forma de defesa (Wink, 2003). As estruturas dos compostos defensivos são similares às estruturas de moléculas endógenas inatas do metabolismo animal e agem tanto por efeito agonista, ligando-se aos receptores celulares com ação similar ao composto inato, quanto por efeito antagonista, bloqueando os receptores celulares (e.g. Wink et al., 1998; Badio & Daly, 1994).

A presença de compostos defensivos evoluiu independentemente em diferentes linhagens, desde as esponjas-do-mar (Taylor et al., 2007) e as grandes linhagens de invertebrados (*e.g.* Cimino & Ghiselin; 1998, Laurent et al., 2003) aos vertebrados (*e.g.* Noguchi et al., 2006; Ligabue-Braun et al., 2012; Ligabue-Braun & Carli, 2015). Atualmente se conhecem milhares de tipos diferentes de compostos, com grandes variações estruturais entre espécies (Wink, 1993; Daly et al., 2005). Além da variação entre diferentes espécies, os compostos defensivos de um único indivíduo também podem ter uma grande diversidade, contendo mais de um tipo de substância (*e.g.* Jeckel et al., 2015), os quais conferem proteção e ação a diferentes tipos de ameaças e moléculas, respectivamente (Wink, 2003). Ademais, essa variedade pode ter importância funcional, interferindo na viscosidade da substância (Blum et al., 1973); ou um papel sinergético, onde a soma de duas ou mais substâncias combinadas é mais eficaz do que a ação de apenas uma (Pasteels, 1983), ou até mesmo como forma de evitar adaptações por parte do predador (Barnett et al., 2014). Alguns compostos são específicos de determinados grupos taxonômicos, podendo ser utilizados como fontes de evidência para inferir hipóteses de relacionamento evolutivo entre grupos, sendo, por isso, usados como evidência na taxonomia e sistemática (*e.g.* Pasteels, 1993; Cei et al., 1967; Grant et al., 2017).

Apesar de normalmente relacionarmos a defesa química com toxicidade letal, a grande maioria dos compostos químicos presentes nesses organismos não são fatais aos predadores nas quantidades apresentadas. Parte da consequência da estratégia de defesa é que os predadores sobrevivam, sejam capazes de aprender e evitem atacar em encontros futuros (Brower et al., 1968; Servedio, 1999). Por isso, os compostos defensivos geralmente causam algum tipo de desconforto, como gosto ruim (e.g. Skelhorn & Rowe, 2006; Bolton et al., 2017), alteração na pressão sanguínea ou no ritmo cardíaco (e.g. Clarke 1997), indigestibilidade e desconforto gastrointestinal (e.g. Brower et al., 1968; Wouters et al.,

2016) ou até mesmo mudanças temporárias de comportamento (e.g. Jumar et al., 2014; Zou et al., 2016). Uma hipótese para explicar a importância desse aprendizado pelos possíveis predadores é o surgimento de colorações brilhantes e disruptivas que servem como aviso e reforço de aprendizado para predadores visualmente orientados, uma estratégia chamada aposematismo (revisão em Mappes et al., 2005). Essa hipótese vem sendo empiricamente comprovada ao longo dos anos para muitos grupos de organismos (*e.g.* Schmidt & Blum, 1977; Rubino & McCarthy, 2004; Saporito et al., 2007). Entretanto, aposematismo e defesa química nem sempre protegem esses organismos de ataques ou predação e/ou infecção por seus inimigos naturais. Muitos predadores adquiriram resistência aos compostos e são capazes de superar o arsenal químico (Geffeney et al., 2005; Despres et al., 2007; Pittendrich et al., 2013; Ujvari et al., 2015).

A resistência a compostos químicos pode ser alcançada através de detoxificação química por enzimas generalistas ou específicas (Hartmann & Ober, 2000; Heidel-Fischer & Vogel, 2015). Predação quase sempre envolve ingestão da presa e, consequentemente, os compostos defensivos estarão expostos ao metabolismo do sistema digestivo do predador. O metabolismo de compostos ativos é amplamente estudado em mamíferos, principalmente por interesses farmacêuticos e econômicos. Em humanos, por exemplo, a concentração total de xenobióticos (compostos químicos estranhos a um organismo, como medicamentos e pesticidas) é diminuída consideravelmente pelo fígado antes de atingir a circulação sistêmica (Pond & Tozer, 1984). As enzimas do sistema do citocromo P450 (CYP450) são as principais responsáveis por modificarem compostos tóxicos em compostos mais estáveis e hidrofílicos, facilitando a excreção no rim. Mamíferos herbívoros, como ovelhas e *hamsters*, e insetos que se alimentam de plantas com defesa química, possuem um arsenal de enzimas CYP450, além de enzimas da família monooxigenase contendo flavina, que permitem que se alimentem dessas plantas para proporcionar estabilização e excreção das toxinas (Ehmke et al., 1990; Miranda et al., 1991; Huan et al., 1998; Hartmann et al., 1999).

Outra forma de resistir às defesas químicas das presas é através de insensibilidade das moléculas alvo, geralmente canais iônicos, através de substituições nas sequências de aminoácidos nos sítios de ligação (Wang & Wang, 1999; Geffeney et al., 2002; Tarvin et al., 2016). Porém, essa substituição pode ter um custo, o que faz com que essa forma de resistência seja menos comum e, quando existe, os aminoácidos substituídos são bastante conservados e convergentes entre diferentes espécies (Feldman et al., 2012; Ujvari et al., 2015). Um caso clássico é o da serpente *Thamnophis sirtalis* (Linnaeus, 1758) que é resistente à toxina da salamandra *Taricha granulosa* (Skilton, 1849), a tetrodotoxina (TTX),

uma das toxinas conhecidas mais potentes, a qual age bloqueando canais de sódio (Mosher et al., 1964). Essas serpentes são capazes de predar essas salamandras devido a uma variedade de mutações no gene que codifica a região de afinidade do TTX com os canais de sódio, a região NaV1.4 (Geffeney et al., 2002). A variedade em tipos e quantidades de mutações de aminoácidos nessa região confere às serpentes diferentes graus de resistência, permitindo se adaptar às concentrações de toxinas das salamandras em populações específicas (Hanifin et al., 2008; Williams et al., 2010). Por exemplo, em localidades onde a salamandra possui concentrações menores de TTX, as mutações são diferentes das mutações em serpentes em regiões onde as presas possuem mais TTX (Hanifin et al., 2008). Isso acontece porque essas mutações têm um custo muito elevado, pois interfere na capacidade de locomoção e reação das serpentes após ingestão da toxina (Feldman et al., 2012). Essa relação é um clássico exemplo de corrida armamentista na coevolução dessas duas espécies (Brodie III et al., 2005). Adaptações para resistir às toxinas das presas parecem ser uma estratégia importante, principalmente para animais que desenvolveram uma dieta especializada.

Procedência dos compostos em organismos quimicamente defendidos

Compostos defensivos podem ser (1) sintetizados pelo próprio organismo (biossintetizados), (2) adquiridos por simbiose ou (3) adquiridos do ambiente. As fontes desses compostos não são mutuamente exclusivas de modo que, por exemplo, o mesmo indivíduo pode sintetizar substâncias e também adquirir do ambiente (*e.g.* Jeckel et al., 2015). Dentre os compostos biossintetizados, alguns são produtos de metabolismo secundário, usando esteroides ou aminoácidos como substrato (Erspamer, 1954; Pasteels, 1983), enquanto outros são proteínas e peptídeos, que são codificados geneticamente (König et al., 2015). Esses compostos são produzidos em tecidos específicos, sendo armazenado ou não em glândulas. A biossíntese, seja por tradução direta das proteínas ou por síntese *de novo* (i.e. síntese de moléculas complexas a partir de moléculas menores) das moléculas, parece ser a forma mais comum dentre as defesas químicas conhecidas nos animais, sendo muito comum em artrópodes terrestres (Whitman et al., 1990) e anuros (Erspamer, 1994).

A outra fonte de compostos de defesa pode ser por associação simbiótica com microrganismos (Flórez et al., 2015). Por exemplo, toxinas encontradas em esponjas das Filipinas, *Theonella swinhoei* (Gray, 1868), são produzidas por bactérias simbióticas filamentosas e unicelulares (Bewley et al., 1996). Porém, o caso mais famoso e ainda controverso é o das bactérias produtoras de TTX encontradas em diversas espécies de peixes

baiacus (revisão por Noguchi et al., 2006) e outros invertebrados marinhos (Chau et al., 2011). Apesar de vários estudos demonstrarem que bactérias produtoras de TTX vivem em simbiose com inúmeras espécies que contém TTX, a rota biossintética ou os genes codificantes de TTX ainda não foram descritos em nenhuma dessas bactérias. Além disso, este tipo de bactérias não foi encontrado em nenhum vertebrado terrestre que possui TTX, como o caso de algumas espécies de anfíbios (Mosher et al., 1964). Acredita-se que em salamandras, o TTX é produzido endogenamente, sem interação simbiótica para a síntese (Lehman et al., 2004). Porém, ainda não existem evidências empíricas que suportem essa hipótese.

Finalmente, a terceira fonte de compostos é por aquisição do ambiente. Alguns gafanhotos, por exemplo, se alimentam de plantas com defesa química e ganham certa proteção enquanto os compostos nocivos estão no trato digestivo. Porém, assim que o trato é esvaziado e/ou a fonte da alimentação modifica, os gafanhotos perdem a proteção química (Sword, 1999). Alternativamente, os compostos podem ser sequestrados da dieta, ou seja, os compostos são ingeridos, absorvidos, transportados e armazenados em tecidos especializados.

Insetos herbívoros evoluíram a capacidade de sequestrar como forma a superar o grande arsenal de metabólitos secundários produzido pelas plantas em resposta à herbivoria (Pasteels, 1983; Wink, 1993). Muitas espécies de insetos são capazes de tolerar a toxicidade e utilizar o composto tanto para defesa quanto para outras funções fisiológicas e ecológicas (Dussourd et al., 1989; Brückmann et al., 2000; Erb & Robert, 2016). Por exemplo, o sequestro de cardenolídeos, um tipo de esteroide do grupo dos glicosídeos cardíacos, é conhecido em diversas ordens de insetos como Lepidoptera (Brower et al., 1984; Black, 1976; Nishio, 1980; Cohen & Brower, 1982), Coleoptera (Dobler et al., 1998; Isman et al., 1977b; Duffey & Scudder, 1972; Nishio et al., 1983), Hemiptera (Rothschild et al., 1970; Duffey & Scudder, 1972; Duffey et al., 1978) e Orthoptera (von Euw et al., 1967). Um caso clássico é o do sequestro de cardenolídeos de plantas do gênero *Asclepsias* pelas larvas de borboletas monarcas (Malcom & Brower, 1989). Os compostos sequestrados enquanto larvas são armazenadas de forma que os protegem mesmo depois da metamorfose, até a fase adulta (Brower et al., 1968). Outro sistema de sequestro em insetos muito estudado é o dos alcaloides pirrolizidinas (AP). Diversas espécies de Lepidoptera (Nickisch-Rosenegk & Wink, 1993) e de Coleoptera (como espécies do gênero *Oreina*, Rowell-Rahier, et al 1991) se alimentam de plantas produtoras de AP e sequestram o alcaloide, transportando-o para glândulas especializadas ou simplesmente acumulando-o na hemolinfa (Hartmann & Ober, 2000).

Dentre os vertebrados, algumas linhagens de aves, serpentes e anfíbios, também adquiriram a capacidade de sequestrar compostos da dieta. A serpente *Rhabdophis tigrinus* (Boie, 1826), por exemplo, alimenta-se de anuros da família Bufonidae (Hutchinson et al., 2007). Esses sapos possuem nas suas glândulas cutâneas altas concentrações de um tipo de glicosídeo cardíaco, o bufodienolídeo, que possui grande afinidade com Na^{+}/K^{+}-ATPase, molécula que mantém o potencial de membrana das células (Agrawal et al., 2012). A *R. tigrinus* tem a capacidade de sequestrar esses esteróis e armazenar em glândulas especializadas na região da cabeça (Hutchinson et al., 2007). Outro exemplo são as espécies de aves da Nova Guiné, dos gêneros *Pitohui* e *Ifrita* que sequestram batracotoxina (BTX) de besouros do gênero *Choresine* (Melyridae; Dumbacher et al., 1992, 2009; Ligabue-Braun & Carli, 2015). A BTX é um dos alcaloides mais tóxicos encontrados na natureza, tendo uma grande afinidade com canais de sódio de membranas de músculos e células nervosas, mantendo-as bloqueadas (Daly et al., 1965). A alta toxicidade da BTX já era conhecida por sua presença em outros vertebrados que sequestram alcaloides: as rãs-de-veneno. As rãs-de-veneno são o grupo de vertebrados mais estudado que sequestra compostos defensivos da dieta. Eles sequestram alcaloides principalmente de formigas e ácaros e os acumulam em glândulas de veneno da pele (Daly et al., 1994, 2005; Smith et al., 2002; Hantak et al., 2013).

O sequestro de compostos químicos da dieta adiciona mais um nível de interação trófica entre os organismos (Fig. 1). O organismo quimicamente defendido, que precisa sequestrar os compostos defensivos, é o predador de um outro animal quimicamente defendido, ambos necessariamente devem ter desenvolvido resistência não só à ação da

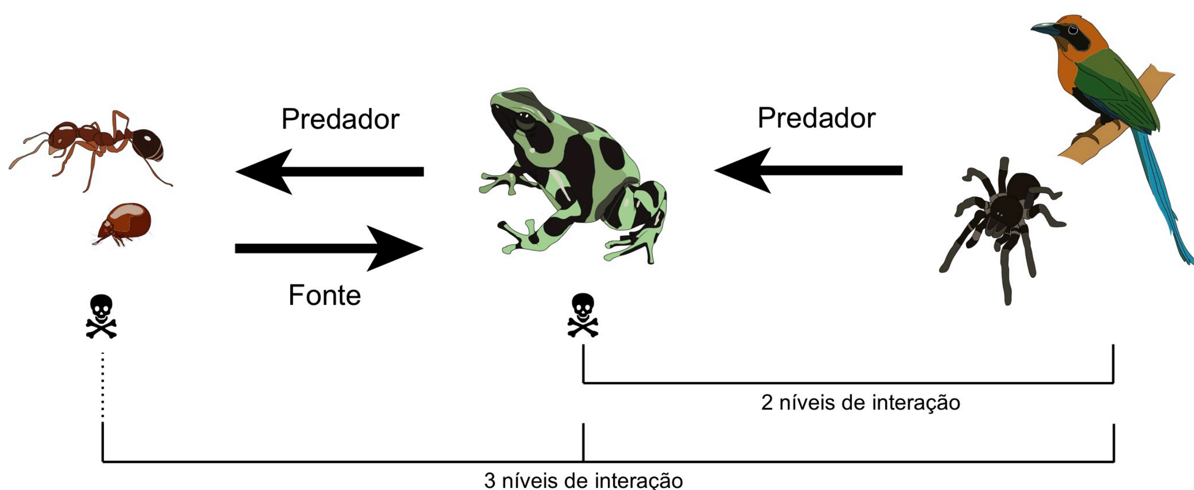

Figura 1. Interações ecológicas em um sistema de presa-predador de animais quimicamente defendidos. Em um sistema onde existe o sequestro de compostos defensivos, uma das presas é a fonte da toxina para o organismo que sequestra.

toxina, mas também ao acúmulo deliberado desses compostos no próprio corpo. Por exemplo, as formigas, que são fonte de alcaloides para as rãs-de-veneno, são produtoras de uma grande variedade de tipos de alcaloides (Edcoubas & Blum, 1990). Devido à toxicidade e função repelente, esses alcaloides são utilizados pelas formigas durante interações agressivas e competitivas (Adams & Traniello, 1981; Adams et al., 2013; Obin & Vander Meer, 1985). As rãs-de-veneno, por sua vez, parecem ter desenvolvido mecanismos que permitem se alimentar desses compostos e acumular sem nenhuma adversidade. Pelo contrário, eles adquiriram vantagens ao sequestrarem esses compostos e se defenderem dos seus próprios predadores. O mesmo sistema acontece em insetos que sequestram metabólitos secundários das plantas. Esses compostos provem proteção às folhas e outros órgãos da planta e têm função repelente para a grande maioria de herbívoros generalistas (e.g Pasteels, 1983; Detzel & Wink, 1995; Wouters et al., 2016). Como forma de inativar ou reduzir a toxicidade dos metabólitos, e ainda proporcionar uma adaptação contra predadores, insetos especialistas em certas espécies de plantas possuem mecanismos que permitem que tais compostos sejam absorvidos e transportados pelo corpo em uma forma estrutural não tóxica. Cada espécie que sequestra um determinado tipo de composto químico desenvolveu mecanismos e estratégias específicas que permitem que esse sistema funcione de forma eficiente.

Adaptações fisiológicas e moleculares do sequestro

A capacidade de sequestro evoluiu independentemente em diversos grupos animais (Daly et al., 1994; Duffey, 1980; Dumbacher et al., 1992; Hutchinson et al., 2007) e, apesar do mecanismo em si variar entre espécies e tipos de compostos químicos, todos eles tiveram que adaptar certos pontos em comum para que a estratégia de defesa fosse eficiente. Essa convergência em diferentes grupos poderia ser resultado de um menor custo fisiológico ao sequestrar um composto já existente ao invés de biosintetizar (Zvereva et al., 2016). Assim, diversas rotas metabólicas não precisariam existir. Entretanto, quando parâmetros fisiológicos e ecológicos são observados, o sequestro não é necessariamente menos custoso ou mais simples (Duffey, 1980; Zvereva et al., 2016). Na verdade, diversas adaptações são necessárias, incluindo mecanismos fisiológicos para absorver e transportar os compostos ao mesmo tempo evitando autointoxicação (Duffey, 1980) e mecanismos comportamentais, como forrageamento, já que dependem da dieta para adquirir a proteção química (Termonia et al., 2001; Darst et al., 2005; Agrawal et al., 2012). As adaptações para evitar autointoxicação são similares ou até iguais a mecanismos comentados anteriormente sobre

predadores adaptados a animais quimicamente defendidos. A diferença no caso de animais que sequestram é que a resistência deve ser eficiente o suficiente para transporte e armazenamento de grandes quantidades do composto defensivo.

Uma das estratégias de se evitar a autointoxicação é por insensibilidade da molécula alvo através de substituições nas sequências de aminoácidos nos sítios de ligação (Wang & Wang, 1999; Tarvin et al., 2016). No entanto, a insensibilidade à toxina pode afetar a sensibilidade dos sítios de ligação ao seu ligante endógeno (Tarvin et al., 2017). Por isso, em proteínas tão conservadas quanto canais iônicos, as substituições possíveis são restritas, resultando em substituições na mesma posição de aminoácido (Ujvari et al., 2016). É o caso da resistência a glicosídeos cardíacos, que surgiu várias vezes independentemente entre invertebrados e vertebrados (Dobler et al., 2012; Bramer et al., 2015; Ujvari et al., 2016; Mohammadi et al., 2016; Holzinger & Wink, 1996; Petschenka et al., 2012) . Os dois tipos de glicosídeos cardíacos já citados, os cardenolídeos e os bufodienolídeos, interagem com a ubíqua enzima de membrana Na^+/K^+-ATPase, bloqueando o transporte de íons (Agrawal et al., 2012). A região mais importante para a ligação desses esteroides com a enzima fica no *loop* extracelular entre os dois primeiros segmentos transmembrana (H1-H2), dentre dez existentes, da subunidade alfa (Agrawal et al., 2012). Estudos revelaram que substituições em dois aminoácidos, nas posições 111 e 122 do loop do H1-H2 são suficientes para conferir baixa afinidade da enzima aos esteroides e consequente resistência às toxinas em diversas espécies de insetos (Dobler et al., 2012), especialmente Lepidoptera (Holzinger & Wink, 1996; Petschenka et al., 2012; Bramer et al., 2015), e em serpentes com dieta especializada em bufonídeos (Ujvari et al., 2015; Mohammadi et al., 2016).

Em rãs-de-veneno, espécies do gênero *Phyllobates* tem insensibilidade ao alcaloide presente nas suas glândulas da pele, o BTX, enquanto outras espécies da mesma família não. Essa insensibilidade se deve a algumas substituições no segmento 6 do domínio I e IV da subunidade alfa de canais de sódio voltagem-dependente (Wang & Wang, 1999; Tarvin et al., 2016). Presume-se que substituições nesses mesmos segmentos também podem conferir insensibilidade à pumiliotoxina e histrionicotoxina, outras duas classes de alcaloides comumente encontrados em anfíbios que sequestram alcaloide (Fig. 2; Tarvin et al., 2016). Insensibilidade à epibatidina, outro alcaloide encontrado em rãs-de-veneno, também foi elucidada recentemente (Tarvin et al., 2017). Este alcaloide interage com receptores nicotínicos de acetilcolina e o sítio de ligação do alcaloide é exatamente no mesmo sítio que a acetilcolina. Apenas uma substituição de aminoácidos nesse sítio foi encontrada, o suficiente para diminuir a sensibilidade do sítio à epibatidina, mas também à acetilcolina. Encontraram,

então, que outras substituições em regiões diferentes do receptor resgataram a capacidade da acetilcolina de se ligar, mantendo a insensibilidade ao alcaloide (Tarvin et al., 2017).

Apesar de substituições de aminoácidos aos alvos moleculares desses compostos proverem insensibilidade permitindo resistência e sequestro, elas podem não ser suficientes para alguns táxons devido a grande e complexa variedade de compostos que um mesmo indivíduo pode possuir (Pasteels, 1983; Jeckel et al., 2015a). Um exemplo desta grande

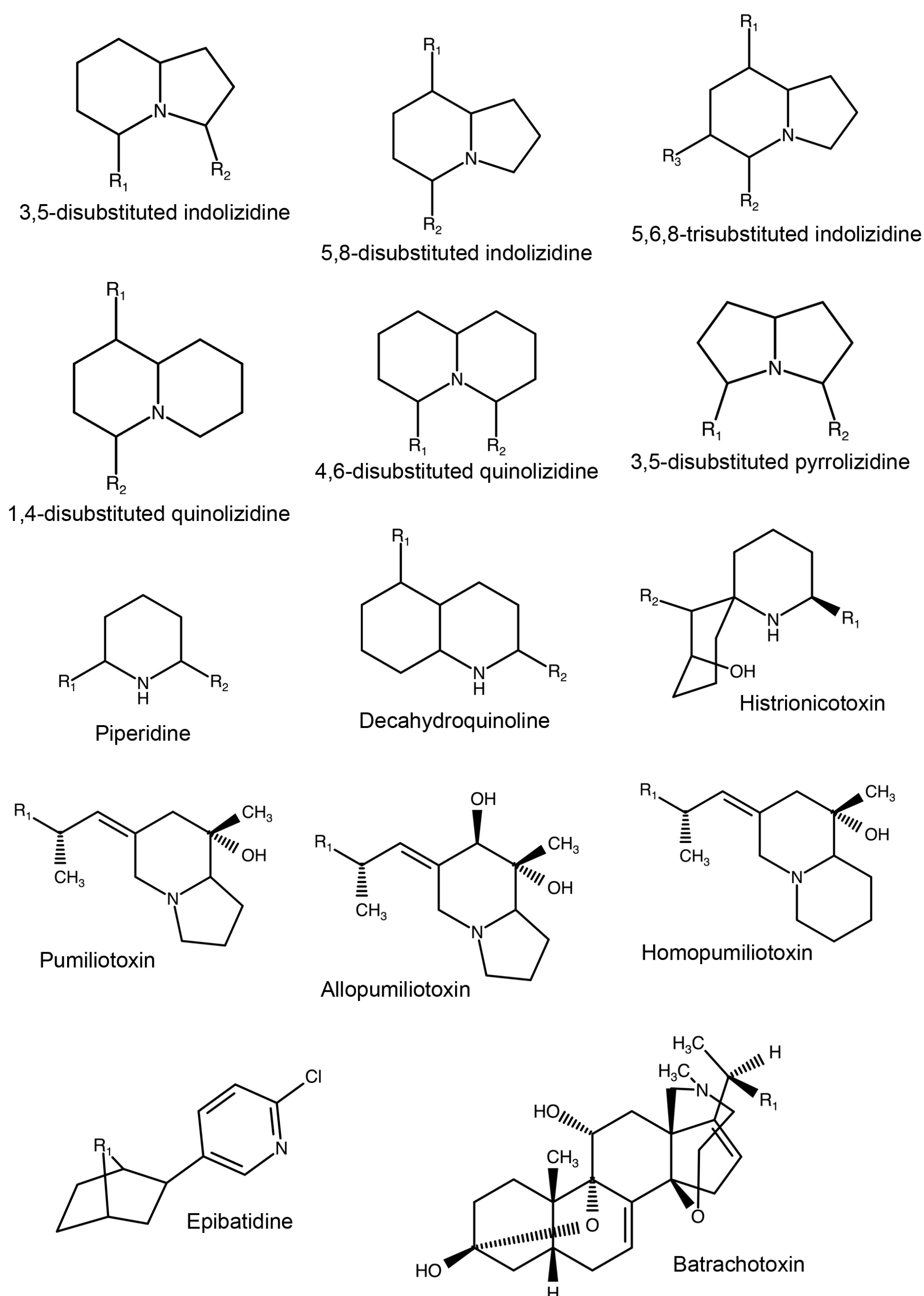

Figure 2. Estrutura química dos alcaloides comumente encontrados em rãs-de-veneno.

variação são as próprias rãs-de-veneno, que podem apresentar na sua pele dezenas de tipos de alcaloides diferentes, que podem ter ações variadas em diferentes moléculas-alvo (revisão em Daly et al., 2005). Por isso, além da insensibilidade, vias fisiológicas de resistência e metabolização dos compostos podem ter um papel importante na prevenção da autointoxicação.

Algumas estratégias de metabolização já foram descritas para insetos herbívoros. Essas estratégias servem para armazenar o composto em uma forma menos nociva ou para facilitar o sequestro, modificando a polaridade dos compostos, já que o transporte transmembrana de compostos apolares é mais fácil, e a manutenção de compostos polares no citosol é menos custoso (Duffey, 1980; Lindigkeit et al., 1997). Um exemplo bem estudado é o das larvas de algumas espécies de mariposas. As plantas possuem alcaloides pirrolizidina (AP) na forma N-oxidada (hidrofílica), que é a forma não tóxica. Quando ingerido, o AP é reduzido para sua forma de alcaloide terciária (lipofílica) no trato digestivo, ficando susceptível à ação das enzimas monooxigenase P450. As enzimas P450 participam na metabolização de xenobióticos lipofílicos, convertendo-os em metabólitos excretáveis. Porém, no caso de APs, eles convertem o alcaloide para uma forma instável, bioativando o composto e tornando-o tóxico. Os insetos capazes de sequestrar AP possuem as enzimas flavoproteicas que rapidamente N-oxidam o alcaloide para sua forma não tóxica, permitindo acúmulo e armazenagem (Hartmann e Ober 2000). Essa forma de estabilização dos compostos para sequestro também ocorre com a lagarta-da-raiz do milho (*Diabrotica virgifera virgifera* [LeConte, 1868]). Eles sequestram benzoxazinoides (BXD) presentes na planta do milho e acumulam principalmente na hemolinfa e na quitina do exoequeleto (Robert et al., 2017). As plantas armazenam BXD na sua forma glicosilado, ou seja, com uma molécula de glicose acoplada. Quando atacada por um herbívoro, no próprio citoplasma da planta a BXD é deglicosilada, se tornando tóxica e fazendo com que o herbívoro cesse a alimentação (Wouters et al., 2016). As lagartas, por sua vez, se alimentam desses compostos e glicosilam os BXD no seu próprio trato digestivo, impedindo a ação tóxica e acumulando os compostos para defesa contra seus próprios entomopatógenos (Robert et al., 2017).

Diferente da detoxificação de compostos nocivos, o sistema de sequestro necessita que os compostos mantenham sua estrutura química básica intacta para manter também a sua funcionalidade. Por isso, existe uma grande variedade de sistemas em que cada espécie apresenta formas únicas de lidar com compostos específicos, permitindo absorção, transporte e armazenamento evitando a autointoxicação, mas mantendo a natureza defensiva do

composto sequestrado. Assim, o foco deste estudo é o sistema de sequestro de alcaloides por uma das linhagens de rãs-de-veneno, as rãs da família Dendrobatidae.

Rãs-de-veneno

Aproximadamente 160 espécies de anuros são capazes de sequestrar alcaloides da dieta, formando um conjunto polifilético de ampla distribuição geográfica chamado "rãs-de-veneno" (Fig. 3A), que inclui membros das famílias Bufonidae (*Melanophryniscus*; Daly et al., 1984), Dendrobatidae (gêneros *Ameerega*, *Epipedobates* e subfamília Dendrobatinae; Myers et al., 1978), Eleutherodactylidae (parte do grupo de espécies de *Eleutherodactylus limbatus*; Rodriguez et al., 2010), Mantellidae (*Mantella*; Daly et al., 1984) e Myobatrachidae (*Pseudophryne*; Daly et al., 1984). Todos esses gêneros não-relacionados compartilham diversas características, que podem estar ligados à capacidade de sequestro de alcaloides. As rãs-de-veneno são diurnas (Santos & Grant, 2010); são micrófagas, ou seja, se alimentam de presas pequenas (Toft, 1995; Bonanseira & Vaira, 2007; Moskowitz et al., 2018); tem tamanhos similares (15–45mm de comprimento rostro-cloacal); habitam normalmente a serapilheira dos ambientes; e geralmente apresentam coloração apossemática, ou seja, coloração que sinaliza presença de químicos defensivos para predadores visualmente orientados (Saporito et al., 2007b; Noonan & Comeault, 2008; Bordignon et al., 2018).

Dentre os dendrobatídeos, a capacidade de sequestrar alcaloides parece ter surgido pelo menos quatro vezes ao longo da filogenia da família (Grant et al., 2017), incluindo 10 dos 16 gêneros descritos (*Adelphobates, Andinobates, Ameerega, Dendrobates, Epipedobates, Minyobates, Oophaga, Paruwrobates, Phyllobates* e *Ranitomeya*; Fig. 4), e somando quase metade das 200 espécies da família (Frost, 2020). Apesar da monofilia do grupo ser bem suportada (Grant et al., 2017), a sua posição em relação a outros anuros ainda parece variar muito (e.g. Pyron 2014; Feng et al. 2017; Jetz and Pyron 2018). Os dendrobatídeos tem uma distribuição geográfica ampla, desde a Nicarágua passando pela bacia amazônica da Bolívia e sudeste brasileiro, até as Guianas (Frost, 2020).

Esse grupo é famoso por suas colorações brilhantes (Fig. 3B) e por seu diverso e muitas vezes complexo modo reprodução e cuidado parental. Diferentemente do modo clássico de reprodução de anfíbios em grandes corpos d'água, os dendrobatídeos desovam nas folhas da serapilheira ou em fitotelmos de bromélias e outras cavidades naturais. Além disso, algumas espécies possuem modos elaborados de cuidado parental, que podem envolver desde comportamento como o transporte de girinos no dorso para fitotelmos, até provisão de

ovócitos nutritivos como a única fonte de alimentação da larva. Outra característica desse grupo, que também pode estar relacionado com a reprodução, é a grande variedade de polimorfismo em algumas espécies. Estudo mostraram que as fêmeas podem basear a sua escolha de parceiro pela coloração do macho, e que a sua preferência pode ser resultado do *imprinting* durante a fase larval, enquanto foi carregado pelos seus progenitores (Yang et al., 2019). Todas as rãs desse grupo são diurnos e são forrageadores ativos, sendo que as espécies que sequestram são especialistas em formigas e ácaros (Santos et al., 2003; Darst et al., 2005), principal fonte dos alcaloides defensivos.

Mais de 1200 alcaloides de 28 classes estruturais diferentes já foram reportados para o grande grupo das rãs-de-veneno (Fig. 2; Daly et al., 2005; Jeckel et al., 2019). Acredita-se que os ácaros e formigas fornecem quase todos os tipos de alcaloides encontrados na pele desses anfíbios (Saporito et al., 2004, 2007). Devido a origem dos compostos defensivos, uma característica importante desse sistema é a grande variação inter e intraespecífica de tipos, quantidade e composição de alcaloides. Fatores como a localização geográfica (Saporito et al., 2006, 2007), a estação (Saporito et al., 2010a), a idade e os estágios de vida (Daly et al., 2002; Stynoski et al., 2014; Jeckel et al., 2015) e o tamanho corporal (Saporito et al., 2010b) interferem diretamente na composição e quantidade de alcaloides presentes em cada indivíduo e população.

Além das causas dessa grande variação, as consequências também são importantes para entender como, quanto e se essa característica funciona como um mecanismo de defesa contra os inimigos naturais das rãs-de-veneno. A relação da presença de alcaloides com a coloração é sinalizada através da coloração aposemática desses animais para seus predadores visualmente orientados, em especial as rãs da família Dendrobatidae (Saporito et al., 2007b; Noonan & Comeault, 2008). A relação direta entre parâmetros visuais e a diversidade de alcaloides, porém, ainda é controversa. Alguns estudos demonstraram que indivíduos de populações com parâmetros de brilho ou tonalidade mais altas, tendem a ser mais “tóxicas” do que indivíduos com a pele com coloração mais opaca (Summers & Clough, 2001; Maan & Cummings, 2012). Entretanto, outros estudos encontraram relação inversa ou até mesmo nenhuma relação entre esses fatores (Daly & Myers, 1967; Wang, 2011; Lawrence et al., 2019). Independentemente dos valores dos parâmetros visuais, as colorações dão um sinal

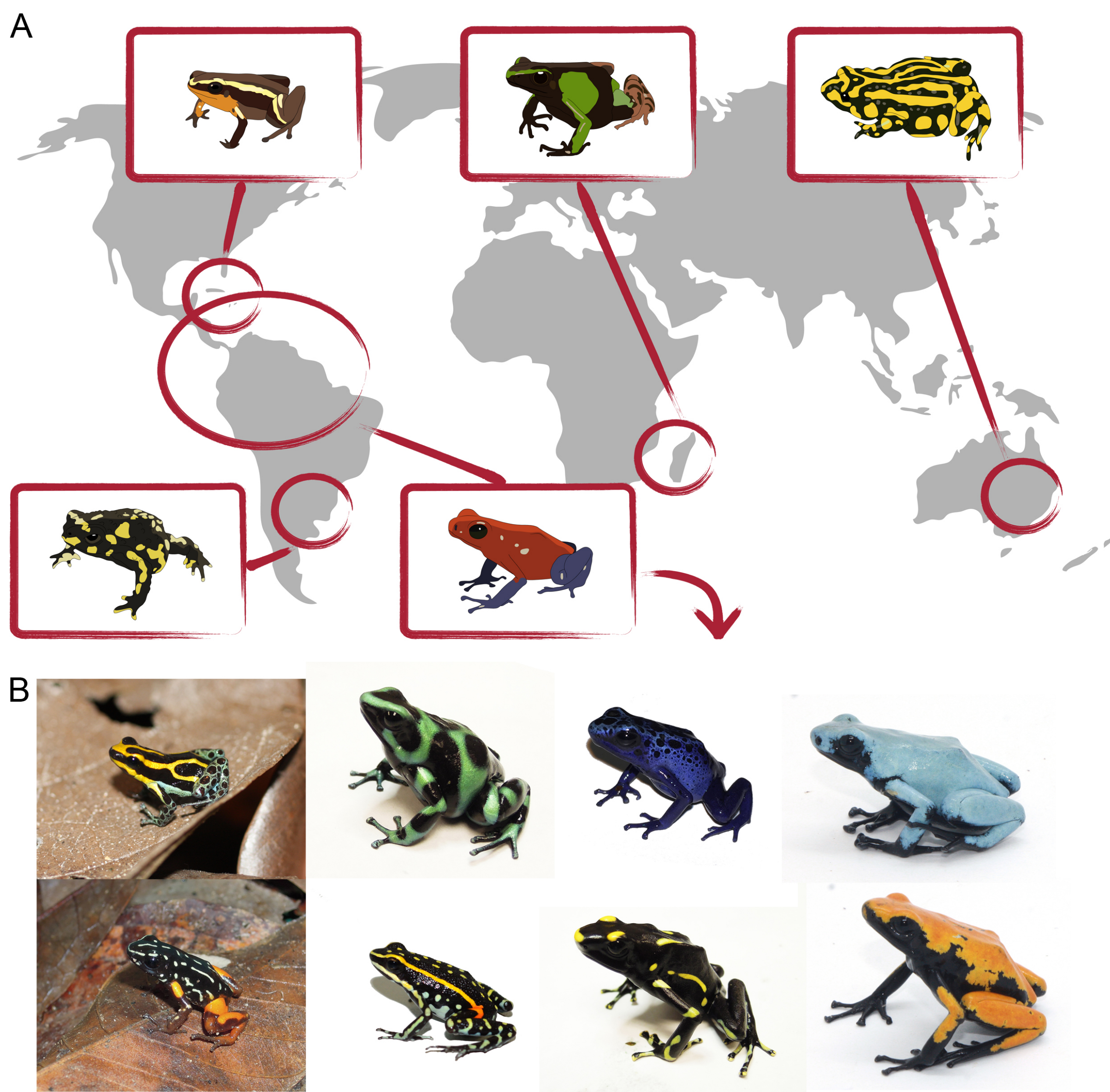

Figura 3. A) Distribuição geográfica das cinco famílias de anuros que sequestram alcaloides: Eleutherodactylidae (parte do grupo *Eleutherodactylus limbatus*) de Cuba, Mantellidae de Madagascar, Myobatrachidae da Austrália, Bufonidae da região sul da América do Sul, e Dendrobatidae, da região Neotropical das Américas Central e Sul. B) Rãs-de-veneno da família Dendrobatidae. Da esquerda para direita, de cima para baixo: *Ranitomeya amazônica, Dendrobates auratus, Dendrobates tinctorius, Adelphobates galactonotus, Adelphobates castaneoticus, Ameerega flavopicta* (foto de M. Anganoy-Criollo), *Dendrobates tinctorius, Adelphobates galactonotus.*

honesto da presença de compostos nocivos para os seus predadores visualmente orientados (Stuckert et al., 2014, 2018).

Parte da variação encontrada entre indivíduos, populações e espécies também pode ser consequência de variações no mecanismo de sequestro. Apesar de se intuir que o mecanismo deva ser similar, se não igual, para as espécies que sequestram dentre os

Dendrobatidae, estudos com alimentação controlada de alcaloides mostram que nem sempre o sistema responde de forma igual a todos os tipos de alcaloide. Nos primeiros experimentos de alimentação com alcaloides, diferenças no sequestro entre tipos de alcaloides já eram evidenciados. Por exemplo, *Dendrobates auratus* (Girard, 1855) prontamente sequestrou as decahidroquinolinas, pirrolidinas, indolizidinas, quinolizidinas e histrionicotoxinas oferecidas na alimentação, mas não sequestrou a pirrolidina 2,5-disubstituída nem a piperidina 2,6-disubstituída (Daly et al., 1994). Modificações de alcaloides também evidenciam que nem toda diversidade de alcaloides é diretamente resultado da disponibilidade na dieta. Por exemplo, *Adelphobates castaneoticus* (Caldwell & Myers, 1990)*, A. galactotnotus* (Steindachner, 1864) e *D. auratus* sequestram e hidroxilam o alcaloide PTX **251D** em um composto cinco vezes mais tóxico, a alopumiliotoxina (aPTX) (+)-**267A**, enquanto que *Epipedobates anthony* e *Phyllobates bicolor* sequestram PTX **251D** sem modificações (Daly et al., 2003). Além disso, as mesmas espécies que modificam o PTX **251D** parecem não sequestrar eficientemente o alcaloide decahidroquinolina (DHQ) **233F** (Daly et al., 2003). A variação e a especificidade do sistema ficam ainda mais complexas quando são oferecidos alcaloides que não são encontrados naturalmente em certas espécies. Por exemplo, *Oophaga pumilio* (Schmidt, 1857) sequestra uma forma não natural de DHQ (apenas o esqueleto da estrutura básica do alcaloide, sem as substituições naturais nas posições 2 e 5), armazenando o alcaloide nas glândulas da pele e até transferindo o alcaloide para os ovócitos de alimentação para seus girinos (Saporito et al., 2019). Um outro exemplo é a epibatidina, um tipo de alcaloide encontrado apenas em espécies do gênero *Ameerega* e *Epipedobates* (Spande et al., 1992). Quando ela foi oferecida para duas espécies do gênero *Dendrobates*, apenas *D. auratus* sequestrou o alcaloide, enquanto *D. tinctorius* (Cuvier, 1797) não sequestrou (Sanchez et al., 2019). Esses exemplos demonstram que todos os alcaloides presentes na pele desses animais provêm da dieta, mas nem todos os alcaloides disponíveis na dieta são sequestrados e armazenados nas glândulas da pele.

Neste contexto, o objetivo geral da presente tese de doutorado foi avaliar como o mecanismo de sequestro pode interferir na variação de tipos, quantidade e composição geral de alcaloides encontrados em rãs-de-veneno da família Dendrobatidae.

Apresentação dos capítulos da tese

Devido à grande variedade de colorações exuberantes e ao seu comportamento de atividade diurna, existe um grande mercado para criadores desses anfíbios como animais de estimação nos países europeus e norte-americanos, ou como atrações em zoológicos e

aquários do mundo inteiro. Independente de questões éticas e legais, como o possível incentivo à biopiratária, o movimento de criação *ex situ* de rãs-de-veneno produziu uma gama de informações sobre a manutenção e reprodução das mesmas em cativeiro (Lötters et al., 2010), facilitando a sua criação também para fins científicos. Como as rãs-de-veneno adquirem as toxinas da alimentação, os animais nascidos em cativeiro são totalmente desprovidos de qualquer tipo de alcaloide na pele. Esta característica permite que experimentos de alimentação controlada de alcaloides possam ser feitos em laboratório para testar diversas hipóteses sobre o sequestro e, também, sobre a relação do alcaloide com outros fatores, como a coloração da pele e o comportamento.

Para a primeira parte deste estudo, foi eleita a espécie *Adelphobates galactonotus* como modelo, por ser uma espécie brasileira que metaboliza um tipo de alcaloide (Daly et al., 2003). Essa espécie está distribuída na região das florestas de várzea ao sul do rio Amazonas, a leste do rio Tapajós e até a foz do rio Amazonas (Frost, 2020), e tem uma grande variedade de coloração dorsal, com populações que variam de amarelo, laranja e vermelho a azul claro, todas elas contrastando com a coloração ventral preta (Hoogmoed & Ávila-Pires, 2012). Em janeiro de 2017, foram coletados indivíduos adultos de *A. galactonotus* na Floresta Nacional de Caxiuanã, Pará, Brasil. Na região da baía de Caxiuanã, ocorrem dois morfotipos de coloração contrastante, o laranja e o azul claro, cada tipo ocorrendo em lados opostos da baía (Hoogmoed & Ávila-Pires, 2012). A fim de identificar os alcaloides presentes naturalmente nos indivíduos dessas duas populações e, para garantir o sucesso da reprodução em cativeiro independente do morfotipo, foram coletados indivíduos das duas populações e transportados para as dependências do Biotério do Laboratório de Biologia Celular do Instituto Butantan, em colaboração com o Prof. Carlos Jared e Profª Marta Maria Antoniazzi. Após identificação de todos os indivíduos através de foto-identificação e determinação do sexo, através da dilatação relativa da região distal da última falange dos dedos da mão (Lötters et al., 2010), estabeleceram-se casais para serem mantidos em terrários apropriados para reprodução (Lötters et al., 2010). Dez indivíduos (5 de cada morfotipo) foram utilizados para avaliar a variação de composição de alcaloides e de palatabilidade entre as duas populações. Esta fase foi importante para a determinação dos tipos de alcaloides sequestrados nessas populações e para verificar possíveis diferenças de quantidade ou predominância de algum determinado tipo de alcaloide. Esse estudo resultou no **capítulo 1** desta tese, intitulado "*Geographically separated orange and blue populations of the Amazonian poison frog* Adelphobates galactonotus *(Anura, Dendrobatidae) do not differ in alkaloid composition or palatability*", publicada na *Chemoecology*, em novembro de 2019.

Para a segunda parte deste estudo, e a primeira parte experimental, o objetivo foi investigar se o sequestro de alcaloides, tanto no tipo quanto na quantidade, é limitado pela disponibilidade de alcaloides na dieta ou pelo próprio mecanismo de sequestro. Para responder à pergunta, foram testados dois tipos de alcaloides, oferecidos em três concentrações diferentes de cada tipo para as rãs. A hipótese era de que o mecanismo de sequestro seria o mais eficiente possível, sequestrando todo o alcaloide disponível, independente da quantidade. Isso porque, como essas rãs dependem do alcaloide disponível na alimentação para sequestro, os sistemas de absorção, transporte e armazenagem seriam quase que 100% eficientes. Mas, antes da finalização desta etapa do experimento, desenvolveu-se no laboratório um novo método de alimentação de alcaloides de uma forma que fosse possível quantificar o alcaloide oferecido e sequestrado para cada indivíduo experimental.

Apesar dos aspectos ecológicos relacionados aos alcaloides tegumentares de rãs-de-veneno serem estudados desde os anos 1960, o sequestro só foi confirmado em meados de 1990. O método utilizado para experimentos de alimentação, desde então, é a indução da ingestão de alcaloides através de uma mistura de alcaloide em pó com a vitamina de dieta pulverizada em drosófilas (Daly et al., 1994; Hantak et al., 2013). Os grãos ficam grudados em pequenas quantidades nas moscas e os sapos se alimentam e ingerem os alcaloides. O sequestro é então comprovado depois de alguns dias ou semanas de alimentação, analisando a pele desses animais, através de métodos de separação e identificação de compostos, como cromatografia líquida ou gasosa acompanhada de análise de espectrometria de massas. Esse método de alimentação de alcaloides foi e ainda é amplamente utilizado em experimentos de sequestro com as diversas linhagens de rãs-de-veneno (Hantak et al., 2013; Saporito et al., 2019; Sanchez et al., 2019). A desvantagem desse método é que não permite a quantificação do alcaloide ingerido, permitindo comparar eficiência de sequestro apenas indiretamente, comparando proporção de dois ou mais alcaloides sequestrados em relação a proporção oferecida (Hantak et al., 2013).

No **capítulo 2**, foi descrito o novo método de administração de alcaloide em rãs-de-veneno que consiste em, utilizando uma micropipeta, injetar uma solução de alcaloide dissolvido em álcool 50% diretamente na parte posterior da boca do animal. Já que esse método permite a administração de quantidades exatas de alcaloide no organismo, outro método descrito foi de determinação da concentração de alcaloide a ser administrada por vez. Como o objetivo desse experimento era testar a eficiência de sequestro, a quantificação de alcaloide administrada por dia deveria ser biologicamente relevante. Era importante não

exceder na quantidade administrada por dia, pois não havia informação se isso poderia desencadear outro tipo de reação do animal, como uma reação de detoxificação. Assim que os protocolos de alimentação foram estabelecidos, neste capítulo, foi demonstrado que o mecanismo de sequestro responde de forma diferente para cada alcaloide testado e que a eficiência difere dependendo da concentração administrada por dose. O capítulo, intitulado *"Dose dependent sequestration efficiency in poison frogs"*, será submetido à revista *Physiological and Biochemistry Zoology*.

O terceiro aspecto investigado foi a distribuição espaço-temporal do alcaloide no corpo depois de ingerido. Ao avaliar quais órgãos e tecidos entram em contato com o alcaloide antes de ser armazenado na pele, pode-se inferir que tipo de transportadores estariam envolvidos no sequestro e onde ocorreriam os mecanismos como a modificação de alcaloides. A distribuição anatômica dos alcaloides em animais coletados da natureza foi reportada para poucas espécies, demonstrando presença de alcaloides em tecidos como fígado, músculo e ovário (Grant et al., 2012; Stynoski et al., 2014). Esse tipo de avaliação não permite sugerir o envolvimento desses tecidos com o sequestro em si, pois em animais coletados da natureza não se sabe quando e o que comeram pela última vez. Com o objetivo de determinar a distribuição dos alcaloides em determinados tempos depois da administração, utilizou-se o método de imageamento através de espectrômetro de massas (*Mass Spectrometry Imaging* - MSI). Esse método separa, ioniza e detecta as substâncias diretamente do espécime, produzindo um conjunto de espectros de massa representando a composição molecular de um pixel de uma secção de um tecido (McDonnell & Heeren, 2007). Através de um software especializado, os pixels são combinados para montar a imagem final do corte analisado. A vantagem desse método sobre outros como histoquímica ou imunohistoquímica é que, além de permitir detectar mais de um tipo de composto por vez, também não necessita de marcadores com afinidade específica a determinados tipos de moléculas. Dependendo da técnica de espectrometria de massas, é dispensado qualquer tipo de tratamento ao tecido alvo, minimizando contaminação ou lavagem da molécula (Cooks et al., 2006). O método específico empregado foi o chamado imageamento por espectrometria de massas com ionização de dessorção por *eletrospray* (DESI-MSI; Wiseman et al., 2008). O treinamento e a obtenção dos dados com essa técnica, de julho de 2018 a janeiro de 2019, foram possíveis com a concessão da Bolsa de Estágio de Pesquisa no Exterior (BEPE) pela FAPESP e pela acolhida no laboratório do Dr. Demian Ifa, na York University, em Toronto, Canadá. Durante esse período, desenvolveu-se um protocolo de criossecção e de detecção de alcaloides em cortes de corpo inteiro em rãs, que resultou, em abril de 2020, em uma

publicação de descrição do método na *Journal of Mass Spectrometry* intitulado "*Use of whole-body cryosectioning and desorption electrospray ionization mass spectrometry imaging to visualize alkaloid distribution in poison frogs*" (**capítulo 3**).

Ao combinar o método de MSI com o método já comumente empregado pelo nosso grupo de pesquisa, o GC-MS, foi possível o mapeamento e a quantificação (em diferentes tempos após a administração) de alcaloides no corpo de rãs que receberam uma dose única de alcaloides. Dessa forma, o objetivo desse capítulo foi determinar a distribuição espaço-temporal dos alcaloides após a ingestão. Com esses dois métodos pudemos avaliar a rapidez de sequestro, além da eficiência de uma dose única. Nesta terceira etapa do projeto, desenvolvida totalmente na York University (DESI-MSI) e na John Carroll University (GC-MS), utilizou-se como modelo a espécie *Dendrobates tinctorius.* Esta espécie foi usada para estudos anteriores com alimentação de alcaloide e é facilmente adquirida no mercado de animais de estimação nos Estados Unidos. O **capítulo 4**, intitulado "*Sequestration timeframe and systemic distribution of two alkaloids in a Dendrobatid poison frog*", sugere pela primeira vez que o mecanismo de sequestro é ainda mais rápido do que anteriormente sugerido e que a distribuição anatômica do alcaloide não varia entre os dois alcaloides testados. Este capítulo será submetido ao *Journal of Experimental Biology*.

O principal aspecto que interfere na presença de alcaloides, obviamente, é a capacidade de sequestrar ou não os alcaloides disponíveis na dieta. Na família Aromobatidae, o grupo irmão de Dendrobatidae (Grant et al., 2017), existem espécies simpátricas com várias espécies de rãs-de-veneno, porém nenhuma análise detectou acúmulo de alcaloide nessas espécies. Além disso, dentro da família Dendrobatidae, existem diversos gêneros em que nenhum alcaloide foi detectado na pele (Fig. 4). Em um estudo (Daly et al., 1994), *Allobates talamancae* (Cope, 1875; Aromobatidae) e *Colostethus panamansis* (Dunn, 1933; Dendrobatidae) foram alimentados com dieta contendo alcaloides por cinco semanas, o mesmo tratamento que as rãs-de-veneno *Dendrobates auratus* e *Phyllobates bicolor* Bibron, 1840 (Dendrobatidae). As duas espécies de rãs-de-veneno sequestraram eficientemente o alcaloide provido na dieta, enquanto o *Allobates* e *Colostethus* não acumularam nada do alcaloide na pele. Mesmo assim, não foi reportado nenhum tipo de efeito adverso nessas duas

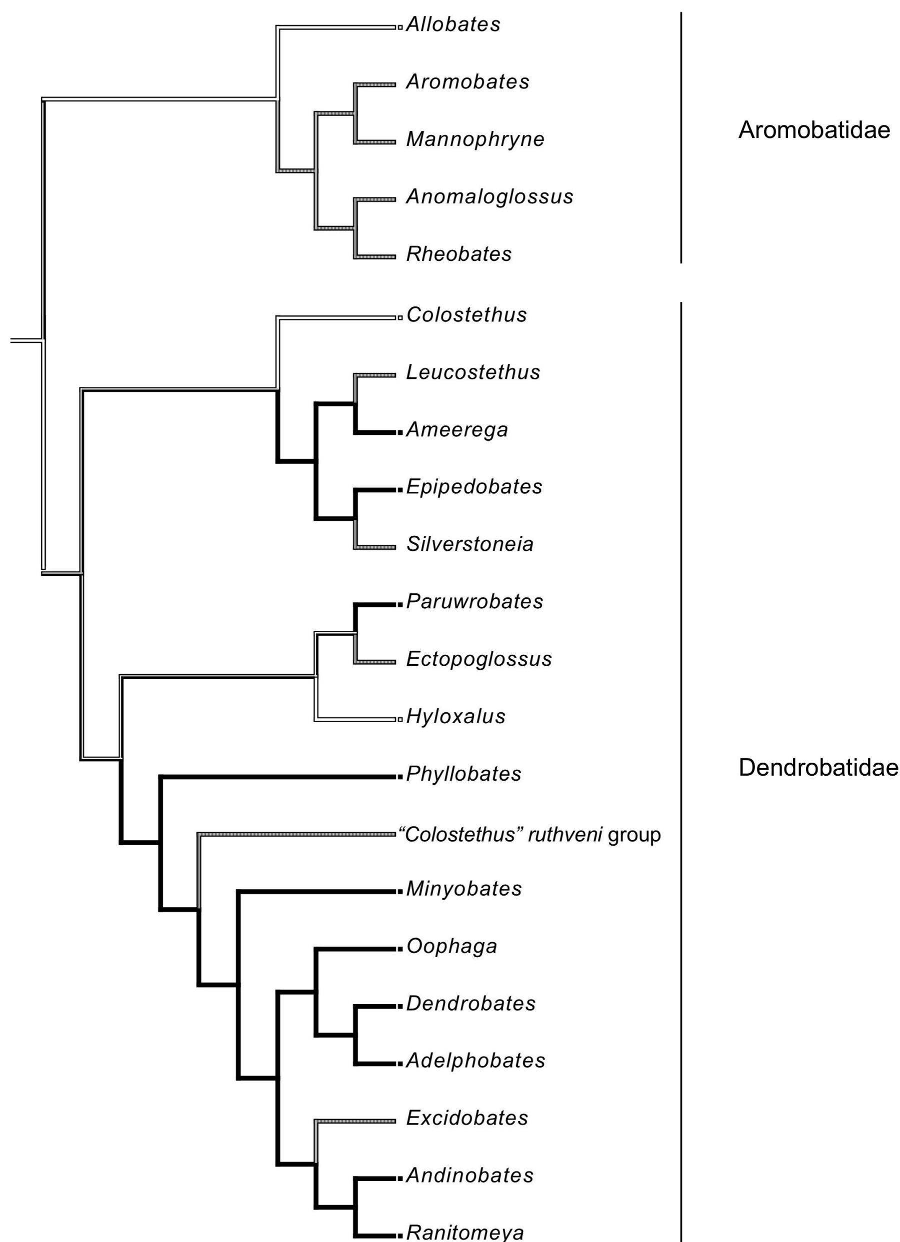

Figura 4. Distribuição filogenética da presença e ausência de alcaloides na pele. Em preto, os gêneros em que foram testados e foram detectados alcaloides; em branco, os gêneros que foram testados e não foram encontrados alcaloides; e em cinza, os gêneros sem informação sobre presença de alcaloides. Topologia retirada de Grant et al 2017.

espécies. Como o alcaloide provém da dieta, esses compostos devem estar expostos a caminhos farmacocinético de um xenobiótico qualquer, sofrendo os mecanismos necessários para excreção. Independentemente do mecanismo de resistência à autointoxicação, presume-se que a resistência tenha surgido antes da capacidade de sequestro (Darst et al., 2005; Mohammadi et al., 2016). Provavelmente, nos animais que sequestram, esse caminho metabólico é modificado para possibilitar o sequestro e o armazenamento dos compostos.

Esse caminho metabólico, porém, pode resultar também em modificação de determinados alcaloides, como comentado anteriormente. Enzimas específicas modificam alcaloides sequestrados da dieta em outros alcaloides (Daly et al., 2003), podendo ter um papel na diversidade dos alcaloides encontrados na pele de diferentes espécies. A última etapa do projeto então, foi testar se a resistência a alcaloides é plesiomórfica para rãs-de-veneno e seus parentes que não sequestram, como membros da família Aromobatidae. Além disso, comparou-se a eficiência de sequestro e a capacidade de modificação de alcaloide em diferentes espécies de rãs-de-veneno em diferentes experimentos de alimentação de alcaloide. Este capítulo sugere a existência de um mecanismo plesiomórfico em anuros para enfrentar os efeitos nocivos dos alcaloides, e que, aparentemente, foi diminuído em rãs-de-veneno, permitindo a evolução da capacidade de sequestro. O **capítulo 5** é intitulado "*The evolution of sequestration of alkaloids in poison frogs*" e será submetido para a *Proceeding of the National Academy of Sciences*.

Todos os capítulos serão formatados em estilo de manuscrito a ser submetido aos jornais citados. Por questões estéticas, as formatações como títulos, margem e espaçamento de linhas seguirão o padrão sugerido pelo modelo de dissertações do Departamento de Zoologia do Instituto de Biociências da Universidade de São Paulo.

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Capítulo 1 - Geographically separated orange and blue populations of the Amazonian poison frog *Adelphobates galactonotus* (Anura, Dendrobatidae) do not differ in alkaloid composition or palatability

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Abstract:
As is typical of chemically defended animals, poison frogs present high variability in their alkaloid-based defenses. Previous studies have shown that geographically separated color morphs of *Oophaga* and *Dendrobates* species differ in both alkaloid composition and arthropod palatability. Here, we tested the generality of that finding by studying the alkaloid composition and palatability of geographically separated blue and orange morphs of the splash-backed poison frog, *Adelphobates galactonotus*. We identified and quantified the alkaloid composition of each individual frog using GC-MS and evaluated the palatability of individual secretions to arthropods conducting feeding trials with *Drosophila melanogaster*. Despite their conspicuous differences in color and separation on opposite sides of a large aquatic barrier, the two morphs did not differ in alkaloid composition or palatability. This result shows that both color morphs are equally chemically protected and suggests that the color variation is not driven by predator selection.

Key-words: Aposematism, chemical defense, polychromatism, GC-MS, Caxiuanã Bay

AMJ, RAS, and TG contributed to the study conception and design. Specimens were collected by AMJ and TG. Chemical analysis was performed by AMJ and RAS. Palatability tests were performed by SK and RAS. The first draft of the manuscript was written by AMJ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Introduction

Poison frogs have evolved the ability to sequester defensive alkaloids from dietary arthropods (Saporito et al. 2009, 2012). As is typical of chemically defended animals (Speed et al. 2012), the alkaloid-based defenses of poison frog skin secretions are highly variable within species and even among individuals of the same population (e.g. Daly et al. 2008; Jeckel et al. 2015a). Alkaloid variation is related to multiple factors, including genetic or epigenetic differences in uptake (Daly et al. 2003; Hantak et al. 2013), availability of alkaloid-containing prey (Daly et al. 1994), size and abundance of granular glands (Saporito et al. 2010a), age (Jeckel et al. 2015b), sex (Saporito et al. 2010b), season (Saporito et al. 2006), habitat type (Andriamaharavo et al. 2010), and geographic location (e.g. Saporito et al. 2006, 2007a; Daly et al. 2008).

Alkaloid variation has also been associated with variation in skin coloration in poison frogs. Most poison frog species present gaudy, presumably aposematic coloration, and chromatic polytypism is common in this group (e.g. Silverstone 1975; Myers and Daly 1976; Brusa et al. 2013; Hoogmoed and Ávila-Pires 2012; Patrick and Sasa 2013; Noonan and Comeaut 2008). Variation in alkaloid composition among polytypic populations has been studied most extensively in *Oophaga pumilio*, a dendrobatid poison frog distributed in lowland rainforests of the Caribbean slope in southern Nicaragua, Costa Rica, and northwestern Panama (Frost 2019). Throughout most of its range, populations of *O. pumilio* are similar in color, but the insular populations of the Bocas del Toro Archipelago, Panama are characterized by highly localized chromatic polytypism. Populations located on different islands differ wildly in coloration (Maan and Cummings 2012), alkaloid profiles (Saporito et al. 2006), toxicity (Daly and Myers 1967; Maan and Cummings 2012), and palatability (Bolton et al. 2017). Similar findings have also been reported for other polytypic species of *Oophaga*, such as *O. histrionica* (Myers and Daly 1976) and *O. granulifera* (Wang et al. 2011).

Although *Oophaga* is the most well-studied polytypic genus of poison frog, chromatic polytypism also occurs in several other poison frog lineages and is especially common among the toothless dendrobatines (Dendrobatini; Grant et al. 2017) of the ADO clade, composed of *Adelphobates*, *Dendrobates*, and *Oophaga* (Grant 2019). Lawrence et al. (2019) recently found that two morphs of the chromatically polytypic species *D. tinctorius* differ in both alkaloid composition and palatability, thereby matching previous findings in *Oophaga*. However, no studies have examined polytypic species of *Adelphobates*. As such, to test the generality of findings in *Oophaga* and *Dendrobates* in this clade, we investigated the

defensive alkaloids of two geographic color morphs of the splash-backed poison frog, *Adelphobates galactonotus*, a chromatically polytypic species distributed south of the Amazon River in Brazil (Hoogmoed and Ávila-Pires 2012). Specifically, to determine if geographically separated color morphs differ in skin alkaloid composition and palatability, we compared the alkaloid composition and arthropod palatability of secretions from the blue morph, known exclusively from the eastern side of Caxiuanã Bay, and the widespread orange morph, collected on the western side of the bay.

Materials and Methods

Sample collection

We collected adult individuals of *A. galactonotus* in January 2017 in Pará state, Brazil (Fig.1), including 5 (2 males, 3 females) of the orange morph collected on the western side of Caxiuanã Bay at a locality inside a protected area (Caxiaunã National Forest, 1°48'16.87" S, 51°26'45.31" W), and 5 (2 males, 3 females) of the blue morph collected on the eastern side of the bay, near riverside plantations (1°57'43" S, 51°25'09" W). We based our sample size on the results of previous studies that analyzed differences in alkaloid composition among populations (Saporito et al. 2006, 2007a; Andriamaharavo et al. 2010; Grant et al. 2012). In

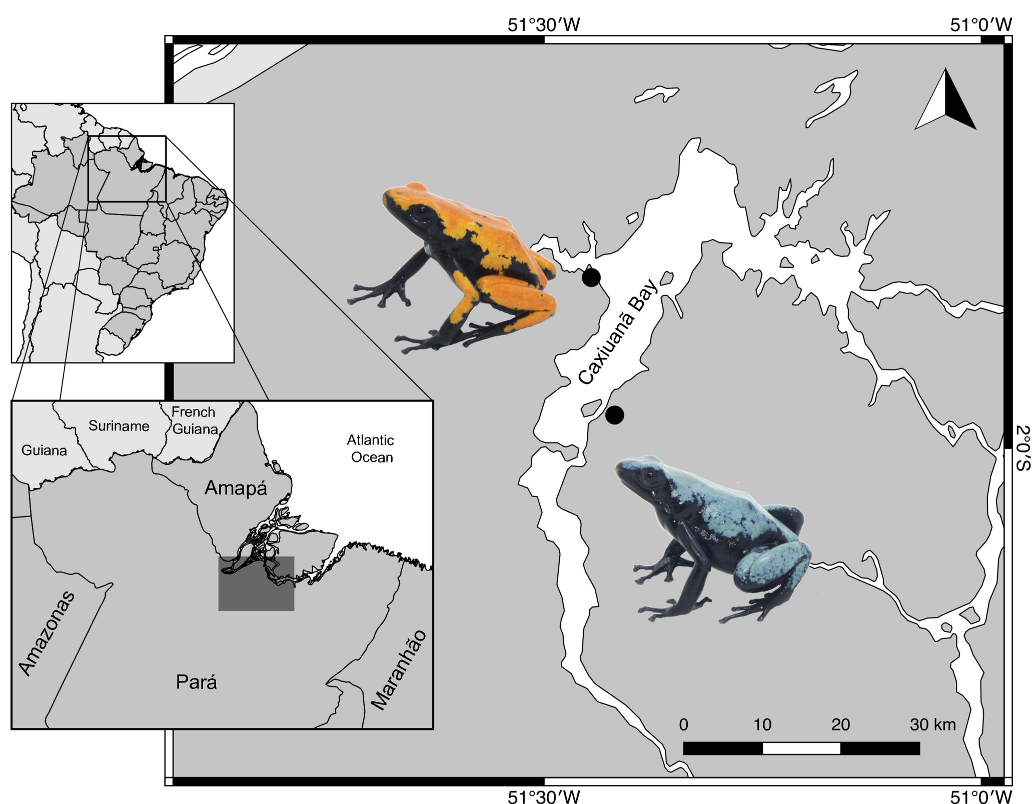

Figure 1. Map of *Adelphobates galactonotus* collection localities around Caxiuanã Bay, in Pará, Brazil.

order to avoid interference of most commonly used anesthetics to euthanize amphibians (Saporito and Grant, 2018), we euthanized all frogs by fast freezing in liquid nitrogen (e.g. Navas et al. 2007; Lillywhite et al. 2017). Following euthanasia, we removed and weighed the entire skin to 0.1 mg and examined gonads to determine sex. We stored skins in individual 4 mL glass vials containing 100% methanol and sealed with Teflon-coated lids and deposited specimens in the amphibian collection of the Museum of Zoology of the University of São Paulo under voucher numbers MZUSP A158924–33.

Alkaloid extract preparation

We isolated alkaloids from individual methanol extracts using an acid-base extraction following Saporito et al. (2010b) and Jeckel et al. (2015a). For each individual frog skin, we performed two extractions: one for alkaloid analysis and another for palatability assays. For the extractions used in alkaloid analyses, we added nicotine as an internal standard and resuspended the alkaloids in 100 µL of 100% methanol. For the extractions used in palatability assays, we resuspended the alkaloids in 100 µL of 20% sucrose/50% ethanol solution without adding nicotine.

Alkaloid identification and quantification

We identified alkaloids by comparing the observed mass spectrometry (MS) properties and gas-chromatography (GC) retention times (Rt) with those of previously reported anuran alkaloids (e.g. Daly et al. 2005). Most anuran alkaloids have been assigned code names that consist of a bold-face number corresponding to the nominal mass and a bold-face letter to distinguish alkaloids of the same nominal mass (Daly et al. 2005). We tentatively identified isomers of previously characterized alkaloids on the basis of their electron impact (EI) and chemical ionization (CI) mass spectral data and GC retention times. Following the methods of Garraffo et al. (2012), we considered alkaloids to be new isomers if they shared identical EI-MS data with a previously identified alkaloid but differed in Rt by at least 0.15 min (Daly et al. 2005). We analyzed each individual frog skin extract in three chromatographic replicates and determined the average quantity of defensive compounds by comparing the observed alkaloid peak areas to the peak area of the nicotine internal standard, using Varian MS Workstation v.6.9 SPI.

Palatability test

In addition to visually oriented vertebrate predators, chemically oriented arthropods also predate poison frogs (Fritz et al. 1981; Szelistowski, 1985; Gray et al. 2010; Santos and Cannatella 2011; Stynoski et al. 2014a,b; Murray et al. 2016). *Drosophila melanogaster* is commonly used as a model to study arthropod taste perception and specifically to understand alkaloid perception by arthropods (Devambez et al. 2013; Lee et al. 2015; Meunier et al. 2003; Sellier et al. 2010), making it a suitable proxy to assess how arthropod predators might perceive variation in alkaloid defenses (Bolton et al. 2017).

To evaluate the palatability of *A. galactonotus* secretions to arthropods, we conducted feeding trials in which common fruit flies (*Drosophila melanogaster*) were allowed the option to feed on two different sucrose solutions (Bolton et al. 2017). In this assay, we added red food coloring to the control solutions (sucrose without alkaloids) and blue food coloring to the treatment solutions (sucrose with alkaloids) in order to distinguish between feeding preferences during trials. Previous studies have used *D. melanogaster* in multiple choice feeding trials and have demonstrated that fruit flies show no preference for different colored solutions (Meunier et al. 2003; Sellier et al. 2010; Bolton et al. 2017). Fruit fly abdomens are transparent, which enabled us to determine which colored solution they fed on or if they consumed a mixture of both colored solutions.

Following the procedures of Bolton et al. (2017), we made two stock solutions for use in the palatability assays, one for the control solution (no alkaloids) and one for the treatment solution (alkaloids). Each stock solution contained 20 mL of 20% sucrose/50% ethanol. For the control solution, we added 100 µL of red food coloring (Market Pantry®) to one stock solution. For the alkaloid treatment solution, we added 50 µL of blue food coloring (Market Pantry®) to the other stock solution. We ran separate experiments for each of the 10 frog skins so that each treatment solution reflected an individual frog's naturally occurring alkaloid defenses. In order to determine if alkaloid palatability is dose-dependent, we tested three alkaloid concentrations for each individual frog, comprising, respectively, 2.5%, 1.25%, and 0.625% of the total quantity of the alkaloids present in each individual frog skin samples.

Each fruit fly palatability assay used 10 individual *D. melanogaster* (wingless, wild type, Carolina Science) that were 3–11 days old (average 5 days), grown on standard fruit fly media (Formula 4-24® Plain, Carolina Science), and starved for 24 hours prior to the experiment. We placed these 10 fruit flies in a 9 cm Petri dish (Fisherbrand, 100 mm x 15 mm, sterile, Polystyrene) lined with filter paper dampened with deionized water (to provide moisture for the fruit flies) and containing 10 µL each of the control and treatment solutions on plastic cover slips (22 mm Fisherbrand® 2R Plastic Cover Slips). Following the methods

of previous studies (Sellier et al. 2010; Devambez et al. 2013; Bolton et al. 2017), we allowed the fruit flies to feed on the solutions for 2 hours in the dark, and then euthanized then by freezing.

In order to quantify feeding preference, we used a dissecting microscope to examine the fruit flies and counted the individuals with red, blue, and purple (mixed) solutions in their abdomens. From this count, we calculated a palatability index for each assay to determine the relative palatability of each alkaloid solution. The palatability index is a value that ranges from −1 to +1, where zero and positive values represent a palatable alkaloid solution and negative values indicate an unpalatable alkaloid solution relative to the control (Bolton et al. 2017). This index was calculated as followed: (# of blue fruit flies – # of red fruit flies – 0.5 * # of purple fruit flies) / (total # of fruit flies). We included each alkaloid extract from an individual frog in four independent replicate assays at each of the three concentrations (n = 12 for each individual frog skin extract).

Statistical analysis

We used non-metric multidimensional scaling (nMDS) to visualize and compare alkaloid composition (richness, type, and quantity of alkaloids) and one-way analysis of similarity (ANOSIM) to test for differences. Both nMDS and ANOSIM analyses were based on Bray-Curtis similarity matrices. We tested for differences in the quantity and richness of sequestered alkaloids between color morphs, sizes (SVL and mass), and sexes using Wilcoxon rank sum tests, and examined the relationship between alkaloid quantity and richness using linear regression. To test if frog alkaloids were considered unpalatable to fruit flies at each of the three concentrations, we performed one-tailed independent samples *t*-tests. Palatability index scores of zero or greater are considered palatable, and therefore average palatability indices for frogs were compared to a hypothesized mean of zero (Dyer et al. 2003; Bolton et al. 2017). To determine if there was a dose response in palatability among alkaloid concentrations, we used linear regression. To test for differences in alkaloid palatability, we performed an independent samples *t*-test. To examine the relationship between palatability and alkaloid quantity and alkaloid richness, we used linear regression. nMDS and ANOSIM were performed in PRIMER-E version 6, comparisons of alkaloid composition between morphs and sexes were performed using the statistical package R-3.6.0 (R Core Team 2019), and statistical analyses for the palatability assays were conducted using GraphPad Prism Software version 8.0.0 for Windows.

Results

Alkaloid composition

Alkaloid composition did not differ significantly between the two color morphs of *A. galactonotus* (alkaloid quantity [W = 15, p= 0.69], skin mass corrected alkaloid quantity [W = 14, p = 0.84], richness [W = 14, p = 0.84], and total composition analysis [Global R = 0.12, p = 0.198]; Fig.2a–d). The total number and quantity of dietary alkaloids varied among individual skin extracts, including among individuals of the same population (Table 1). Females and males did not differ in size (W = 18, p = 0.26), and there was no difference in alkaloid quantity (W = 12, p = 1), skin mass corrected alkaloid quantity (W = 11, p = 0.91), and richness (W = 19, p = 0.17; Fig.2e–f). Total quantity and richness of alkaloids were not

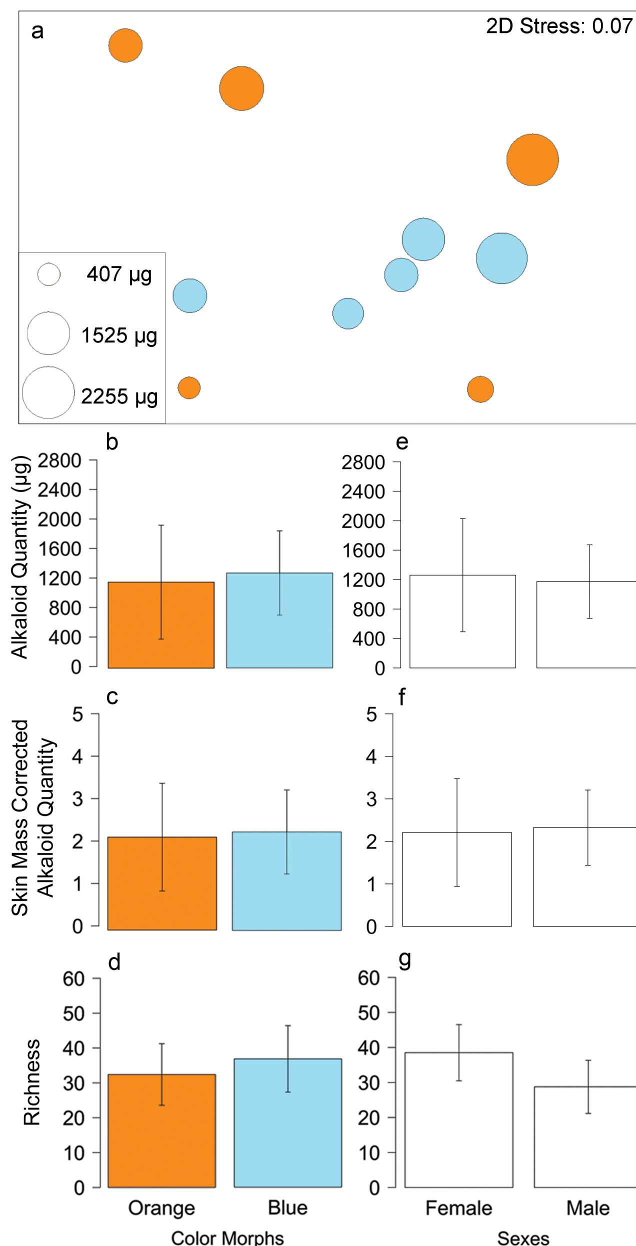

Figure 2. Comparison of alkaloid composition between morphs and sexes of *Adelphobates galactonotus*. (a) nMDS plot of alkaloid composition between blue and orange morphs. Each circle represents an individual frog, and the distance between symbols represents the relative difference in alkaloid composition. The diameter of each circle is proportional to the quantity of alkaloids present in that frog (µg per frog skin). (b–d) Comparison of alkaloid quantity (µg), mass corrected alkaloid quantity, and alkaloid richness, respectively, between blue and orange morphs. (e–g) Comparison of alkaloid quantity (µg), mass corrected alkaloid quantity, and alkaloid richness, respectively, between males and females of both color morphs.

significantly related ($F_{1,8} = 4.5$, $p = 0.07$), even when corrected by wet skin mass ($F_{1,8} = 2.4$, $p = 0.16$).

We identified 89 alkaloids (including isomers) representing 16 structural classes (Table 2). Seven alkaloids are new, and we also identified several tentatively new isomers of

Table 1. Summary of alkaloid variation of Adelphobates galactonotus (±1 S.D.).

	Blue Morph	Orange Morph	Blue + Orange Morphs
Total Quantity (µg per skin)	1285 ± 571 µg	1166 ± 772 µg	1225 ± 643 µg
Corrected Quantity (µg per mg skin)	2 ± 1 µg	2 ± 1 µg	2 ± 1 µg
Richness (per skin)	37 ± 10	32 ± 9	35 ± 9
Quantity Range (µg per skin)	800–2180 µg	406–2255 µg	407–2255 µg
Richness Range (per skin)	28–48	18–41	18–48

previously characterized alkaloids. The MS data and Rt for all seven new alkaloids are shown in Online Resource 1 and Rt for all of the new isomers are included in Online Resource 2.

Overall, the most abundant alkaloid in *A. galactonotus* was histrionicotoxin (HTX) **259A** (348.8 ± 413.1 µg per skin), with 3 times the amount of the second most-abundant alkaloid, HTX **261A** (103.6 ± 81.5 µg per skin). Both alkaloids were present in all individuals of both color morphs. Allopumiliotoxin (aPTX) **337D** and 5,6,8-indolozidine (5,6,8-I) **259C** were also present in all individuals of both populations, but in smaller amounts (26.1 ± 22.5 µg and 52.1 ± 43.7 µg per skin, respectively). 5,6,8-I **231B** and **249C**, aPTX **305A**, decahydroquinoline (DHQ) *trans*-**243A**, HTX **285B** and Izidine (Izi) **211C** were present in all but one individual, and aPTX **323B** was present in all but two individuals.

Among the 89 alkaloids in total, 46 are shared between the two populations. Among the 26 alkaloids that are unique to the blue morph, 17 are present in only one individual, 6 are present in two individuals and 2 are present in three individuals. The only alkaloid present exclusively in all individuals of the blue morph is DHQ *5-epi-trans*-**243A**, which is an isomer

of DHQ *trans*-**243A** found in all individuals of both morphs. In the orange morph, we found 15 unique alkaloids, 10 of which are present in only one individual, 3 in two individuals, and 2 in three individuals. All alkaloids present in each of the color morphs are listed in Table 2. Although only 46 of the 89 alkaloids are shared among the two populations, the amount of the exclusive alkaloids in each color morph add up only to 7.2% of the total alkaloids found in blue morph population and 3.2% of the total alkaloids in orange morph population.

Palatability test

Frog alkaloids were significantly unpalatable to fruit flies at all three concentrations ($p \leq 0.001$ for all comparisons). There was no statistically significant dose response in palatability among concentrations for either morph (Orange: $F_{1,13} = 3.43$, $p = 0.087$; Blue: $F_{1,13} = 1.37$, $p = 0.264$); however, on average, the higher concentration of alkaloids were more unpalatable (Fig.3). Being conservative, the lowest concentration of 0.6% was used for all of the remaining analyses. There were no significant differences in palatability between the orange and blue populations of *A. galactonotus* ($t = 0.43$, $p = 0.681$; Fig.4). There was no relationship between alkaloid palatability and alkaloid quantity for either morph (Orange: $F_{1,3} = 5.27$, $p = 0.106$; Blue: $F_{1,3} = 0.477$, $p = 0.539$; Fig.5a) or alkaloid richness (Orange: $F_{1,3} = 0.763$, $p = 0.447$; Blue: $F_{1,3} = 1.43$, $p = 0.318$; Fig.5b); however, there was a trend towards a decrease in palatability with an increase in alkaloid quantity and richness (Fig.5).

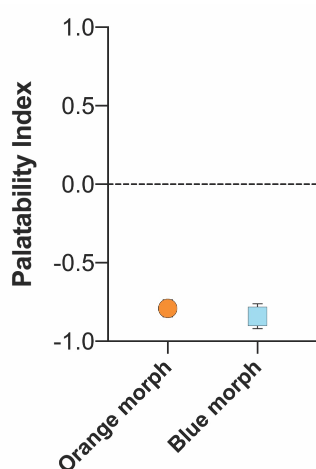

Figure 3. Mean palatability (±1 S.E.) between orange and blue morphs of *Adelphobates galactonotus*. The dotted line represents the point at which the solution of alkaloids is considered palatable.

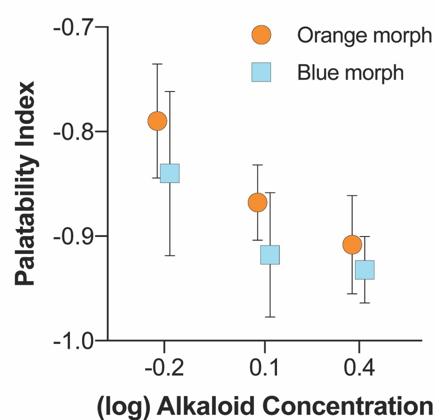

Figure 4. Dose response of mean palatability (±1 S.E.) for each of the three alkaloid concentrations tested between orange and blue morphs of *Adelphobates galactonotus*. Each data point is offset + or - 0.02 units for clearer visualization of data.

Discussion

In the only previous study that examined the alkaloids of wild-caught *A. galactonotus*, Daly et al. (2009) analyzed a single specimen from Tucuruí (reported as "Tucurvi"), Pará. Although Daly et al. (2009) did not provide color information, only orange frogs are known from that region (Hoogmoed and Ávila-Pires 2012). Among the four alkaloids we observed in all individuals, Daly et al. (2009) also detected HTX **259A** (trace amount), HTX**261A** (minor constituent), and 5,6,8-I **259C** (major constituent); however, they did not detect any aPTX **337D**. Daly et al. (2009) also reported HTX **291A**, aPTX **253A** and **267A**, DHQ *trans*-**243A**, and 5,6,8-I **249C** as major constituents; in our results, HTX **291A** was present in both blue and orange populations, aPTX **253A** and **267A** were absent from both populations, DHQ *trans*-**243A** was only present in the blue population, and 5,6,8-I **249C** was present in both populations. We did not perform statistical comparisons of Tucuruí population with the blue and orange populations we studied because only one individual from that population has been analyzed. However, given that the orange morph has a broad distribution and additional morphs exists (Hoogmoed and Ávila-Pires 2012), future studies should address additional comparisons across multiple population to test the generality of our findings in this species.

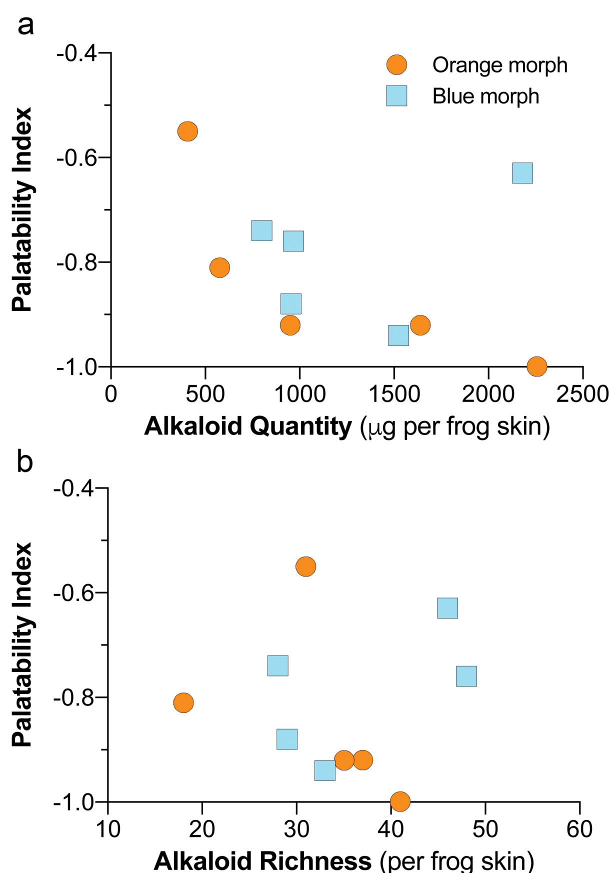

Figure 5. The relationship between palatability and (a) alkaloid quantity or (b) alkaloid richness for orange and blue morphs of *Adelphobates galactonotus*

Alkaloid composition does not differ between the orange and blue populations of *A. galactonotus*, despite their conspicuously different coloration and geographic separation on opposite sides of a significant aquatic barrier. This result contrasts with those of previous studies of other species of poison frog that found significant differences (Saporito et al. 2006, 2007a; Daly et al. 2008; Grant et al. 2012; McGugan et al. 2016; Lawrence et al. 2019). The two main reasons for the lack of significant differences are: 1) the high amount of shared alkaloids between the populations, including the two most abundant alkaloids (HTX **259A** and HTX **261A**), and 2) the low amounts and occurrence of unshared alkaloids. The difference between results found in *A. galactonotus* and other species could be due to lack of variation in alkaloid-containing arthropods on either side of Caxiuanã Bay and variation among localities of other species. For example, in a study of *Oophaga sylvatica*, McGugan et al. (2016) attributed differences among populations to differences in arthropod availability. Unfortunately, data on arthropod availability are lacking for our study sites. Alternatively, the difference could be due to genetically or epigenetically determined alkaloid uptake being the same in orange and blue morphs of *A. galactonotus*, which might be different than other poison frog species. The physiological and genetic aspects of alkaloid sequestration are not understood, but experiments suggest that they play a role in the variation of alkaloid composition (Daly et al. 1994, 2003; Hantak et al. 2013).

The unpalatability of alkaloid defenses in *A. galactonotus* is consistent with previous studies of alkaloid palatability in poison frogs (Schulte et al. 2016; Bolton et al. 2017; Lawrence et al. 2019). Given the lack of differences in alkaloid composition between blue and orange morphs, the lack of differences between the two color morphs in palatability is expected. Previous studies have found that conspicuousness of dorsal skin coloration in poison frogs is an honest indicator of alkaloid presence for visually oriented predators (Stuckert et al. 2014, 2018), and native predators are able to recognize and avoid aposematic coloration (e.g. Saporito et al. 2007b; Noonan and Comeaut 2008). However, variation in visual cues and alkaloid levels are not necessarily correlated (Daly and Myers 1967; Wang 2011; Stuckert et al. 2014, 2018; Crothers et al. 2016; Bolton et al. 2017) and do not predict differences in predation risk (Hegna et al. 2011; Stuckert et al. 2014). In *A. galactonotus*, the orange morph is brighter than the blue morph (Rojas et al. 2015); however, the lack of differences in alkaloid composition and palatability illustrate that this difference in brightness and hue is not a qualitative indicator of toxicity in this species.

The lack of differences in the defensive chemicals and palatability of the orange and blue morphs of *A. galactonotus* suggests that the color polytypism in this species is not

related to predation. Indeed, our results are consistent with previous findings that the frequency of attacks by visually oriented predators on paraffin models representing these two color morphs does not differ in either population (Rojas et al. 2015). Dietary accumulations of pigments have been shown to be unrelated to defensive chemical in other poison frogs as well (e.g. Crothers et al. 2016). Also, our personal observations in captive breeding *A. galactonotus* from both morphs established that, in this species, the color polytypism is a genetic feature. Instead, we suggest that the different color morphs in *A. galactonotus* might be related to female mate preference. Nothing is known about mate preferences in this species; however, females of *O. pumilio* prefer males of the same color morph (Maan and Cummings 2008) through parental imprinting (Yang et al. 2019). If female *A. galactonotus* also imprint on parental coloration and prefer males of the same color morph, then assortative mating could be the main driver of the distinctive color polytypism in *A. galactonotus*.

Acknowledgments

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Conflict of Interest: The authors declare that they have no conflict of interest.

Table 2. Alkaloids identified in blue and orange morphs of *Adelphobates galactonotus*, arranged by structural class. Alkaloids present in both morphs are underlined.

Blue Morph

Structural classes	3,5-I	3,5-P	4,6-Q	5,6,8-I	5,8-I	aPTX	Epiq	DHQ	HTX	Izidines	Pip	PTX	Tri	Unclass	New
	195B	trans-**223M**	195C	209C	217A	293K	196	*cis*-195A	235A	211C	269C	277B	193C	227	209
	5E, 9E 223AB	*cis*-223M		231B(2)	243C	<u>305A</u> (3)		195J	239H			323F		271B	213
	5E, 9Z 223AB	249I		*cis*-**249C** (2)		323A		5-epi-trans-**219A**	259A (2)						245
	5Z, 9E 223AB	251K (2)		259C		<u>323B</u> (3)		2-epi-cis-**219A**	261A (3)						251
	5Z, 9Z 223AB			261B		337D		*cis*-219A	265E						265
	223R			<u>263A</u> (2)		341A		5-epi-trans-**243A** (2)	283A						
	275C			<u>265U</u> (2)				*cis*-243A	285B (2)						
				273A				*trans*-**243A** (2)	291A						
				281M				245Q							

Orange Morph

Structural classes	3,5-I	3,5-P	4,6-Q	5,6,8-I	aPTX	Epiq	DHQ	HTX	Izidines	PTX	Pyr	SpiroP	Unclass	New
	5E, 9Z 223AB	trans-**223M**	195C	207C	293K	196	*cis*-195A	235A	211C	277B	211T	222	223DD	209
	5Z, 9E 223AB	251K (2)		<u>231B</u> (3)	<u>305A</u> (4)		*cis*-243A	239H		323A		236		232
	5Z, 9Z 223AB			233G	307C		*trans*-**243A** (2)	259A (2)		323F				245
				249C (2)	<u>323B</u> (4)		245Q	261A (3)						293
				<u>259C</u> (2)	337D			265E						
				261B				283A						
				263A				285B (2)						
				263D				291A						
				265U										

273A

281M

The number of isomers detected for each alkaloid is indicated in parentheses. The retention times for these tentatively new isomers are in Table 1 of the Supplemental Information. Abbreviations for alkaloid structural classes are as follows: 3,5-I (3,5-disubstituted indolizidine); 3,5-P (3,5-disubstituted pyrrolizidine); 4,6-Q (4,6-disubstituted quinolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); 5,8-I (5,8-disubstituted indolizidine); aPTX (allopumiliotoxin); Epiq (Epiquinamide); DHQ (2,5-disubstituted decahydroquinoline); HTX (histrionicotoxin); Pip (2,6-disubstituted piperidine); PTX (pumiliotoxin); Pyr (2,5-disubstituted pyrrolidine); SpiroP (Spiropyrrolizidine); Tri (tricyclic); Unclass (unclassified as to structure); New (New alkaloids - Figure 1 of the Supplemental Information).

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Capítulo 2 - Dose dependent sequestration efficiency in poison frogs

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Abstract

Sequestration of chemical defensive compounds depends on the type and quantity of compounds available in the environment, but selectivity of the mechanism also plays an important role in the sequestration efficiency. Dendrobatid poison frogs that sequester alkaloids from their diet have also been shown to have differences in alkaloids sequestration efficiency. However, the absence of a specific alkaloid does not necessarily mean that the species does not sequester the alkaloid. In this study, we tested two alkaloids from different classes decahydroquinoline (DHQ) and histrionicotoxin (HTX). Although these two classes usually occur together, only one dietary source of HTX have been described so far, in contrast to several ant species known to produce DHQ. The aim of this study was to quantify the extent to which alkaloid sequestration and modification are limited by either alkaloid availability or the sequestration mechanism by administering different dose concentrations of synthetic DHQ and HTX **235A** to captive-bred individuals of *Adelphobates galactonotus*. We found that *A. galactonotus* has different dose-dependent efficiency response to different classes of alkaloids. Whereas HTX **235A** sequestration rate increased at higher doses, DHQ sequestration rate remained constant. In addition, we describe for the first time the *N*-methylation of sequestered DHQ. The high variation in alkaloid composition among individuals and species, is also affected by the type of alkaloid and amount of each alkaloid available.

Introduction

Multiple invertebrate and vertebrate lineages have independently evolved the ability to sequester defensive chemicals from their diet—a process that involves the absorption, transport, and storage of extrinsic molecules for protection from predators and/or pathogens (Pasteels 1983, Dussourd et al. 1989, Savitzsky et al. 2012). The types of chemicals that a species can sequester are limited (e.g., monarch caterpillars sequester cardenolides [Brower et al. 1968]; chrysomelid leaf beetles sequester alkaloids [Rowell-Rahier et al. 1991]; poison frogs sequester alkaloids [Saporito et al. 2012]), and, even though they are dependent on the quantity and types available in the environment, there is evidence of selectivity within those chemicals. For example, flea beetles do not sequester all types of alkaloids available in a plant (Dobler et al. 2000), and chrysomelid leaf beetles are better efficient to sequester the alkaloids found in their main hostplant (Hartmann et al. 1997). Sequestration of chemicals from the diet also involves enzymatic modifications to better capture compounds or convert them to new structures that are less toxic (Hartmann et al. 1990, 1997, Lindigkeit et al. 1997). The selection and modification of compounds suggest the evolution of physiological mechanisms that are unique to particular lineages and types of chemicals.

Sequestration of defensive chemicals appears to be rare among vertebrates (Savitzky et al. 2012). Nevertheless, the ability to sequester lipophilic alkaloids from dietary arthropods evolved no fewer than four times within the Neotropical poison frog family Dendrobatidae (Saporito et al. 2012; Santos et al. 2012; Grant et al. 2017). Dendrobatid poison frogs are microphagous and appear to sequester most of their alkaloid defenses from ants and mites (Saporito et al. 2009, 2011, 2015; Jones et al. 2012).

Laboratory experiments have demonstrated the ability to sequester alkaloids in several species of dendrobatid poison frogs and have revealed variation in the efficiency with which different species sequester different alkaloids (Daly et al. 1994, 1997, Hantak et al. 2013). For example, in laboratory experiments, *Dendrobates auratus* readily sequesters decahydroquinolines (DHQ), indolizidines, quinolizidines and histrionicotoxins (HTX), but does not uptake 2,5-disubsituted pyrrolidines or 2,6-disubstituted piperidine (Daly et al. 1994). However, the absence of a particular alkaloid in a natural population or species does not necessarily mean individuals are incapable of sequestering that alkaloid. For example, although epibatidine is found

naturally only in species of *Ameerega* and *Epipedobates* (Spande et al. 1992), *D. auratus* is capable of sequestering it, even though the closely related congener *D. tinctorius* cannot (Sanchez et al. 2019). Similarly, HTX is absent from wild-caught *Oophaga lehmanni* but is readily sequestered when administered in feeding experiments, suggesting that the arthropod source of the alkaloid is absent from the species' natural environment (Garraffo et al. 2001).

Although HTX has been detected in nearly 40 species of Dendrobatidae distributed among multiple genera (Grant et al. 2017: supplementary material), HTX appears to be rare in the environment, having been detected in only one ant species (Jones et al. 2012). A possible explanation for the apparent inconsistency between the apparent environmental scarcity of HTX and its abundance among so many poison frog species would be the upregulation of a transporter protein to capture more molecules when the alkaloid is scarce (Caty et al., 2019). In this case sequestration efficiency would decrease with increasing dosages. However, upregulation to increase efficiency of less available alkaloids would not allow the high intraspecific variation known for most poison frog populations (Saporito et al 2006, 2007, Jeckel et al 2019, Lawrence et al 2019).

In addition to sequestering defensive chemicals unchanged, some species are known to modify them following ingestion. *Dendrobates* and *Adelphobates* species hydroxylate pumiliotoxin (+)-**251D** to allopumiliotoxin (+)-**267A**, whereas *Epipedobates* and *Phyllobates* species sequester it unchanged (Daly et al. 2003). Therefore, it remains possible that some alkaloids for which a source has not yet been identified are modified from the ingested alkaloid. For example, the *N*-methyl DHQ class of alkaloids are naturally found in several species of *Ameerega* but are absent in sympatric species of *Adelphobates*, suggesting that *Ameerega* species might have the uniquely evolved ability to *N*-methylate decahydroquinolines derived from ants (Daly et al., 2009).

The aim of this study was to quantify the extent to which alkaloid sequestration and modification are limited by either alkaloid availability or the sequestration mechanism (i.e., the organism's physiological ability to process alkaloids) by administering different dose concentrations of synthetic DHQ and HTX **235A** to captive-bred individuals of *Adelphobates galactonotus*. By administering known quantities of alkaloid, we were able to quantify both sequestration efficiency

and rate of conversion as well as the proportional distribution of alkaloid stored in different organs and eliminated in feces.

Materials and Methods

Poison frogs

Adelphobates galactonotus were captive bred at Butantan Institute, São Paulo, Brazil. All frogs used in this study were 1-year old sub-adults F1s bred by us from adults collected in Floresta Nacional de Caxiuanã, Pará, Brazil in January 2017. Prior to commencing experiments, all frogs were maintained in proper terraria with recommended humidity, temperature and light cycle (12 h photoperiod; Lötters et al. 2010) and fed 3 times a week with fruit flies and crickets dusted with vitamin powder. During experiment, each frog was transferred to an individual plastic container (15 cm x 15 cm) with holes for ventilation, wet paper towel substrate, a water bowl, and objects for concealment. Humidity, temperature, light cycle and feeding routine were kept as described above.

Experimental design

Alkaloids

We purchased synthetic racemic DHQ (97%) from Sigma-Aldrich. Racemic HTX **235A** was synthesized as described in Matsumura et al. (2018).

Alkaloid concentration calculation

Although the DHQ used in our experiment is a synthetic compound unknown in nature, a variety of naturally occurring DHQs and HTXs have been detected in ants consumed by dendrobatid poison frogs, including the genera *Monomorium* (Daly et al. 1994) and *Solenopsis* (Daly et al. 2000, McGugan et al. 2016). Each *Monomorium* ant contains 0.5–2.0 µg of alkaloid (Jones et al. 1982), and each *Solenopsis* ant contains 0.1–1.0 µg of alkaloid (Jones et al. 1996). As a conservative but biologically relevant estimate of the amount of alkaloid per arthropod, we averaged the lowest amounts of alkaloids reported for the two ant species, giving 0.3 µg of alkaloid per ant.

To estimate the rate of ant consumption by frogs, we focused on dietary studies of *Adelphobates galactonotus* and *Dendrobates* spp. (*sensu* Grant et al. 2006; Silverstone 1975, Caldwell 1996, Born et al. 2010, Cajade et al. 2010, Passo-Palaez et

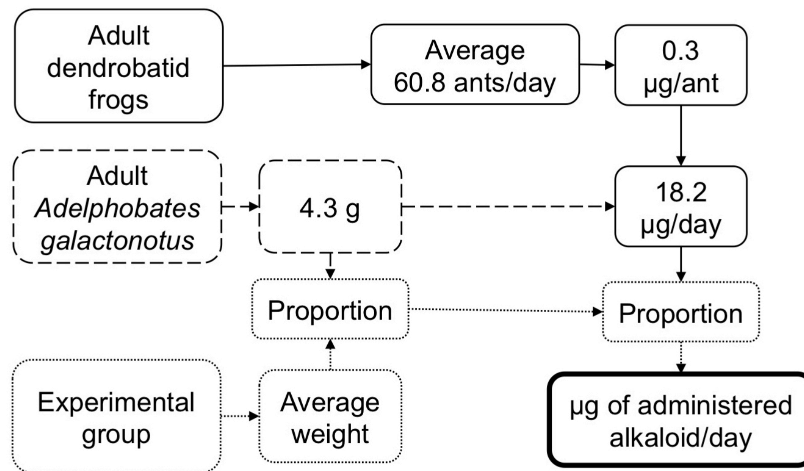

Figure 1. Fluxogram of calculation of daily amount of alkaloid administered to each experimental group.

al. 2017) because they are closely related and have similar adult snout–vent lengths (Grant et al. 2006, 2017).

In order to feed the conservative amount of alkaloids, we used the average of the lowest amount of alkaloids per frog per species (average number of ants per stomach = 61; average amount of alkaloid per adult per day: 18.24 µg). We corrected for the difference in average body mass between adult *A. galactonotus* (4.3 ± 0.5 g, average weight from collected adults [n = 3]) and the subadults used in our study (2.3 ± 0.4 g), resulting in a full daily dosage of 9.8 µg of alkaloid, which we rounded to 10.0 to facilitate preparation of alkaloid solutions (Fig.1).

We administered three dosage regimes. The first experimental group received a low dosage (10% of the full dosage, 1.0 µg), the second experimental group received a medium dosage (30% of the full dosage, 3.0 µg), and the third group received the full dosage (10.0 µg), each diluted in 5 µL of 50% ethanol solution. Each experimental group (n = 3) was administered with an ethanol solution with only one of the alkaloids (DHQ or HTX **235A**). The control individual received 5 µL doses of 50% ethanol without alkaloid. To ensure the frogs would not be negatively affected by the 50% ethanol solution, we evaluated loss of appetite, emaciation, lethargy, and/or uncoordinated movements as signs of intoxication or disturbance in the metabolism (Lötters et al. 2010). Table 1 shows the total amount of alkaloid that each experimental group received over the course of the experiment.

Daily Administration of Alkaloids

The experimental design that Daly et al. (1994) and consecutive studies used involved a mix of powder vitamin and alkaloid that was sprinkled on fruit flies. The frogs ate the flies with grains of vitamin and alkaloid that attaches to their body, allowing its ingestion and consecutive sequestration. Although this method has been useful for alkaloid feeding purpose, it does not allow precise quantification of sequestration efficiency. Hantak et al. (2013) was able to show differences in alkaloid sequestration efficiency by comparing the proportional amounts of different alkaloids given to the individuals and showed that some alkaloids are sequestered more efficiently than others. Yet, they were not able to quantify the total amount of alkaloid ingested and to compare to the total amount sequestered. In order to quantify the exact amount of alkaloids that each frog was ingesting, we administered 5 µL of the alkaloid solution orally for 14 days using a micropipette with the aid of a plastic Pasteur pipette to force open the mouth.

Previous amphibian pharmacokinetics studies have reported a half-life of 1.2 hours to 2 days for the drugs administered (Fox and Russell 1987, D'agostino et al. 2007, Guénette et al. 2007, Guénette et al. 2008, Howard 2010, Howard 2010, Lalonde-Robert et al. 2012, Rifkin et al. 2017). As such, to ensure that unsequestered alkaloid had cleared, we euthanized frogs 7 days after the last treatment.

During the 21 days of the experiments (14 days of alkaloid administration + 7 days of latency), we fed the frogs 3 times per week with fruit flies and crickets dusted with vitamin powder (NEKTON-Rep, Nekton Produkte, Germany). Previous studies of vertebrate herbivores have shown that excretion of toxins through the feces can impact in the bioavailability of the toxin (Patey et al. 2020) and that the toxins can be excreted without modification by gut mucosal metabolism (Sorensen et al. 2004, Thacker et al. 2012). As such, we collected all feces during the experiment to determine to quantify alkaloid excretion.

After euthanasia, we collected the skin, liver, kidney and muscle. All organs and feces were stored at room temperature in 1 mL 100% methanol in a 4 mL glass vial with Teflon coated lids until preparation for analysis.

Gas Chromatography-Mass Spectrometry analysis

We isolated alkaloids from individual methanol extracts using an acid-base extraction with a nicotine internal standard, following Saporito et al. (2010b) and Jeckel et al. (2015a). We analyzed each individual organ and feces extract in three

chromatographic replicates and determined the average quantity of each alkaloid by comparing the observed alkaloid peak areas to the peak area of the nicotine internal standard. Gas chromatography mass spectrometry (GC-MS) was performed on a Varian Saturn 2100T ion trap MS instrument coupled to a Varian 3900 GC with a 30 m x 0.25 mm i.d. Varian Factor Four VF-5ms fused silica column. GC separation of alkaloids was achieved using a temperature program from 100 to 280°C at a rate of 10°C per minute with Helium as the carrier gas (1 mL/min). Alkaloids were analyzed with both electron impact MS (EI-MS) and chemical ionization MS (CI-MS) with methanol as the reagent gas. A Varian MS Workstation v.6.9 SPI was used to generate MS spectra. We identified alkaloids by comparing the observed MS properties and GC retention times with control runs of both DHQ (Rt: 4.60 min, base peak of 98 *m/*z, and major peak at 139 *m/z* [16%]) and HTX **235A** (Rt: 12.24 min, base peak at 194 *m/z* and fragmentation peaks at 96 *m/z* [50%], 176 *m/z* [40%], 150 *m/z* [27%], and 220 *m/z* [18%]).

DHQ methylation methods

To confirm the presence of the *N*-methylated DHQ in the skin extracts of the experimental poison frogs, we analyzed synthetically produced *N*-methyl DHQ extracts in GC-MS and Vapor phase Fourier-transform infrared spectral data (GC-FTIR).

GC-MS and GC-MS-FTIR analysis. Gas chromatography mass spectrometry (GC-MS) was performed as described above. Vapor phase Fourier-transform infrared spectral data (GC-FTIR) were obtained with a Hewlett-Packard model 5890 gas chromatograph, with a 30m x 0.32 mm i.d. Phenomenex Zebron ZB-5 capillary column, using the same temperature program as above, coupled with an Hewlett-Packard model 5965B (IRD) narrow band (4000-750 cm^{-1}) infrared detector and Hewlett-Packard model 5971 mass selective detector (MSD). A Hewlett-Packard ChemStation was used to generate FTIR spectra.

N-methylation of decahydroquinoline. Synthetic (+)/(-) decahydroquinoline (DHQ) was produced by Acros Organics, New Jersey, USA. Following similar methods to Daly et al. (2009), the *N*-methylation of DHQ was performed "on-line" with the Saturn GC-MS and Hewlett-Packard GC-MS-FTIR instrument by injecting (**a**) a 1 μL methanol solution of DHQ or (**b**) 1 μL of frog skin extract (post-feeding experiment) with 0.5 μL of aqueous formaldehyde and 0.5 μL of formic acid. The

methylation reaction occurs in the injection port (i.e., "on-line") with the products being observed after gas chromatography at either the MS or FTIR detector. Methylation of synthetic DHQ (MW 139) resulted in the formation of synthetic *N*-methyl DHQ (MW 153).

Results

We quantified the alkaloids in the methanol extracts of the organs and feces and detected both DHQ and HTX **235A** in skin and liver extracts of all individuals of *A. galactonotus*. We restrict our analysis of sequestration efficiency to alkaloids detected in skin extracts, as these are the alkaloids that were transported and stored in the skin poison glands. The rate of HTX **235A** sequestration increased with higher concentrations, whereas DHQ was sequestered poorly, independent of concentration (Fig 1, Table 1). The ratios that the groups sequestered were different to the ratio administered; thus, the sequestration rates were not proportional to the amount administered (Table 1). The percentage detected in the liver was < 6%, and it was inversely related to the dosage of alkaloids (Fig 1).

Table 1. Total amount and ratio of alkaloids (Histrionicotoxin [HTX] **235A** and decahydroquinoline [DHQ]) administered to and sequestered by each experimental group. Mean ± sd (percentage from total administered).

	Experimental group	Total alkaloid administered	HTX **235A** sequestered	DHQ sequestered
Amount of alkaloids	Low	14.1 µg	3.8 ± 0.4 µg (27.3%)	0.9 ± 0.1 µg (6.6%)
	Medium	42.2 µg	13.4 ± 0.8 µg (31.8%)	3.1 ± 0.5 µg (7.4%)
	Full	140.6 µg	67.4 ± 13.8 µg (48%)	10.3 ± 0.4 µg (7.3%)
Ratio	Full:Low	10	17.5	11.1
	Medium:Low	3.3	5	3.3
	Full:Medium	3	3.8	3.4

We did not detect alkaloids in muscle or kidney extracts, suggesting that there were not significant amounts of circulating alkaloids in the body and that all alkaloids

were either excreted or transported to the storage glands after 7 days. Feces of most experimental groups did not have detectable amounts of alkaloid, except for the full dosage group of DHQ; however, excretion in feces accounted for less than 0.2% of total alkaloid administered (Fig 1), suggesting the lack of elimination of unmodified alkaloids. The control frog did not exhibit any signs of intoxication following administration of 50% ethanol.

GC-MS analysis of all frog skin extracts revealed the presence of DHQ and HTX **235A**. All HTX **235A** was unmodified, but 2.7 ± 0.9 % of the DHQ in the skin and 0.8 ± 0.2 % of DHQ in the liver were *N*-methylated (Fig. 2), as confirmed by comparison of the GC-MS and GC-FTIR spectral data properties of the extracted compounds to those of the synthetic DHQ and synthetic *N*-methyl DHQ (Fig. XX). Furthermore, "on-line" methylation of frog skin extracts resulted in complete conversion of DHQ (derived from the alkaloid feeding solution) to *N*-methyl DHQ also confirming the conversion. Additional MS comparisons with *N*-methyl DHQs obtained from other dendrobatid frogs (e.g., DHQ **233C**, **257A**, **263R**) provide further support for an *N*-methyl substituent (Daly et al. 2006, 2009). On the basis of IR data, both the extracted and synthetic skin extract *N*-methyl DHQ exhibited stronger

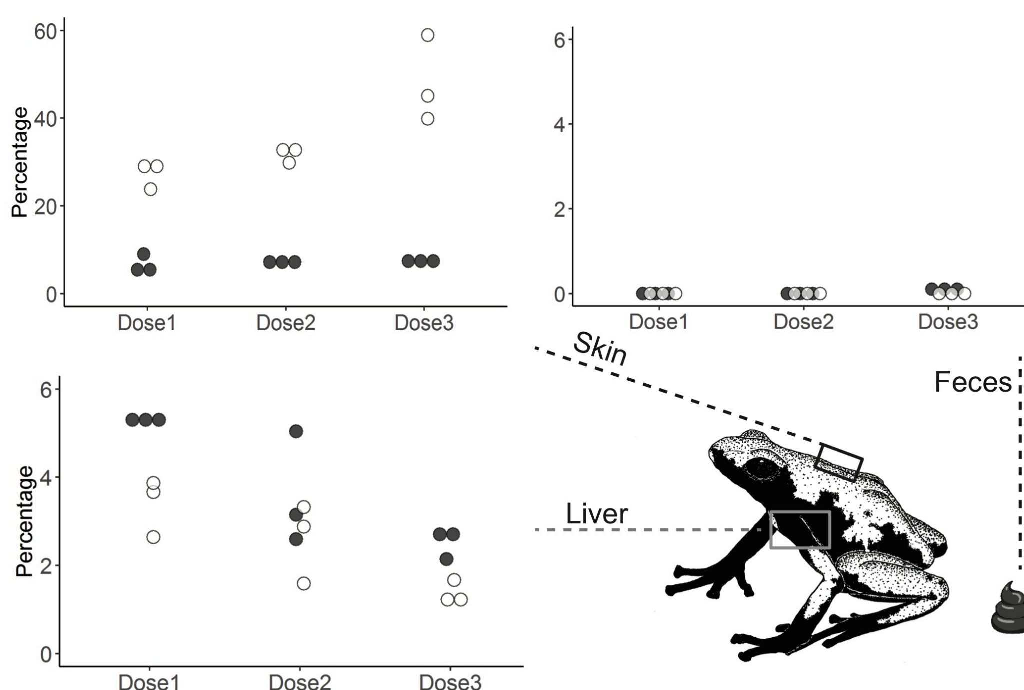

Figure 2. Percentage of alkaloid detected in the skin, liver and feces of the experimental groups. Each circle represents an individual of each group. Black circle: decahydroquinoline; white circle: histrionicotoxin **235A**.

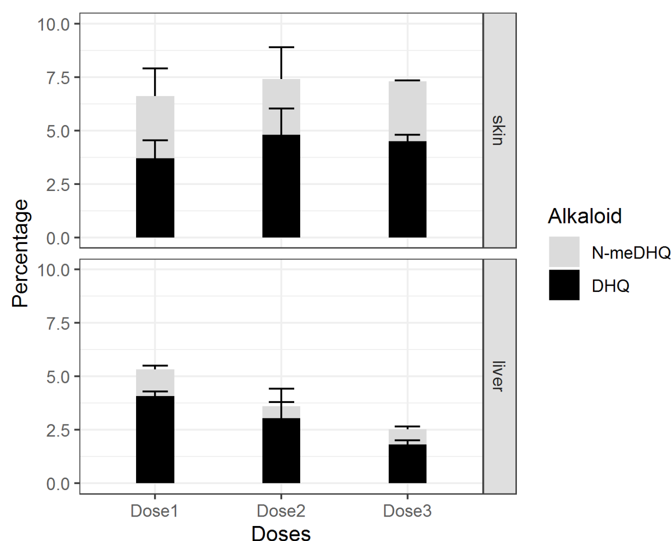

Figure 3. Mean percentage of decahydroquinoline (DHQ) and *N*-methyl decahydroquinoline (*N*-meDHQ) detected in the skin and liver of each experimental group.

Bohlmann bands at 2780 cm^{-1} than the DHQs, which is an expected difference between DHQs and *N*-methyl DHQs (Fig. XX; Tokuyama et al. 1991; Garraffo et al. 1993, 1994; Daly et al. 2009).

Discussion

We found that *A. galactonotus* has different dose-dependent efficiency response to different classes of alkaloids. Whereas HTX **235A** sequestration rate increased at higher doses, DHQ sequestration rate remained constant. When comparing absolute amounts of sequestered alkaloids, both DHQ and HTX **235A** increased sequestration with increased dosage. This means that, the more alkaloid they eat, more alkaloid they sequester. However, the efficiency of sequestration in comparison to the amount ingested appears to be dependent on the type of alkaloid. HTX **235A** sequestration efficiency appears to be dose-dependent, whereas DHQ does not. Thus, our results refute the hypothesis that efficiency increases when alkaloids are scarce because the lowest dosages of alkaloids resulted in lower (HTX **235A**) or similar (DHQ) rates of sequestration, when compared to higher dosages. On the basis of these findings, sequestration appears to be limited by the availability of alkaloids in dietary items, but the mechanism that regulates efficiency of sequestration differs between DHQ and HTX **235A**.

One possible mechanism that could regulate alkaloid sequestration rate are transporters. Transport of alkaloid either to or through the circulatory system is still unknown for poison frogs. Candidate molecules that have been suggested are bile derived blood transporters (Clark et al. 2012), the solute transporter subunit alpha (Caty et al. 2019), and saxiphilin, an amphibian transporter that has high affinity to the neurotoxin saxitoxin (Caty et al. 2019). All these molecules were detected in high association with poison frog's alkaloids and/or has increased expression in wild-collected poison frogs, i.e. frogs that were not submitted to a controlled experiment; therefore, it is still not possible to relate these molecules directly to an alkaloid sequestration function. Caty et al. 2019 suggested that increased expression of saxiphilin might be upregulated by small quantities of alkaloids in the blood in an effort to capture more molecules. However, if this is true, our results suggest that if upregulation of these transporters occurs, it is at concentrations higher. Additional biochemical and regulatory experiments are needed to understand and how sequestration is regulated and transported through the body to the skin poison glands.

Another relevant result in our study was the difference of sequestration rates between HTX **235A** and DHQ, independently of concentration dose. In the low and the medium doses, *A. galactonotus* sequestered approximately 4 times more HTX **235A** than DHQ, and in the full dose, 6.5 times more (Table 1). Difference in sequestration efficiency of different alkaloids has already been shown experimentally for different families of poison frogs (Daly et al. 1994, 2003; Hantak et al. 2014), but this is the first time that it was possible to quantify the absolute amount and proportions of alkaloids administered and sequestered. Our findings suggest that differences in uptake efficiency are important factors determining the variation of alkaloid profiles among individuals, populations, and species of poison frogs.

Differences in alkaloid sequestration between DHQ and HTX **235A** could result as an effect of molecular physiochemical properties of the compounds. For example, the synthetic DHQ used in this study is not a structure naturally found in poison frogs. The alkaloids of the DHQ class found in poison frogs have substitutions in the C-2 and C-5 positions, which could be important for the selectivity of the sequestration system. Those substitutions can also interfere in the lipophilicity of the chemical compound. Lipophilicity is a propriety that determines important parameters such as solubility, and it is measured with the pH-dependent descriptor log*D*. Based on the predicted log*D* curve, DHQ is less lipophilic than HTX **235A** in all pH

variation of a gastrointestinal tract of amphibians. In a fed frog, the pH can vary from approx. 6.5 (esophagus), to approx.4.5 (stomach), and back to ca. 7.5 (small intestine) (Yang et al. 2019). In a fasting frog, the pH of the stomach may get to pH 3 (Takeuchi et al. 1983). The difference in lipophilicity could translate in differences in sequestration efficiency.

Animals that are dependent on their food for chemical protection usually present mechanisms to prevent autointoxication. These mechanism varies from insensibility to target molecules like ion channels (*e.g.* Wang & Wang 1999, Tarvin et al. 2016, Ujvari et al. 2016) or physiological mechanisms that regulates the quantity of compounds available in the body, such as induced mucosal and hepatic enzymatic detoxification (Gordon et al. 2000) or gastrointestinal motility that lower the amount of toxin available for absorption (Camara 1997). These mechanisms may grant the trade-off balance between the costs associated with self-intoxication and the benefits gained from protection (Gossmann et al. 2010, Blennerhassett et al. XX, Morgenstern and King XX). However, there is a limit of concentration (high or low) of compounds in the ingested items that play an important role in the sequestration or the detoxification mechanism efficiency. For example, systems that allows efficient sequestration for an adapted low–medium concentration chemicals, but higher concentrations are detrimental to the animal (Camara 1997, Celorio-Mancera et al. 2011). By analyzing the feces of the poison frogs, we wanted to test whether some percentage of the alkaloids were eliminated before it had the chance to be absorbed, as many herbivores that are specialists in toxic plants eliminate part of the toxins ingested as a strategy to avoid self-intoxication (Ehmke et al. 1990, Amidon et al. 1995, Bruckmann et al. 2000, Sorensen et al. 2004). However, that was not the case for poison frogs because we did not detected alkaloids in their feces (Figure 1)—at least not unchanged alkaloids. Probably the percentage of alkaloids not detected was metabolized for detoxification and elimination, or attached to a transporter, restraining detectability by our methods.

In herbivores specialized in toxic plants, self-intoxication is prevented by degrading the toxic compound by antagonistic CYP450 enzymes (Ahmad 2002, Fogleman 2000, Celorio-Mancera et al. 2011). Higher concentration doses of toxic compounds upregulated genes of different CYP450 members when compared to lower concentrations (Celorio-Mancera et al. 2011), and different compounds had different regulation responses of CYP450 enzymes (Celorio-Mancera et al. 2012).

Recent studies analyzing transcripts of different organs in experimental and wild poison frogs have reported high expression of genes of the CYP450 enzymes (Caty et al. 2019, Sanchez et al. 2019), including an ortholog of the human CYP3A4, that is known to metabolize most of the drugs used as medication. Even though CYP450 enzymes might be present in almost all tissues and organs, and might be influenced by many different factors (age, sex, diet, etc.), it is also the main promising system of to analyze for future studies focusing in the alkaloid metabolism by poison frogs because of their importance in xenobiotics metabolism in other vertebrate and invertebrates.

Alkaloid-based defenses in dendrobatids appear largely as the result of uptake and sequestration of chemically unchanged alkaloids from dietary arthropods (Saporito et al. 2009, 2012). To date, the only known published exception is for members of the genus *Dendrobates* and *Adelphobates*, which hydroxylate more than 70% of the dietary pumiliotoxin (+)-**251D** to allopumiliotoxin (+)-**267A**, a modification that appears to be the result of an enantio- and stereoselective hydroxylase (Daly et al. 2003). Herein, we provide experimental evidence that *Adelphobates galactonotus* is capable of an additional modification of an alkaloid, specifically *N*-methylation of orally administered synthetic decahydroquinoline (DHQ). Approximately, 3% of dietary decahydroquinoline was *N*-methylated to *N*-methyl decahydroquinoline. Decahydroquinoline serves as the backbone structure of the over 35 alkaloids in the 2,5-disubstituted decahydroquinoline class yet lacks the 2,5 substitutions that are common to most DHQs present in dendrobatids. Although the mechanism by which *N*-methylation occurs in *A. galactonotus* is not known, it is likely to be enzyme mediated (similar to hydroxylation of pumiliotoxins), possibly by an *N*-methyl transferase, an enzyme class that is common among animals (ADD CIT); however, it is also possible that *N*-methylation is due to the absence of a demethylation process that could be common to other dendrobatids. Although the chemical and/or physiological function(s) of pumiliotoxin hydroxylation has not been studied, subcutaneous injection of aPTX **267A** into mice results in a five-fold increase in toxicity when compared to PTX **251D**, suggesting a possible defensive function. Future studies will need to examine the mechanism by which *N*-methylation occurs, as well as the potential chemical and physiological function(s) of *N*-methylation.

N-methylation of DHQ might explain the presence of *N*-methyl decahydroquinolines (a recently described new class of alkaloids; see Daly et al. 2009

& Saporito et al. 2012), in *A. galactonotus* and possibly other dendrobatids. *N*-methyl decahydroquinolines were originally discovered and characterized in several members of the genus *Ameerega* (Daly et al. 2009), but have since been detected to a lesser extent in some *Adelphobates*, *Oophaga*, *Dendrobates*, and *Ranitomeya* (Stuckert et al. 2014; Hovey et al. 2018; Lawrence et al. 2019; Jeckel et al. 2019). Although the six described *N*-methyl DHQs are considered a unique structural class among frog alkaloids (mainly for classification purposes), they are structurally identical to the 2,5-disubsituted decahydroquinolines, except for the presence of an *N*-methyl substituent (see Daly et al. 2009 for structures). In fact, three *N*-methyl DHQs (**233C**, **257A**, and **263R**) are simply *N*-methylated versions of the DHQs **219A**, **243A**, and **249D**, respectively. An arthropod source has not been discovered for *N*-methyl DHQs, but DHQs and *N*-methyl piperidines have been reported from myrmicine ants (Jones et al. 1982, 1984; Saporito et al. 2009); however, it is not known whether dendrobatids obtain *N*-methyl DHQs from dietary arthropods, or if they metabolize DHQs by *N*-methylation (Daly et al. 2009). The ability of *A. galactonotus* to *N*-methylate dietary provided DHQ, strongly suggests the likelihood that some, if not all, *N*-methyl DHQs are the result of *N*-methylation. A small amount of the *N*-methyl DHQ **245Q** was recently identified in *A. galactonotus* (Jeckel et al. 2019), which could be due to *N*-methylation of some yet unknown DHQ, possibly derived from an ant. *N*-methyl DHQs are best known in members of *Ameerega*, yet there appears to be variation in their presence, which might indicate differences in the ability to *N*-methylate. Of the *Ameerega* species that have been examined, *A. macero*, *A. parvula*, *A. picta*, *A. trivittata*, and an unidentified species of *Ameerega* (closely related to *A. rubiventris*; Grant et al. 2006) were most likely to contain either one or both of the DHQs **219A** and **243A**, as well as the N-methylated versions of these alkaloids (N-methyl DHQs **223C** and **257A**; Daly et al. 2009), suggesting that these species may be capable of *N*-methylating dietary derived DHQs. To a lesser extent, *A. cainarachi* and *A. hahneli* variably contained *N*-methyl DHQs, even though they also contained the corresponding DHQs, possibly suggesting differences in the ability or extent of methylation among these species. Among the remaining *Ameerega*, *A. bassleri*, *A. bilinguis*, *A. petersi*, and *A. pulchripecta* contained DHQs, but not the corresponding *N*-methylated DHQs, suggesting that these species are unable to *N*-methylate, whereas *A. erythromos* and *A. silverstonei* did not contain DHQs or *N*-methyl DHQs. The biological and chemical significance of *N*-methylation, as well as the taxonomic

distribution of *N*-methylation among dendrobatids is not known and will require further study.

Here, we have shown that sequestration of alkaloids in poison frogs seems to be mainly limited by the alkaloid availability in the dietary items, but the efficiency of sequestration may be quantity dependent and differ among different classes of alkaloids. In addition, some alkaloids may suffer some kind of modification, such as the *N*-methylation of a percentage of the sequestered DHQ. Thus, the high variation in alkaloid composition among individuals and species, besides all the known factors (e.g., geographic location [Saporito et al., 2006, 2007], season [Saporito et al., 2010a], age and life stage [Daly et al., 2002; Stynoski et al., 2014; Jeckel et al., 2015]), is also affected by (1) the type of alkaloid and (2) amount of each alkaloid available, both influencing the efficiency. We also describe a new type of modification that could also impact the diversity of alkaloids found in the skin of these frogs. Remains to be known what drives these differences in efficiency and the ecological and physiological consequences of being more efficient and being capable of modifying the alkaloids.

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Capítulo 3 - Use of whole-body cryosectioning and desorption electrospray ionization mass spectrometry imaging (DESI-MSI) to visualize alkaloid distribution in poison frogs

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Short title: Whole-body Cryosectioning and DESI-MSI in Poison Frogs

Abstract

Ambient mass spectrometry is useful for analyzing compounds that would be affected by other chemical procedures. Poison frogs are known to sequester alkaloids from their diet, but the sequestration pathway is unknown. Here, we describe methods for whole-body cryosectioning of frogs and use Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI-MSI) to map the alkaloid histrionicotoxin **235A** in a whole-body section of the poison frog *Dendrobates tinctorius*. Our results show that whole-body cryosectioning and DESI-MSI are an effective technique to visualize alkaloid distribution and help elucidate the mechanisms involved in alkaloid sequestration in poison frogs.

Keywords: Alkaloid, Amphibia, Chemical defense, Dendrobatidae, Histology.

Introduction

Mass spectrometry imaging (MSI) is a technique whereby the analyte is desorbed, ionized, and detected directly from biological samples. A set of mass spectra is acquired, which represents the local molecular composition at known *x*,*y* coordinates of the tissue section and then assembled in an image by a specific software (for review in MSI see [1]). In Ambient Ionization Mass Spectrometry techniques, ionization occurs at ambient conditions with minimal or no preparation of samples.[2] Among ambient ionization techniques, Desorption Electrospray Ionization (DESI) has been applied extensively in pharmaceutical and natural products research to detect both large and small molecules.[3–7] In DESI, a fine spray of charged droplets is directed at the sample surface, generating ions that are directed to the spectrometer.[2] The lack of sample preparation in DESI-MSI allows histological analysis of the same tissue after MSI, providing correlation and confirmation of the morphological structures in the sample.[8] These analytic characteristics allow this technique to be used in a wide variety of research, and, in particular, where mapping of molecules is critical to understanding the location, movement, and/or metabolism of specific compounds.

One example is the study of defensive chemicals in amphibians. The production, storage, and mechanism of secretions have been studied extensively, and the biochemical pathways and distribution of these compounds are known for some molecules and amphibian species. Two studies have used Matrix Assisted Laser Desorption Ionization (MALDI) MSI to study peptides in the skin of two different frog species.[9,10] MALDI utilizes a layer of matrix to assist the ionization and, because the matrix could interfere in the detection of some smaller ions, it is not an ideal technique to analyze smaller molecules found in some species of frogs, such as the lipophilic alkaloids found in poison frogs (150–500 Da).[11]

Poison frogs are a polyphyletic group of anurans that independently evolved the capacity to sequester alkaloids from their diet.[12–15] These compounds are found in dietary arthropods such as ants, mites, and beetles,[16] and laboratory-controlled experiments have shown that these defensive chemicals are stored in the frog's skin glands with little or no chemical modification.[13–15] Although many ecological and evolutionary aspects of the causes and consequences for the presence of lipophilic alkaloids in poison frogs have been studied,[17] little is known about the sequestration mechanism or the organs and tissues involved in the process.

Visualization and mapping of alkaloids in the body of a poison frog could elucidate the pathway alkaloids travel through different organs and tissues. Standard histological procedures have been used to study the morphology and ultra-structure of the skin glands where frog alkaloids are stored after uptake from dietary sources;[18,19] however, there are no histochemical procedures available for staining all alkaloids, which is largely due to the considerable diversity of alkaloids present in poison frogs (more than 1400 alkaloids of 24 classes have been described to date).[17,20,21] For this reason, techniques that allow for visualization of small molecules and metabolites on a surface of interest without specific chemical targets are interesting strategies to analyze poison frog alkaloids.

In this study, we present the use of DESI-MSI to map alkaloids in whole-body sections of poison frogs. To do this, we used an alkaloid from the histrionicotoxin (HTX) class (HTX **235A**) in an experimental procedure that included oral administration of the alkaloid, DESI-MSI, and histology. HTXs are a class of spiropiperidine alkaloids that have highly selective inhibition of nicotinic acetylcholine receptors and are commonly found in dendrobatid poison frogs.[11,22] Here, we describe the method for the whole-body sectioning of a frog and the detection and mapping of HTX **235A**.

Materials and Methods

Poison frog experimental procedures

Since wild poison frogs sequester alkaloids from their natural diet, captive-bred frogs lack alkaloids.[23] For our study, we used two individuals of the poison frog species *Dendrobates tinctorius* (Dendrobatidae). All experimental procedures with live animals were performed at John Carroll University, Ohio, USA (IACUC protocol #1700). The juvenile frogs (approx. 1.5 cm snout–vent length) were purchased in the pet trade (Josh's Frogs, MI, USA), maintained in terraria under controlled temperature, light, and humidity,[24] and fed *Drosophila melanogaster* dusted with multi-vitamin powder *ad libitum*. Using a pipette, one of the frogs was orally administered 5 μL of a solution of 0.64 μg/μL of HTX **235A** suspended in 50% ethanol. After 1 h, the frog was euthanized by fast-freezing in liquid nitrogen. The other frog was used as a negative control for alkaloid presence. The frogs were stored at -18°C until being transported to York University, ON, Canada (CITES permit #18US17079D/9) and prepared for cryosectioning. The racemic HTX **235A** used in

this study was synthesized via $Hg(OTf)_2$-catalyzed cycloisomerization and SmI_2-mediated ring expansion reactions.[25]

Whole-body cryosectioning

We tested three different embedding media: 2% carboxymethyl cellulose (CMC), 5% CMC, and 5% CMC + 10% gelatin based on procedures described for zebrafish in Nelson et al.[26] and Perez et al.[27]. We removed the arms and legs of the frog[8] and placed the whole body in a flexible, peel-away mould with embedding media. We prepared 15 µm tissue sections at -18°C on a Shandon E cryotome, Thermo Fisher (Nepean, ON, Canada) and thaw-mounted each section on a glass microscope slide. The slides were stored in a -18°C freezer until DESI analysis. The tissue sections for DESI-MS were air-dried for 30 min before the procedure. The sections for histological analysis were stained with hematoxylin and eosin (H&E). We pipetted all staining reagents on slides until sections were immersed. In brief, we immersed the sections in hematoxylin for 1 min, washed thoroughly with tap water, immersed sections for a few seconds (approximately 3 sec) in eosin, washed thoroughly with ethanol 95%, and mounted slides with Cytoseal™ 280 (Thermo Scientific).

*DESI-MS of HTX **235A***

We analyzed a 0.5 µg/µL HTX **235A** solution using DESI-MS and DESI-MS/MS in a linear ion trap mass spectrometer (LTQ; Thermo Scientific, USA). We analyzed a 0.5 µL drop of the HTX **235A** solution in a silica plate and a 0.5 µL drop on the tissue section of the control frog. For optimal desorption and ionization, we used the following DESI ion source parameters: incident angle of 52°, 2 mm capillary tip to surface, 3–5 mm distance from mass inlet to capillary tip, nitrogen gas pressure of 100 psi, and flow rate of 4 µL/min of 95% methanol/5% water solvent. We obtained full scan DESI-MS spectra in the mass range of *m/z* 100–1000 and conducted MS/MS on HTX **235A** peaks using collision energies between 25 and 30 arbitrary units (manufacturer's unit). We used Qual Browser Xcalibur 2.0 to process the mass spectral data.

DESI-MSI

The DESI ion source was mounted on a 2D moving stage that automatically moves in the *x*- and *y*-directions for imaging acquisition. The poison frog whole-body section was 1.6 cm long and 0.6–0.7 cm wide. We performed DESI-MSI in positive ion mode with the same DESI ion source parameters described above. Ion injection time was set to 250 ms, summing 3 microscans. Full scan mass spectra were in the mass range of *m/z* 100-1000. Imaging acquisition times varied between 3.0–3.5 h with a spatial resolution of 100 µm for all images. We used ImageCreator ver. 3.0 software to convert the Xcalibur 2.0 mass spectra files (.raw) into a format compatible with BioMap (freeware, http://www.maldi-msi.org/).

Results and Discussion

In Figure 1, we present the summarized method developed to detect HTX **235A** in the longitudinal section of the experimental specimen and to determine the tissues in which the alkaloid was detected by comparison with the H&E-stained section. We determined HTX **235A** peaks through DESI-MS in the silica plate and on the control section (base peak *m/*z 236, Fig. 2A). Figure 2B shows the MS/MS profile of the alkaloid. The major characteristic fragments can be attributed to the neutral loss of water forming the base peak at *m/z* 218, followed by consecutive loss of propene and ammonium forming the ions at *m/z* 176 and 159, respectively. The initial loss of propene forming the ion at *m/z* 194 is also observed. The same MS and MS/MS profile was detected in the experimental poison frog. On the basis of these data, we were able to determine that 1 h after oral administration HTX **235A** was present in the

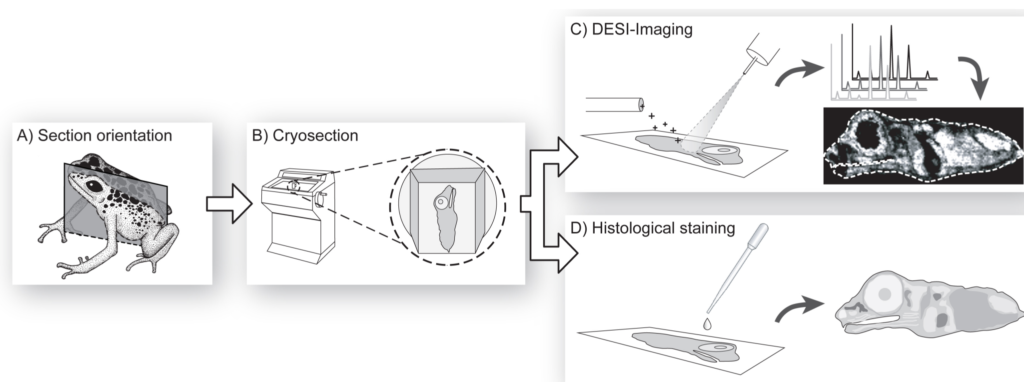

Figure 1. General overview of the major steps in DESI-MSI of whole frog specimens. A) Determine optimal orientation for sectioning. Sagittal sectioning allows simultaneous visualization of as many organs as possible. B) Cryosection whole frog from posterior to anterior (-18°C, 15 µm sections), thaw-mount on glass microscopy slides. C) Perform DESI-MSI, including generation of mass spectra and conversion to image. D) Stain with hematoxylin and eosin for comparison with MSI.

mucous membrane of the mouth, stomach, and, with less intensity, liver (Fig. 3). No peak at *m/z* 236 was detected in the control frog, confirming the absence of HTX **235A**.

Our study is the first to use DESI-MSI to map the distribution of defensive chemicals in frogs. Barbosa et al.[9] and Brunetti et al.[10] used MALDI-MSI in studies of peptide distribution in the skin of a different family of frogs that does not possess alkaloids. Although MALDI-MSI is one of the most commonly used methods of mass spectrometry for *ex vivo* molecular view of targeted organs or whole-body sections from an animal,[28,29] its reliance on a UV-absorbing matrix to ionize molecules can hinder the detection of small molecules. More specifically, any matrix-related peaks that fall within similar mass ranges to alkaloids of interest could prevent their detection.[30] The peptides present in the frogs analyzed by Barbosa et al.[9] and Brunetti et al.[10] are approximately 3–10-times the molecular weight of our target alkaloid, which allows for the use of a matrix for crystallization and formation of ions. DESI, on the other hand, ionizes the molecules directly on the surface of interest with a fine spray of charged droplets, picking up desolvated ions from both small and large molecules without the mediation of a matrix,[2] making it a more appropriate technique for studying alkaloids.

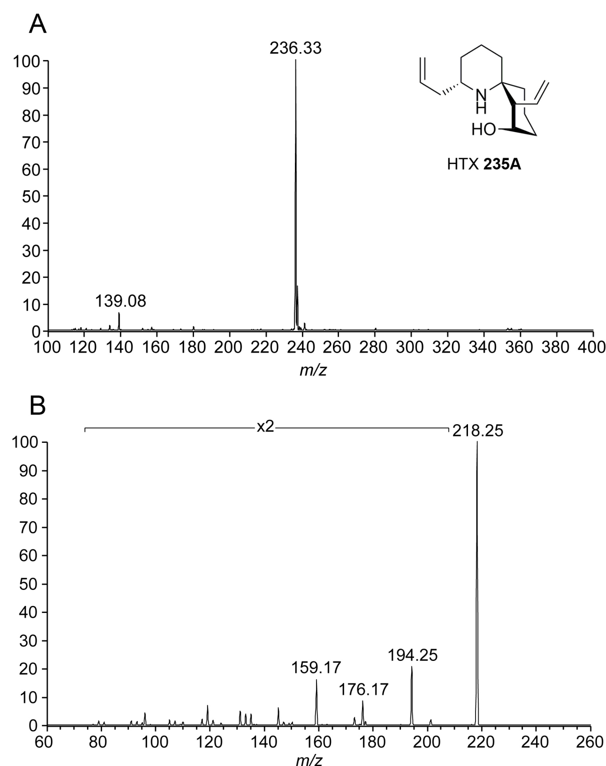

Figure 2. A) Full DESI-MS and chemical structure and B) MS/MS spectra of HTX **235A**.

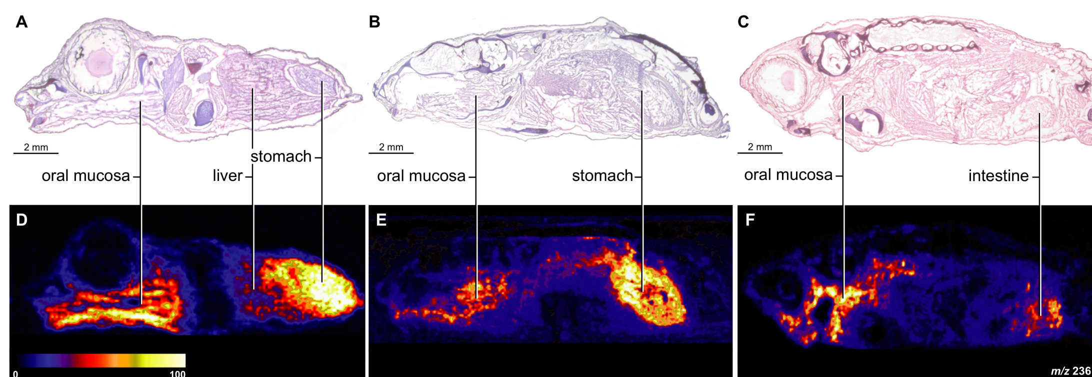

Figure 3. A) Histological section and B) DESI-MSI of Dendrobates tinctorius after 1 h of oral administration of HTX **235A**. Organs and areas of the body where HTX **235A** was detected are identified.

One of the difficulties of mapping the distribution of target molecules in vertebrates is whole-body cryosectioning because it involves sectioning both soft (e.g., skin, muscle) and hard (e.g., bone) tissues simultaneously, which requires careful selection of embedding media and specific attention to other practical techniques during sectioning. The optimal medium for whole-body cryosectioning used in the present study was 5% CMC + 10% gelatin. This was the only medium that was not too hard to tear the section, disintegrating the tissues and hindering the histological analysis, but hard enough to maintain rigidity at a proper temperature. The solution was also the most pliable and stable medium for frog cross-sectioning, in agreement with similar results found for zebrafish cryosectioning.[26] The minimum section thickness we achieved was 15 µm. Also, given that whole-body sectioning involves different tissue densities, the best orientation of the body relative to the blade was found to be posterior to anterior (Fig 1B), which resulted in soft tissues being cut before most bony structures (e.g., skull, vertebral column, pectoral girdle). Even though we experimented and optimized whole body sectioning, the sections obtained in our histological analysis are not suitable for cellular level analysis; nevertheless, they permitted identification of major tissues and organs, which was our main goal.

With the positive results in detecting and mapping poison frogs alkaloids in whole-body sections, it might be possible to study the metabolomics of frog alkaloids. For example, the use of stable labeled alkaloids would allow metabolized products of frog alkaloids to be detected. Some poison frogs are known to metabolize certain alkaloids (e.g., Daly et al. 2003), but the physiological role of metabolism and the organs and tissues involved in these modifications are still unknown.

Our results show that whole-body cryosecting and DESI-MSI are an effective tool to study alkaloid sequestration in poison frogs. Nevertheless, whole-body mapping of alkaloids in poison frogs using DESI-MSI is a first step toward further studies aiming to understand the pharmacokinetics and biochemical mechanisms of alkaloid sequestration. For example, some poison frogs are known to metabolize certain alkaloids,[13] but the physiological role of metabolism and the organs involved in these modifications are still unknown. In a controlled oral administration experiment with the specific alkaloid that is metabolized, DESI-MSI could show the tissues involved in biomodification. Further, the use of stable labeled alkaloids would allow a metabolomic study of these compounds and structural elucidation of metabolites. Also, there is no information about the detailed location of alkaloids in specific parts of the body (e.g., different types of skin glands). Given the challenges involved in whole-body cryosectioning and consequent lack of resolution, after initially mapping the molecule in whole-body sections and targeting the regions or organs of interest in one experimental specimen, one could remove the target organs from another experimental specimen and section them separately. This approach would allow for cellular level analysis, as well as a higher resolution during DESI-MSI acquisition.

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Capítulo 4 – Sequestration timeframe and systemic distribution of two alkaloids in a Dendrobatid poison frog

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Abstract

Sequestration involves the ingestion, transport, and storage of the chemical compounds to use against their own enemies. However, the bioavailability of orally ingested compounds can be affected by many factors, such as metabolism and excretion mechanisms. Understanding the anatomical pathway of a compounds can help understand which tissues or organs may play important roles in the sequestration mechanism. Poison frogs sequester alkaloids from their dietary items and are known to store these compounds in the specialized skin glands. Although it is known that most of these alkaloids are stored unchanged, little is known about the actual mechanism. In this study, we investigated the systemic distribution of two alkaloids (decahydroquinoline and histrionicotoxin **235A**) in a poison frog over the course of 24 h after a single dose administration. We used gas chromatography coupled with a mass spectrometry to quantify the alkaloids in different organs of the body and desorption electrospray ionization mass spectrometry imaging (DESI-MSI) to map the alkaloids in different organs and tissues of the body). In the 1 h experiment, there was high quantities of alkaloids in the stomach, but a percentage was already present in the skin. However, in the 24 h, no alkaloid was detected by DESI-MSI and the amount of alkaloid in the skin remained similar from the 1 h experiment. This is the first study to quantify alkaloid sequestration in the skin in such a small timeframe after alkaloid ingestion.

Introduction

A variety of chemically defended organisms independently evolved the ability of acquiring toxins from their diet through a mechanism called sequestration (Pasteels, 1983; Dussourd et al., 1989; Savitzsky et al., 2012). These animals are able to ingest, absorb, transport, and store these defensive chemicals, while largely maintaining their structure and function. Orally ingested compounds have many factors that can influence the bioavailabiliy, such as absorption, metabolism of enzymes, and gastrointestinal motility (Patey et al. 2020). Thus, one of the main aspects studied in sequestration mechanism is the anatomical and biochemical pathway of defensive compounds during food intake (Narberhaus et al., 2004).

Poison frogs are a polyphyletic group of anurans that independently evolved the ability to sequester alkaloids from their diet (Myers et al., 1978; Daly et al., 1984; Rodriguez et al., 2010). The amount and diversity of alkaloids present in the skin glands of an adult poison frog represents the alkaloids accumulated throughout their lifetime of sequestration of alkaloids from dietary item such as ants and mites (Daly et al., 1994; Saporito et al., 2004, 2007, 2012; Jeckel et al., 2015). The total quantity present in the skin has an important role on the protection against predators and microbes (Bolton et al., 2017), thus, sequestration is presumably an efficient process in order to sequester the low amounts of alkaloid that each dietary item possess (Jones et al., 1982, Jones et al., 1996; Saporito et al., 2011). Some blood transporters of lipophilic molecules have been targeted as candidate transporters, such as a bile acid-associated transporter (Clarke et al., 2012) and saxiphilin, an amphibian-specific transferrin (Caty et al., 2019); however, much about these possible transporters remain unknown, such as the specific transporter, which organ(s) these transporters are expressed in, and how long the transportation system takes to carry alkaloids to skin glands.

To understand the process of alkaloid uptake in frogs following ingestion, one needs to first uncover the anatomical pathway an alkaloid takes from mouth to skin gland. Some wild-caught poison frogs, such as *Melanophryniscus simplex* and *Oophaga pumilio* have considerable amounts of alkaloids in non-integumentary tissues of the body, such as liver, muscles, and reproductive organs (Grant et al., 2012; Stynoski et al., 2014). Accumulation of defensive chemicals in specific organs of the body may have important ecological significance, for example in *O. pumilio*, with respect to transferring alkaloids to the offspring through trophic eggs (Stynoski

et al., 2014; Saporito et al., 2019); however, poison glands located in the dermis are the main storage tissue for the sequestrated compounds (Jared and Toledo, 1995).

In this study, we investigated the systemic distribution of alkaloids in a poison frog over the course of 24 h after a single dose administration. We wanted to determine the anatomical pathway of ingested alkaloid and the timeframe of alkaloid distribution in the body after ingestion. Based on previous studies (Grant et al., 2012; Stynoski et al., 2014; Saporito and Grant, 2018), we hypothesized that alkaloids would be rapidly dispersed and widespread in the body tissues in addition to accumulation in the skin. We investigated the distribution of two alkaloids, decahydroquinoline (DHQ) and histrionicotoxin (HTX) **235A**, by mapping and quantifying them in different tissues of the frogs' bodies in different times after alkaloid ingestion. Alkaloids from DHQ and HTX classes are commonly found in the Neotropical Dendrobatid poison frogs (Grant et al., 2006, 2017). Even though they are similar in structure, these two specific alkaloids differ in lipophilicity and uptake efficiency (Jeckel et al., Chapter 2), which could result in different uptake timing, quantity, and distribution. In this study, in addition to the routine quantification method by gas chromatography coupled with a mass spectrometry (GC-MS), we used desorption electrospray ionization mass spectrometry imaging (DESI-MSI) to map the alkaloids in different organs and tissues of the body. DESI-MS is an ambient ionization mass spectrometry technique, where ionization occurs at ambient conditions with minimal preparation of samples (Cooks et al., 2006) that has been applied extensively in pharmaceutical and natural products research to detect particularly small molecules, and has successfully detected alkaloids in a poison frog's body section (Jeckel et al., 2020). The results obtained by using these two methods of analysis provided insights into the distribution of alkaloids in different tissues of the frog's body during uptake and the role of different organs to the sequestration system.

Materials and Methods

Frogs – We used 24 juveniles of *Dendrobates tinctorius* purchased in the pet trade (Josh's Frogs). They were maintained in individual plastic terraria (15 cm x15 cm) with recommended humidity, temperature, and light cycle (12h–12h). Until one day before the experiments, they were fed *ad-libitum* with *Drosophila melanogaster* dusted with vitamins to complement nutrition.

Alkaloids – Racemic DHQ (97%) was purchased in Sigma-Aldrich, and racemic HTX **235A** was synthesized via Hg (OTf)2-catalyzed cycloisomerization and SmI2- mediated ring expansion reactions (Matsumura et al., 2018).

Experimental design – The calculation of alkaloids in µg fed for each individual and the oral administration method is specified in Jeckel et al., (Chapter 2). In brief, based on adult *Dendrobates* + *Adelphobates* stomach content, we calculated the proportional amount of alkaloid based on the mean body weight of the experimental frogs (0.7 g). Each experimental frog received a single dose of 3.2 µg of alkaloid dissolved in 5µL of 50% ethanol solution. For each alkaloid, we had 3 experimental groups (n = 4) based on the time of latency after the oral administration of the alkaloids: 1h, 12h, and 24h. During the experiment, which lasted a maximum of 24 hours, we did not feed the frogs, and they did not defecate. After each latency time, we euthanized the frogs by cooling followed by flash freezing in liquid nitrogen. We analyzed 3 individuals of each group by GC-MS and 1 individual by DESI-IMS. The frogs for DESI-IMS were stored at −18°C until transport to York University, ON, Canada. The organs analyzed in GC-MS were skin, tongue, esophagus, stomach, intestine, kidney, liver, muscle, heart, and carcass (remaining of the body after dissection of the cited organs, hereafter "body"). We stored each organ in 100% methanol in a 4mL glass vial with Teflon coated lids.

Whole-Body Cryosection – To obtain whole-body cryosections, we followed protocol described in Jeckel et al. (2020). In brief, we embedded the frogs in 5% CMC + 10% gelatin and cryosectioned in the sagittal orientation. We analyzed three regions of the body through left parasagittal (R1), mid-sagittal (R2), and right parasagittal sections (R3; Fig 1A). Tissue sections of 15 µm were prepared at −18°C on a Shandon E cryotome, Thermo Fisher (Nepean, ON, Canada) using C.L. Sturkey Diamond Disposable Blades (Lebanon, PA, USA; dimensions: 76 × 8 × 0.25 mm) and thaw-mounted on a glass microscope slide. We collected alternated serial tissue sections for DESI-MS and histology in order to allow correlation and organ identification. The sections for histological analysis were stained with hematoxylin and eosin (H&E) following Jeckel et al., (2020) and mounted with Cytoseal 280 (Thermo Scientific). We took digital photographs of the histological sections using a Zeiss Discovery.V12 stereoscope and a Zeiss AxioCam ICc5 camera.

Desorption Electrospray Ionization Mass Spectrometry –We analyzed a 0.5 µg/µL solution of HTX **235A** and DHQ using DESI-MS and DESI-MS/MS in a linear

ion trap mass spectrometer (LTQ; Thermo Scientific, USA). We analyzed a 0.5 µL drop of the alkaloids solution in a silica plate using 95% methanol/5% water solvent (for optimal desorption and ionization specifications, see Jeckel et al., 2020). We obtained full scan DESI-MS spectra in the mass range of m/z 100 to 1000 and conducted MS/MS on alkaloids' peaks using collision energies between 25 and 30 arbitrary units (manufacturer's unit). We used Qual Browser Xcalibur 2.0 to process the mass spectral data.

Desorption Electrospray Ionization Mass Spectrometry Imaging – DESI-MSI analysis followed Jeckel et al., (2020). In brief, tissue sections for DESI-MS were air-

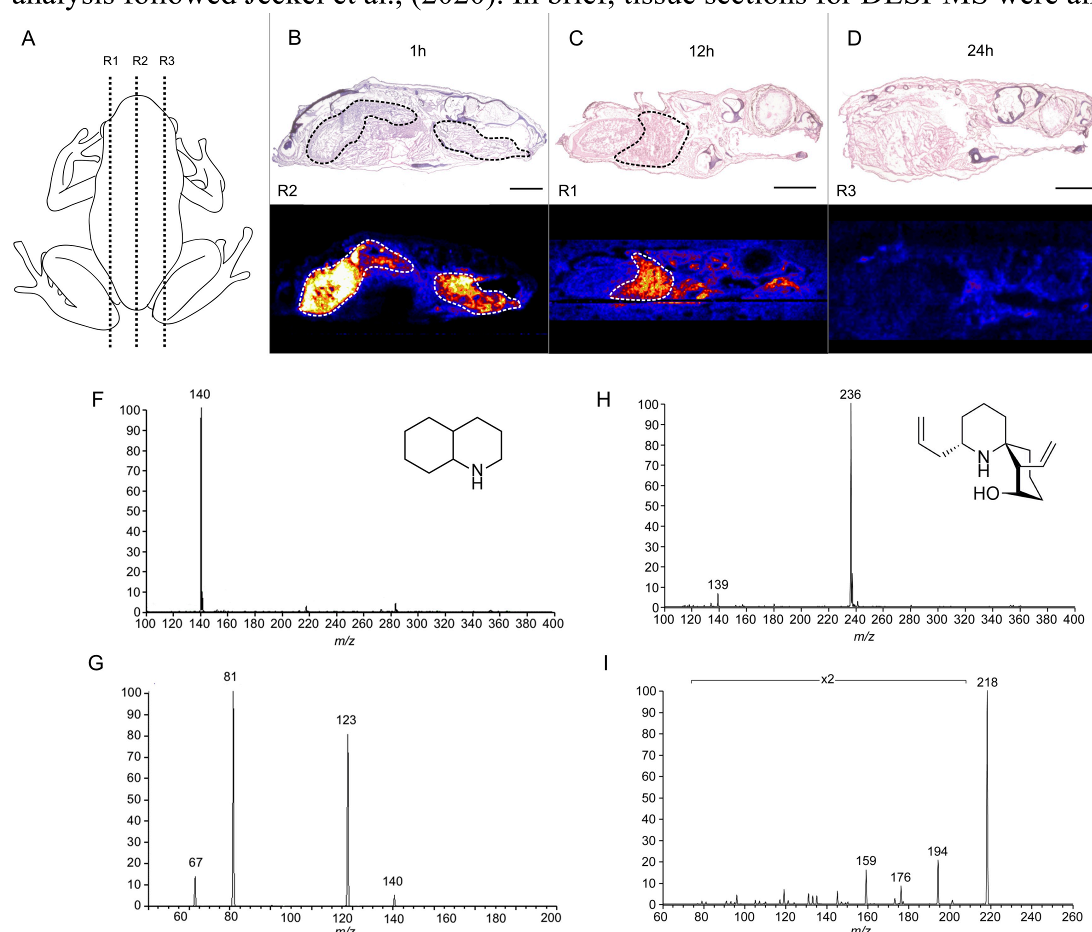

Figure 1. Desorption electrospray ionization mass spectrometry imaging in 3 sagittal sections of Dendrobates tinctorius. A) Left (R1) and right (R3) parasagittal and mid-sagittal (R2) sections analyzed. B) Histological and MSI images of R2 1 hour after administration with HTX **235A**. Dashed lines indicate stomach and oral mucosa. C) Images of R1 12 hours after administration with DHQ. Dashed lines indicate liver tissue. D) Images of R3 24 hours after administration with DHQ. No ionization was detected in this section. F) Full desorption electrospray ionization mass spectrometry imaging (DESI-MS) of decahydroquinoline (DHQ) spotted on silica plate. G) MS/MS spectra of DHQ. H) Full desorption electrospray ionization mass spectrometry imaging (DESI-MS) of histrionicotoxin (HTX) 235A spotted on silica plate. G) MS/MS spectra of HTX **235A**.

dried for 30 minutes before the procedure. We analyzed the sections using DESI-MS and DESI-MS/MS in a linear ion trap mass spectrometer (LTQ; Thermo Scientific, USA; for DESI-MS specifications see Jeckel et al., 2020), with a spatial resolution of 100 μm. We used Qual Browser Xcalibur 2.0 to process the mass spectral data, and ImageCreator ver. 3.0 software to convert the mass spectra files (.raw) into a format compatible with BioMap (freeware, http://www.maldi-msi.org/).

Gas Chromatography-Mass Spectometry – We isolated alkaloids from individual methanol extracts using an acid-base extraction following Saporito et al., (2010b) and Jeckel et al. (2015a), adding nicotine as an internal standard. We analyzed each individual organ extract in three chromatographic replicates and determined the average quantity of defensive compounds by comparing the observed alkaloid peak areas to the peak area of the nicotine internal standard, using Varian MS Workstation v.6.9 SPI. We identified alkaloids by comparing the observed mass spectrometry properties and gas-chromatography retention times with control runs of both DHQ and HTX **235A**.

Results

We detected both DHQ and HTX **235A** using DESI-MSI (Fig. 1B–I). The distribution of the alkaloids in the body was similar for both alkaloids (SInfo1). In the 1 h experiment, we detected high ionization of alkaloids in the mouth mucosa and in stomach and lower ionization of alkaloids in the intestine (Fig 1B, SInfo.1). In the 12 h experiment, we detected high ionization in the liver, but weak ionization remained in mouth and gastrointestinal tract (Fig 1C). In contrast, in the 24 h experiment, almost no ionization was detectable anywhere (Fig 1D), except for some mucosa detected in R1 sections of HTX **235A** frog (SInfo1).

By GC-MS analysis, we detected alkaloids in all frogs of all experimental groups, but not in all organs. We detected alkaloids only in stomach, liver, skin, and body extracts (Fig 2). In the stomach, we detected considerable amounts only in the 1 h experimental group of both DHQ and HTX **235A**, whereas skin and body presented considerable and very similar amounts in all experimental groups for each alkaloid. We detected significantly more DHQ than HTX **235A** (t-test p value = 0.002; Table 1). Although alkaloids were present in detectable amounts in these organs, from the total amount of alkaloid administered, we only detected on average 20.0 ± 5.3 % of DHQ and 9.9 ± 1.9 % of HTX **235A**.

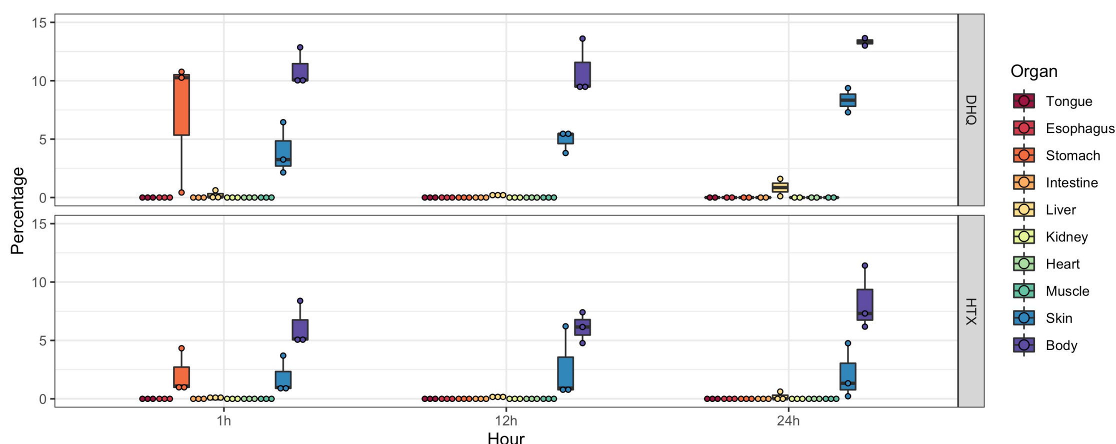

Figure 2. Boxplot representing the percentage of DHQ and HTX **235A** from total administered detected in the extracts of indicated organs per hour after ingestion of alkaloids. Each circle in every organ represents an individual of each experimental group.

Table 2. Percentage of alkaloids from total administered detected in each experimental group per organ extract. Organs that are not listed in the table did not have any alkaloid detected.

		Stomach	Liver	Skin	Body	Total
DHQ	1h	7.2 ± 1.9	0.2 ± 0.1	4.0 ± 1.6	11.0 ± 1.9	22.3 ± 0.3
	12h	0.0	0.2 ± 0.2	4.9 ± 3.1	10.9 ± 1.3	16.0 ± 2.1
	24h	0.0	0.9 ± 0.4	8.3 ± 2.4	13.3 ± 2.8	22.5 ± 2.7
HTX **235A**	1h	2.1 ± 5.8	0.1 ± 0.3	1.8 ± 2.2	6.2 ± 1.6	10.2 ± 6.9
	12h	0.0	0.2 ± 0.2	2.6 ± 1.0	6.1 ± 2.4	8.9 ± 3.1
	24h	0.0	0.2 ± 1.1	2.1 ± 1.5	8.3 ± 0.5	10.6 ± 3.0

Discussion

In the present study, we analyzed the systemic distribution of alkaloids in a frog's body over the course of 24 h after a single dose administration using DESI-MSI, for mapping the distribution in the body, and GC-MS, to quantify the amount of alkaloid in each organ. Besides liver, non-integumentary tissues did not have accumulation of alkaloids after 24h of alkaloid ingestion (Fig. 2). The amounts of alkaloids detected in skin, liver, and body were very similar in 1 h, 12 h, and 24 h after ingestion, except stomach. Apparently, there was a considerable amount of alkaloid in the stomach to be absorbed; however, that quantity did not seem to be relocated to any other organ in the following hours. Thus, the majority of the alkaloid

that was sequestered (i.e. present in the skin), arrived in the first 1 hour after ingestion (Fig. 2, Table 1). This is the first study to quantify alkaloid sequestration in the skin in such a small timeframe after alkaloid ingestion. The transport system from guts to skin glands after alkaloid ingestion appears to be quicker than expected, but apparently not very efficient in the first 24 hours in a single-dose alkaloid ingestion. Nonetheless, the amount of alkaloid in the body did not change significantly 24 hours after ingestion, which could mean that alkaloids are still circulating in the body, and an efficient sequestration is not quick at all, and it takes more than 24 h. It is possible tht, after 24h, the remaining alkaloid would eventually end up being stored in the skin glands. It also possible that they would be degraded or accumulated in the different body tissues. Although some molecules have been targeted as possible alkaloid transporters (Clarke et al., 2012; Caty et al., 2019), most of the questions regarding the mechanism of sequestration will be possible to answer with the description of the transportation system of alkaloids in poison frogs.

We analyzed the entire frogs in this study, but we only detected a maximum of ~25% of alkaloids ingested. The low percentage contrasts with recent study done with captive-bred frogs in a similar experimental procedures of alkaloid quantity calculation and administration (Jeckel et al., chapter 2). One explanation possible is that the remaining 75% of alkaloids were degraded and excreted or were attached to some type of transporters, and were not detectable by DESI or GC-MS methods. Also, the response of the sequestration mechanism could be dependent on the number of doses and quantity of alkaloid ingested. In natural conditions, each dietary item would be considered a dose, and probably induce response of the organism as a multiple dose system, instead of a single dose system. Multiple doses of alkaloids regulate different gene expressions than single-dose administration in poison frogs, including genes of detoxification enzymes, which could probably affect the sequestration efficiency (Sanchez et al., 2019) and explain the unexpected poor sequestration efficiency of either alkaloid. The quantity of alkaloid in each dose has also been shown to elicit different alkaloid sequestration efficiency in poison frogs (Jeckel et al., chapter 2). Differences in sequestration efficiency is multifactorial, including chemical properties (Jeckel et al., in Chapter 2), genetic predisposition (Daly et al., 2003; Hantak et al., 2014), and most probably, number dose and quantity of alkaloid present in each dose.

The lack of alkaloids in non-integumentary tissues (except liver) agrees with findings from other feeding experiments (Daly et al., 1994), but it is contrary to studies on wild-caught poison frogs (Grant et al., 2012; Stynoski et al., 2014). As the diversity of alkaloids in the skin glands are accumulated over the lifetime of the frog (Jeckel et al., 2015), the alkaloids present in different organs and tissues of wild caught frogs are thought to be concentrated in those tissues over time (Grant et al., 2012). Probably, a single low dose is not enough for alkaloid to be accumulated in a detectable amount in non-integumentary tissues. The presence of alkaloids in non-integumentary tissues of poison frogs has been reported in the liver, muscles, and mature oocytes of *Melanophryniscus simplex* (Grant et al., 2012) and in the ovaries of the egg provisioning species *Oophaga pumilio* (Stynoski et al., 2014). In both species, the alkaloids in the non-integumentary tissues were present in the same relative proportion as the ones present in the skin but in lower quantities. In *Oophaga*, the presence of alkaloids in the oocytes has been shown to provision protection to the tadpoles (Saporito et al., 2019), but the mechanism and the proportional amount of alkaloid invested in the oocytes per frog remain unknown.

One of the main difficulties of studying alkaloid sequestration mechanisms is the variety of alkaloid classes that are found in the skin of poison frogs. Currently 24 different classes of alkaloids have been detected in these frogs (Daly et al., 2005), and the uptake system differs in response to different alkaloid types in different species (Daly et al., 2003; Sanchez et al., 2019; Jeckel et al., Chapter 5). In the present study, we tested two different classes of alkaloids that are very common in poison frogs: the decahydroquinolines and the histronicotoxins. As discussed in previous studies (Jones et al., 2012), these two alkaloids might have chemical structure similarities, however, they differ in some physicochemical proprieties (Jeckel et al., in Chapter 2) and they were not sequestered equally by *D. tinctorius*.

By combining a mapping and a quantification method of alkaloid detection, we could determine the spatiotemporal distribution of the compounds during the first 24 hours of the sequestration process. DESI-MSI confirmed the presence of alkaloids in the stomach in the 1h groups and provided evidence of accumulation of alkaloids in the liver in the 12h groups, which we would not be able to attest with GC/MS quantification alone. In addition, mapping also informed that part of the alkaloid apparently lingers for a considerable amount of time in the mouth mucosa. Although we do not know if part of the absorption might occur in the mouth, we can at least

affirm, from DESI-MSI analysis, that it is still present in the area for at least 24 hours after administration (SInfo1).

We have shown here that a small percentage of alkaloids ingested by poison frogs are rapidly transported to the skin, whereas another small part remains circulating in the body and the majority of the ingested alkaloid is undetectable. These results help us understand the spatiotemporal distribution of alkaloids after ingestion and the mechanism that are tied to the sequestration system. The rapid transport of alkaloid show that transporters might be present in the body ready to uptake the alkaloids, but maybe its efficiency might need multiple dosages or higher quantity of alkaloids (Sanchez et al., 2019; Jeckel et al., in prep, Chapter 2). As the alkaloids of a single poison frog are accumulated over a lifetime, the quantity found in each individual frog might be a consequence of small percentages of each dietary item eaten, each item a dose, reflecting the variance of profiles among individuals of the same population. Further studies are needed to unveil how transporters and detoxification enzymes are regulated during alkaloid ingestion, and how they interfere in the sequestration efficiency and uptake in the skin glands.

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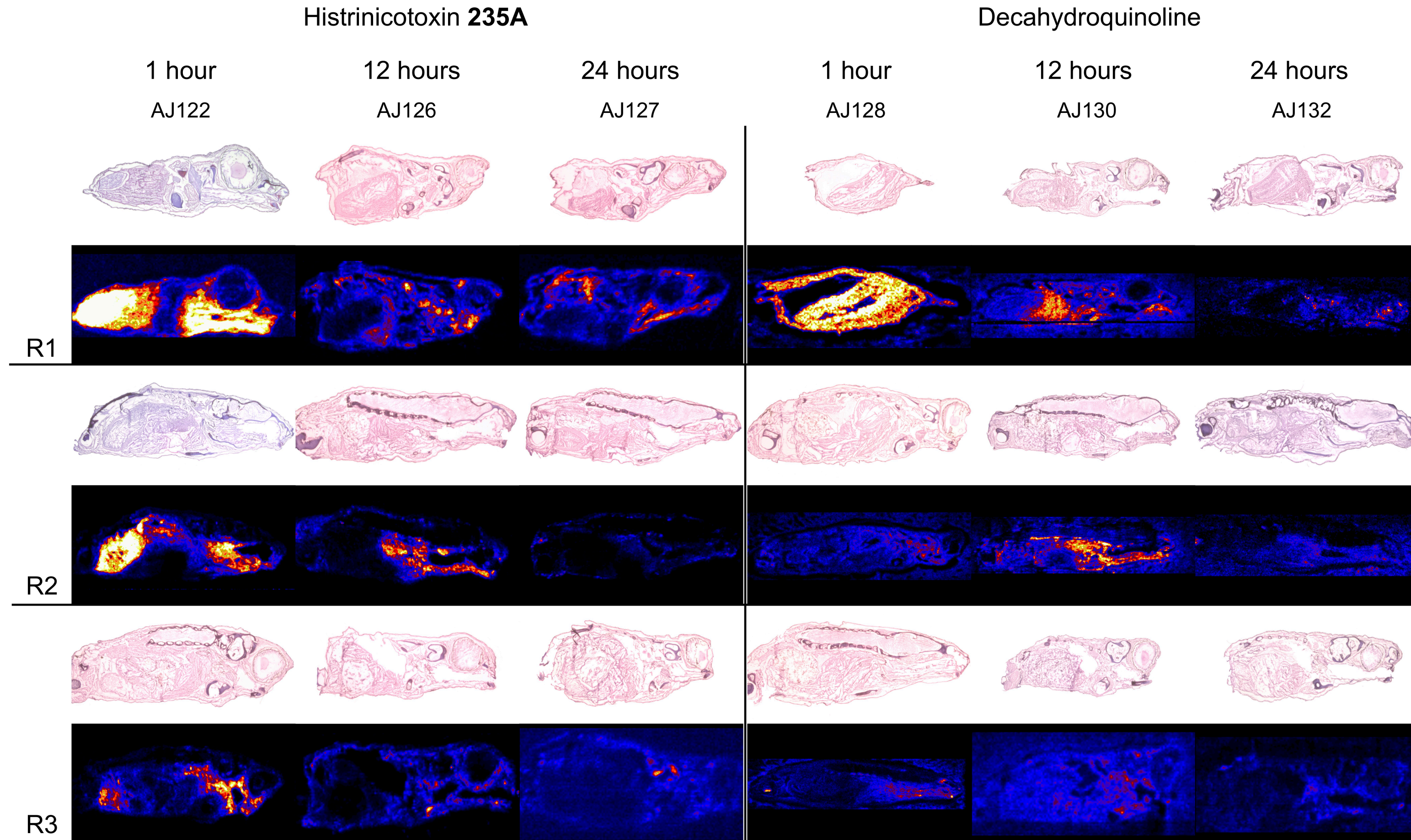
Supplemental Information

SInfo1



Capítulo 5 - The evolution of sequestration in poison frogs

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Abstract

The ability to sequester alkaloids from dietary item has evolved at least 4 times in Dendrobatidae. Some adaptations are known to allow these compounds to circulate in the body without intoxicating the poison frogs, such as amino acid substitutions, and mechanisms of transport and storage of alkaloids in specialized skin glands. Little is known about the sequestration mechanism, but it does not appear to be entirely conserved across dendrobatid lineages. The evidence of differential accumulation of alkaloids among species denote the existence of a specific transportation and/or a storage system, as opposed to a passive uptake and storage system. Also, some non-sequestering species did not present any sign of intoxication after ingesting the alkaloids. The ability to consume alkaloids through diet without injuries could be suggested as a plesiomorphic trait to cope with alkaloid toxicity. In this study we analyzed the different responses of non-sequestering (*Allobates femoralis* and *Hyle cinerea*) and sequestering species (*Adelphobates galactonotus, Dendrobates auratus, D. tinctorius, Phyllobates vittatus* and *Ranitomeya ventrimaculata*) to the ingestion of different classes of alkaloids. Our results show that non-sequestering species did not sequester alkaloids in their skin and the daily ingestion of alkaloids did not result in any sign of intoxication, demonstrating that they were able to tolerate the presence of the alkaloids in the organism. Also, we demonstrated that the poison frogs sequestered different alkaloids with different efficiencies, and that the ability to modify alkaloids is probably more widespread among dendrobatid poison frogs than previously known. We suggest that the detoxification system is plesiomorphic to anurans and it has been under expressed in poison frogs, allowing the sequestration and biomodification of dietary alkaloids.

Introduction

Poison frogs are a polyphyletic group of amphibians known to sequester and store alkaloids in their skin glands as chemical defense against predators and pathogens (Saporito et al., 2009, 2012). Five distantly related families of anurans have independently evolved this ability (Daly et al., 1965, 1984; Rodriguez et al., 2011) and, among them, it has evolved at least four times in Dendrobatidae alone (Grant et al., 2006, 2017). The ability dendrobatid poison frogs have to sequester alkaloids from dietary arthropods (Saporito et al., 2009, 2012) is correlated with other adaptations, such as aposematism (Saporito et al., 2007b; Noonan & Comeault, 2008), defensive behavior (Blanchette & Saporito, 2017; Umber et al., 2017; Savastano et al., 2020), and dietary specialization (Santos et al., 2003; Darst et al., 2005). The capacity to ingest and store relatively high amounts of alkaloids has also been related to specific adaptations that allow these compounds to circulate in the body without intoxicating the poison frogs.

One of the adaptations that provide alkaloid resistance to dendrobatid poison frogs is associated with amino acid substitutions in ion channels that apparently makes them insensible to some of the alkaloids sequestered (Tarvin et al., 2016, 2017; Wang & Wang, 2017; Márquez et al., 2019). Other adaptive mechanisms are probably involved in the alkaloid uptake system, including blood and membrane transporters that deliver the alkaloids to specialized skin glands (Clarke et al., 2012; Caty et al., 2019). However, little is known about the sequestration mechanism, which does not appear to be entirely conserved across dendrobatid lineages, with differences in sequestration ability described among species and genera (Daly et al., 1994, 2003). For instance, some species are able to modify specific alkaloids (Daly et al 2003; Jeckel et al., chapter 2), and others sequester different alkaloids types with varying degrees of efficiency (Daly et al., 1994, 1997; Sanchez et al., 2019). These differences among species and in sequestration and modification specificity denote the existence of a transportation and/or storage systems, as opposed to passive alkaloid uptake and storage.

The inability to sequester alkaloids has been experimentally shown in some genera of the superfamily Dendrobatoidea, such as *Colostethus* and *Allobates* (Daly et al., 1994). In nature, species of these genera do not have detectable amounts of alkaloids (Daly et al., 1994; Grant et al., 2006, 2017) and, in laboratory experiments, they did not uptake the alkaloids administered in their diet (Daly et al., 1994). Interestingly, the authors did not describe adverse consequences of daily ingestion of alkaloids during a 5-week experiment (Daly et al 1994). Detoxification mechanisms, such as oxidative enzymes (e.g. cytochrome P450, flavin

dependent monooxygenases) are shared by most organisms (Nebert et al. 1989) and it has important role in allowing ingestion of dietary items that have chemical defensive compounds (Ehmke et al., 1990; Miranda et al., 1991; Huan et al., 1998; Hartmann et al 1999). Thus, the ability to consume alkaloids through diet without injuries could be a plesiomorphic trait in Dendrobatoidea (Darst et al 2005).

Even though, in nature, consumption of different alkaloid types is regulated by the environment (i.e., availability of prey items; Daly et al., 1992; Garraffo et al., 2001; Saporito et al., 2011), the ability to sequester alkaloids have been used as character for dendrobatid phylogenies, due to its heritability and the differential accumulation of alkaloids among species (Grant et al., 2006, 2017). Laboratory experiments have shown differences in alkaloid sequestration efficiency in some dendrobatid poison frogs (Daly et al., 1994, 1997, 2003), but measurable evidences of differential accumulation among species are lacking.

Contrary to detoxification strategies that degrades and excrete residual metabolites, sequestration requires the maintenance of intact and functional compounds. Hence, specific mechanism should exist so that, in sequestering species, alkaloids can be transported and stored unchanged or, at least, maintain their function. Alternatively, if non-sequestering frogs lack sequestration mechanisms, they should be either susceptible to the alkaloid chemical reactions or be able to detoxify and excrete them. This study aims to analyze the responses of non-sequestering (*Allobates femoralis* and *Hyla cinerea*) and sequestering species (*Adelphobates galactonotus*, *Dendrobates auratus*, *D. tinctorius*, *Phyllobates vittatus*, and *Ranitomeya ventrimaculata*) to the ingestion of different classes of alkaloids. We evaluated sequestration efficiency and possible sign of intoxication (e.g. accumulation of alkaloids in liver) by quantifying accumulation of different alkaloids in different organs of the body. Also, we compared differences in alkaloid sequestration and modification efficiency among the poison frogs, as these parameters translate which and how much a species sequesters and modifies from a given amount of an alkaloid. By comparing different species of poison frogs and alkaloid types, we indirectly tested the presence of an uptake mechanism (e.g. transporters, enzymes) and the differential uptake of alkaloids.

Materials and Methods

Frogs

We performed controlled feeding experiments with seven species from the families Dendrobatidae (*Adelphobates galactonotus*, *Dendrobates auratus*, *D. tinctorius*, *Phyllobates vittatus*, and *Ranitomeya ventrimaculata*), Aromobatidae (*Allobates femoralis*), and Hylidae

(*Hyla cinerea*). All frogs were captive bred in our laboratory or purchased in the pet trade (Josh's Frogs and Back Water Reptiles), except for *A. femoralis* specimens (AJXXX-XXX) that were collected in Fazenda Treviso, Belterra, State of Pará, Brazil (3°09'27" S, 54°51'34" W), on February 04–14th, 2018. Until the beginning of each experiment, we maintained the frogs under constant humidity, temperature, and light cycle (12h-12h cycle; Lötters et al. 2010) and fed daily with fruit flies and crickets dusted with vitamin powder. In total, we did 3 different experiments. We evaluated loss of appetite, emaciation, lethargy, and/or uncoordinated movements as signs of intoxication or disturbance in the metabolism (Lötters et al. 2010).

Experiment 1

We used a total of 28 frogs in this experiment — 6 individuals of *A. femoralis*, *A. galactonotus*, *D. tinctorius*, and *H. cinerea* for the alkaloid feeding treatment, and 1 individual of each species as control group. During experimental days, we maintained each frog in individual 1.5 L plastic container lined with wet paper towel to allow feces collection. The feeding experiment lasted 14 days, with 7 days of latency before euthanasia.

Alkaloid treatment solution. All specimens were fed in a controlled feeding procedure described in Jeckel et al. (Chapter 2). Briefly, the estimated amount of alkaloid ingested per frog per day was based on the stomach content of some dendrobatid species. This number of dietary items was multiplied by the estimated amount of alkaloid in ants and mites, and the amount of alkaloid was calculated proportionally to the average weight of each species. Supplemental Table 1 shows the amount of alkaloid per day and total administered per experimental group. Each species had three experimental groups: one fed with HTX **235A** (n= 3), another with DHQ (n= 3), and a control group fed with 50% ethanol solution (n= 1). All animals of this experiment were fed with this solution for 14 days and had 7 days of latency before euthanized. We collected the feces of the frogs during the 21 days of experiment and stored them in 1 mL of 100% methanol glass vial with a Teflon-coated lid for isolation and characterization.

Experiment 2

We used a total of 12 frogs in this experiment — 4 individuals of *R. ventrimaculata* and 4 *D. auratus* for the alkaloid feeding treatment, and 2 individuals of each species as negative controls (no alkaloid powder). Each treatment group for both species were housed in

separate 44L glass terraria. The feeding experiment lasted 17 weeks and frogs were euthanized 48 hours after last alkaloid feeding.

Alkaloid treatment powder. The alkaloid feeding treatment was prepared by making a 0.5% alkaloid powder that contained equal parts decahydroquinoline (DHQ) and pumiliotoxin (PTX) (+)-**251D** mixed into a vitamin-mineral powder (Nekton, Clearwater, FL, USA). Originally, a 1% alkaloid powder was made, but the concentration of alkaloid was too high and killed the fruit flies upon dusting them with the alkaloid treatment powder. Thus, a 0.5% alkaloid powder was used to dust wingless fruit flies prior to feeding the frogs for the duration of the experiment. Control tanks received wingless fruit flies dusted with vitamin-mineral powder only. All frogs were fed daily with their respective treatment. At the termination of the feeding experiment, all frogs were sacrificed, and skins placed in methanol for alkaloid isolation and characterization.

Experiment 3

We used a total of 10 frogs in this experiment — 3 individuals of *D. auratus* and 3 *P. vitattus* for the alkaloid feeding treatment, and 2 individuals of each species as negative controls (no alkaloid in solution). We housed each frog from both species in a separate 470 ml Pyrex glass terrarium with a glass lid. The feeding experiment lasted 14 days for both species and frogs were euthanized 48 hours after last alkaloid administration.

Alkaloid treatment solution. Prior to beginning the alkaloid experiment, a pilot project was conducted to ensure that 50% ethanol did not cause signs of intoxication to the frogs. A total of 3 *Dendrobates auratus* were given 5 µl of a 50% ethanol solution and monitored for adverse effects at 5 minutes, 4 hours, and 6 hours after oral administration. No adverse effects or changes in the frogs' behavior were observed. All experimental specimens were fed in a controlled feeding procedure described in Jeckel et al. (Chapter 2). For the alkaloid treatment, each frog was given 5 µl of the alkaloid solution (0.125µg/µl DHQ in 50% ethanol) daily, which amounts to 0.625 µg (625 ng) per feeding. The concentration of alkaloid in the feeding solution was estimated to be a conservative quantity of total alkaloid a frog might consume in a single day, assuming its diet was composed of mites only. The average amount of alkaloid in the oribatid mite *Scheloribates laevigatus* was estimated to be 17 ng per mite, making the daily alkaloid intake of the feeding solution in the present study roughly equivalent to consuming 36.7 mites per day. Although this alkaloid quantity is likely an underestimate of the total amount a natural frog is capable of sequestering per day, frogs in the experiment were fed the entire alkaloid amount at one time, whereas natural frogs

would likely consume smaller amounts of alkaloid throughout the day. All control frogs were given 5 μl of only 50% ethanol. At the termination of the feeding experiment, all frogs were sacrificed and had their skin placed in methanol for alkaloid isolation and characterization.

Gas Chromatography-Mass Spectrometry

We isolated alkaloids from individual methanol extracts using an acid-base extraction following Saporito et al. (2010b) and Jeckel et al. (2015a), adding nicotine as an internal standard. We analyzed each individual organ and feces extract in three chromatographic replicates and determined the average quantity of each alkaloid by comparing the observed alkaloid peak areas to the peak area of the nicotine internal standard, using Varian MS Workstation v.6.9 SPI. We identified alkaloids by comparing the observed MS properties and GC retention times with control runs of DHQ, PTX **251D** and HTX **235A**.

Results

Experiment 1

Among the four species tested in this experiment, only the poison frogs (*A. galactonotus* and *D. tinctorius*) sequestered significant amounts of both DHQ and HTX **235A** in the skin (Fig. 1), whereas *A. femoralis* and *H. cinerea* retained only trace amounts of both alkaloids; except for HTX **235A** in *A. femoralis*, which we were not able to detect any traces. DHQ produced a base peak of 98 *m/*z and other fragmentation peak at 139 *m/z* (16%) in a retention time (Rt) of 4.60 min, and HTX **235A** produced base peak at 194 *m/z* and fragmentation peaks at 96 *m/z* (50%), 176 *m/z* (40%), 150 *m/z* (27%), and 220 *m/z* (18%) in Rt 12.24 min. Supplemental Table 1 presents the total quantity of alkaloids each experimental group consumed based on the average weight, and the percentage (mean ± sd) of alkaloid detected in each extract examined. There were no detectable amounts of alkaloids or only trace amounts of alkaloids in the feces of all species analyzed in this experiment. Also, small and trace amounts of alkaloids were detected in the liver of almost all experimental groups, except for *A. femoralis* fed with HTX **235A**, and *H. cinerea* fed with DHQ. None of the individuals from non-sequestering species showed any sign of intoxication while ingesting alkaloids, neither the control individuals while ingesting 50% ethanol. As for the first

experimental group of *A. femoralis* administered with DHQ, because they were the only wild-caught specimens of the experiment, we presume that the stress of handling for administration was responsible for the premature death of the two individuals. The 50% ethanol solution was not the harmful aspect because none of the second experimental group (half quantity) and the HTX **235A** experimental group individuals showed any sign of intoxication (e.g. loss of appetite, lethargy). None of the negative control or the experimental control *A. femoralis* had alkaloids (Supplemental Material 2).

Experiment 2

The skin secretions obtained from all frogs from this experiment (*D. auratus* and *R. ventrimaculata*) contained alkaloids, whereas no alkaloids were detected in those of the control group. Based on the proportion of each alkaloid in the alkaloid/nutritional supplement mixture, the relative abundance differed from the original proportions (PTX:DHQ = 1:1) and differed considerably between the two species (on average *D. auratus*: PTX:DHQ = 18.2:1;

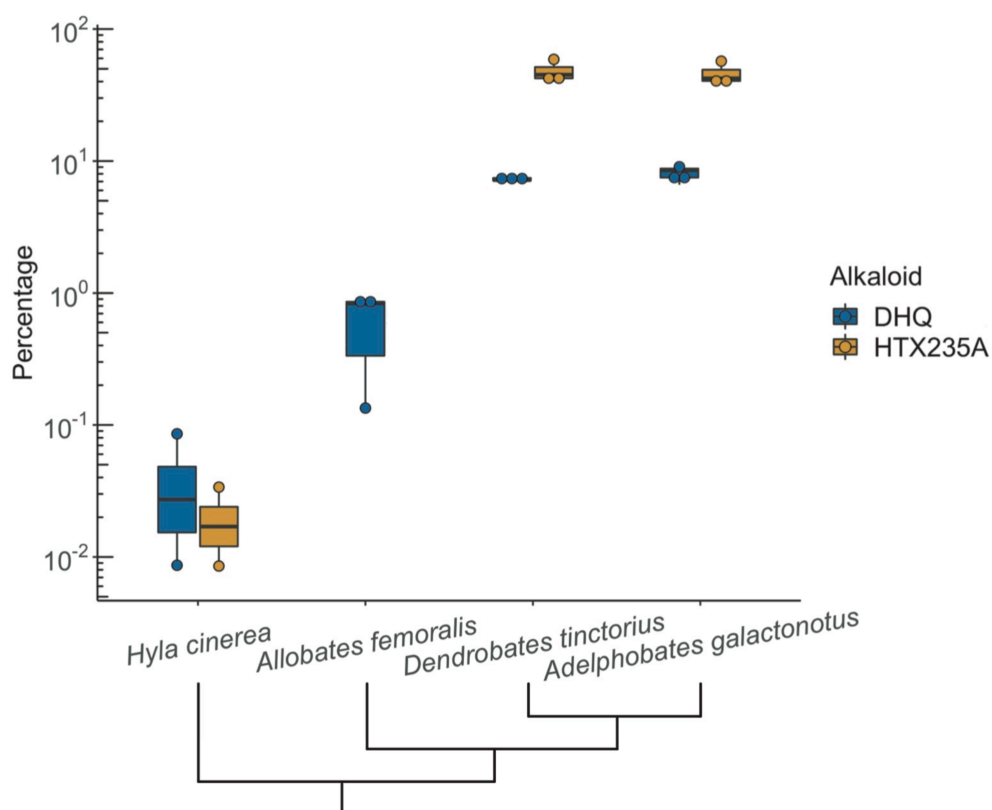

Figure 1. Boxplot shows percentage of DHQ and HTX **235A** sequestered per species of the Experiment 1. Circles represent individual frogs of each experiment and black lines inside boxes represent mean value of the group. Unrepresented individuals (1 of each experimental group of *H. cinerea*, and all individuals of HTX **235A** group of *A. femoralis*) did not have any detectable alkaloid in their skin.

R. ventrimaculata: PTX:DHQ = 2.2:1). Apparently, *D. auratus* was much more efficient in sequestering PTX **251D**, sequestering approximately 7 times more than *R. ventrimaculata* (Table X?). Also, both alkaloids were modified by the frogs: PTX **251D** was hydroxylated and converted to aPTX **267A**, and DHQ was *N*-methylated. The proportion of the converted compounds was similar in both species: in *D. auratus* an average of 89.7 ± 1.8% of PTX **251D** was converted to aPTX **267A** and 52.3 ± 5.1% of DHQ was N-methylated, whereas in *R. ventrimaculata*, 86.8 ± 5.4% was converted to aPTX and 53.0 ± 2.7% of DHQ was N-methylated.

<u>Experiment 3</u>

The skin secretions obtained from all frogs from this experiment (*D. auratus* and *P. vittatus*) contained DHQ, whereas no DHQ was detected in those of the control group. In total, all frogs from the experimental group received 8.75 µg of DHQ throughout the 14 days of experiment. In both species, the percentage sequestered varied considerably among the frogs: *D. auratus* sequestered a range of 11.8–86.4%, whereas *P. vittatus* sequestered a range of 7.9–24.5%. As expected, *D. auratus* *N*-methylated part of the sequestered DHQ (65.3 ± 5.9% *N*-methylated), but *P. vittatus* did not.

Discussion

<u>Sequestering vs non-sequestering frogs</u>

Our results show that non-sequestering species did not sequester alkaloids in their skin. In addition, the daily ingestion of alkaloids did not result in any sign of intoxication in *H. cinerea* and *A. femoralis* experimental groups. The non-intoxication signs and the trace amounts or total absence of alkaloids in the feces and other organs of these two non-sequestering species demonstrated that they were able to tolerate the presence of the alkaloids in the organism. On the basis of our results, we suggest that non-sequestering frogs may have an efficient mechanism to metabolize dietary alkaloids and cope with its toxicity and that might be plesiomorphic to all anurans, not only to Dendrobatoidea. In a similar experiment, another hylid, *Boana bandeirantes*, fed with DHQ and HTX **235A** did not present sign of intoxication either (A. Jeckel, personal obs.). Previous study using feeding experiment with *Allobates talamancae* and *Colostethus panamansis*, both non-alkaloid-containing species, did not show detectable amounts of alkaloids and did not describe any sign of intoxication either (Daly et al 1994). Interestingly, pharmacological studies that injected alkaloids from the HTX

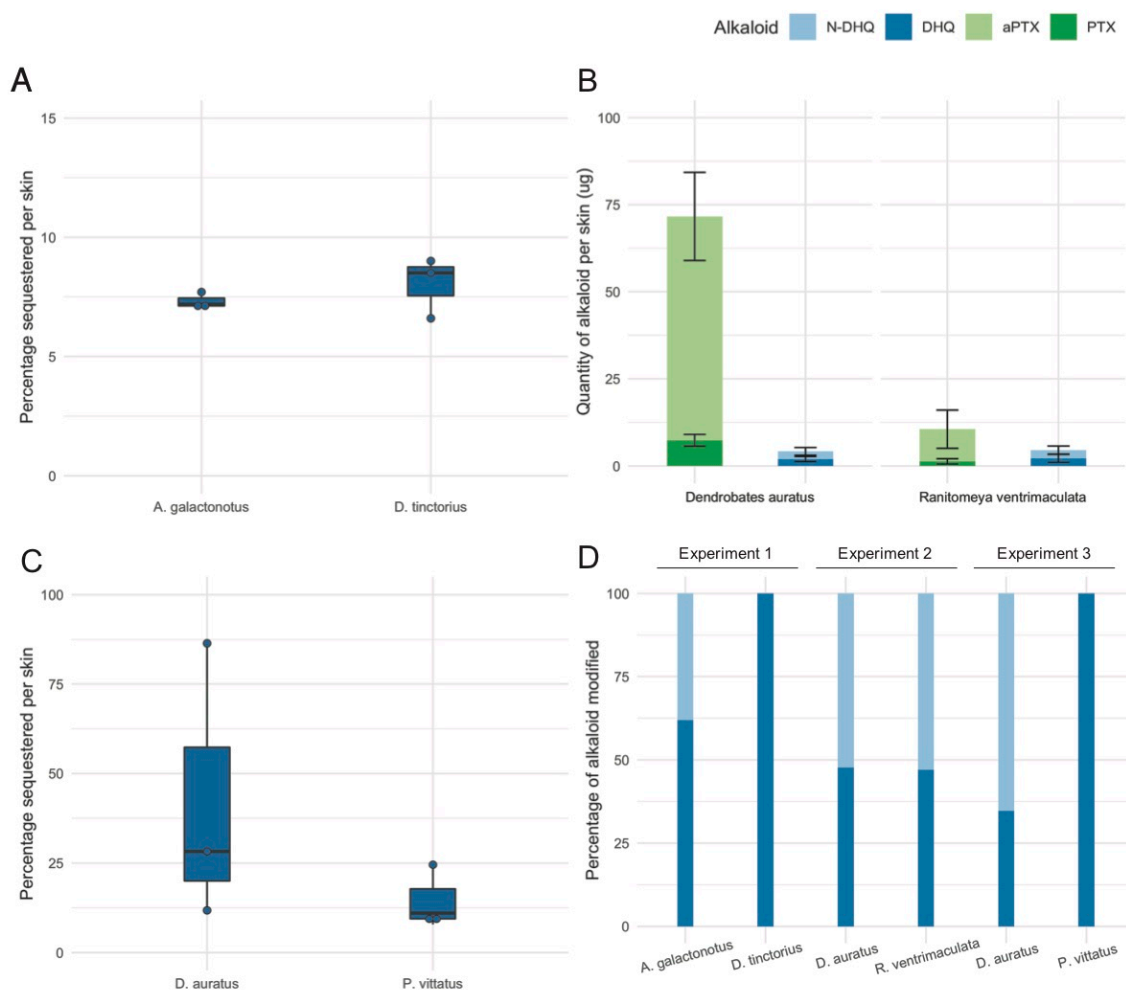

Figure 2. Results of alkaloid sequestration in the poison frog species. (A) Experiment 1: Boxplot of percentage of DHQ sequestered per skin from the total amount administered to *Adelphobates galactonotus* and *Dendrobates tinctorius*. (B) Experiment 2: Mean quantity of PTX **251D** and DHQ sequestered and modified to aPTX **267A** and *N*-methyl DHQ, respectively, per skin in *Dendrobates auratus* and *Ranitomeya ventrimaculata*. Whiskers represent standard deviation. (C) Experiment 3: Boxplot of percentage of DHQ sequestered per skin from the total amount administered to *D. auratus* and *Phyllobates vittatus*. (D) Proportion of DHQ modified to *N*-methyl DHQ in all species from the 3 experiments of the study. Circles in (A) and (C) represent individual frogs per experimental group.

class in *Lithobates pipens*, *L. catesbeianus*, and *Xenopus laevis* frogs (Lapa et al 1975, Ogura & Warashina 1987, Eldridge et al 2018) reported toxic effects in all specimens in different stages of life. Since in our experiments the alkaloids were orally administered (*i.e.* the actual pathway of entrance of dietary alkaloid in the frog's body), these species may have an efficient mechanism of metabolism in the digestive system, including digestive tract and liver, and a mechanism of excretion of alkaloids that was able to eliminate almost 100% of the alkaloid ingested.

Detoxification mechanisms have been thoroughly studied in mammals and other animals of pharmaceutical and economic interest. In humans for example, first pass metabolism enzymes act in both the intestine and liver and act to lower the bioavailability of most ingested drugs. One of the most important first pass enzymes in mammals is a member of the cytochrome P450 (CYP450) system, CYP3A4 (van Herwaaden et al 2007). Two recent studies analyzed transcripts of different organs of wild and captive bred poison frogs. Sanchez et al (2019) found an ortholog transcript of the human CYP3A4 in the skin of an experimental *D. tinctorius* that was single dosed with a mixture of alkaloids normally not found in this species (epibatidine, sparteine, berberine, lupinine, and quinine). Additionally, Caty et al (2019) analyzed skin, liver, and intestine transcripts of wild-caught (presumably in contact with alkaloids) and captive-bred frogs (not in contact with alkaloids). They did not find differential expression of metabolizing enzymes or genes in the skin or intestine but detected over expression of genes involved in small molecules metabolism in the liver of wild-caught frogs, including several CYP450 genes. Specific information about amphibian mechanism of detoxification of small molecules is lacking, but it could be similar to the detoxification system found in mammals and insects. For example, in the guts of herbivore mammals and insects that feed on pyrrolidine alkaloid (PA) producing plants, the non-toxic N-oxide form of PA present in the plant tissue is easily reduced to the toxic free base by CYP450 enzymes. Intoxication is prevented by either (1) suppressing the activity of the CYP450 enzymes (Hartmann et al 1997); (2) degrading the toxic PA by other antagonistic CYP450 enzymes (Huan et al 1998); or (3) by converting the toxic free base back into the N-oxide with a highly specific flavin-dependent monooxygenase (Ehmke et al 1990, Miranda et al. 1991, Huan et al. 1998, Hartmann et al 1999). This mechanism is found in specialized herbivores, but if we consider that the main dietary item of anurans are insects, and that most insects present some kind of chemical defense mechanism (Laurent et al 2005), an efficient mechanism of metabolizing small molecules could be a characteristic present in most anurans, independent of the ability to sequester defensive alkaloids.

Another interesting finding of experiment 1 was the relative amounts of alkaloids detected among the species. The four species tested in experiment 1 differ in alkaloid quantity present in the skin by orders of magnitude (Fig. 1), where poison frogs retained most alkaloids in the skin, and the hylid retained the lowest amounts of alkaloid. These results may suggest that the mechanism of alkaloid metabolism is most efficient in *Hyla*, followed by *Allobates* and less efficient in dendrobatid poison frogs. We suggest that the evolution of alkaloid sequestration in dendrobatids may have been due to a reduction in the metabolism

(degradation) of alkaloids, along with the development of a mechanism to prevent autointoxication, such as insensitivity of target-bonding sites (Wang and Wang 1999, Tarvin et al 2016, 2017, Marquez et al 2018). *Epipedobates* species for instance tolerates the alkaloid epibatidine through amino acid replacements in the β2 subunit of nicotinic acetylcholine receptor, which alters the sensitivity of the receptor to this specific alkaloid (Tarvin et al 2017). In this way, it is possible that all anurans have a mechanism of toxin resistance, which allows eating from a toxic-rich diet, such as insects, but only sequestering lineages developed systems of autoresistance, allowing accumulation of high amounts of chemical compounds for self-defense (Arbuckle et al 2017). Autoresistance mechanisms involve changes that may have pleiotropic effects, which could be one of the reasons that only a few anuran lineages developed a sequestration strategy (Feldman et al 2012). It could also explain why closely related lineages, such as *Allobates,* lack substitutions in the amino-acid sequence of alpha subunit of voltage-dependent sodium channels that provides resistance to dendrobatid poison frogs (Tarvin et al 2016). Santos et al. (2003) suggested that the toxicity evolved previous to aposematic coloration and diet specialization, and similarly, Daly (1998) proposed that the uptake system is a primitive trait over expressed in poison frogs. Here, we suggest a similar but twisted pattern: the detoxification system is primitive to anurans, independent of diet specialization, and it has been under expressed in poison frogs, allowing the sequestration of intact alkaloids.

Modification of sequestered alkaloids

Feeding experiments with poison frogs in controlled environment have progressively increased the suspicion that the alkaloid sequestration system might not be exactly similar among poison frogs from different families and even among members of the same family (Daly et al., 1994, 2003; Smith et al., 2002; Hantak et al., 2013; Jeckel et al., chapter 2). Here, we have shown that different species might sequester the same alkaloid with different levels of efficiency (Fig. 2). In addition, we have also shown that the ability to modify alkaloids is probably more widespread among dendrobatid poison frogs than previously known (Fig. 2). We report for the first time that, in addition to *Adelphobates* and *Dendrobates* species, *Ranitomeya ventrimaculata* is also capable of hydroxylating PTX **251D** to aPTX **267A**. And, in addition to *A. galactonotus*, we add to the list of species that *N*-methylate DHQ, *Dendrobates auratus* and *Ranitomeya ventrimaculata*. In figure 3, we show all the species that have been tested so far, indicating those that sequestered and modified alkaloids, those that only sequestered without modifying them, as well as the species that did

not sequester or modified alkaloids (Daly et al., 2003; Jeckel et al., chapter 2). With the increasing list of species that are able to modify dietary alkaloids, the modification might have more impact on the diversity and variation of alkaloids found in the many species of dendrobatid poison frogs than previously thought.

The capacity to hydroxylate PTX **251D** could explain the presence and absence of aPTX **267A** in some dendrobatid poison frogs. As Daly et al. (2003) observed, in most *Oophaga* species, PTX **251D** is always accompanied by significant amounts of aPTX **267A**, whereas aPTX **267A** does appear in some populations independently of PTX **251D**. This is not a rule for the other dendrobatid genera. In most *Dendrobates* and *Adelphobates* species analyzed, only aPTX **267A** has been detected (Daly et al. 2003 – Table 1, Daly et al. 2009), except for *D. auratus*, which had minor amounts of PTX **251D** in wild-caught animals, and we showed here that they sequester and retain at least a minor amount unchanged in the skin. Recent studies have shown that some populations of *D. tinctorius* and *A. galactonotus* did not contain either of these two alkaloids (Lawrence et al 2019, Jeckel et al 2019). *Ranitomeya* has shown a pattern similar to *Oophaga*: PTX **251D** is usually accompanied by aPTX **267A**, but the former does not necessarily accompany the last. In fact, PTX **251D** was only detected in a single population of *R. variabilis* from San Martin, Peru (Stuckert et al 2014). Even though

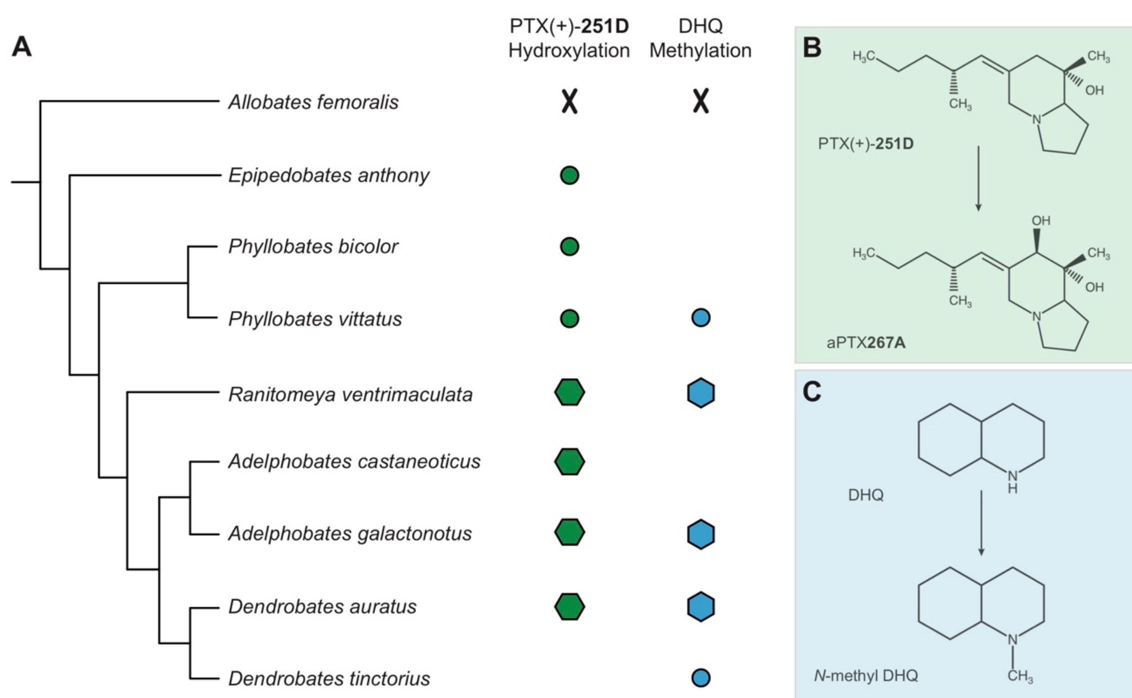

Figure 3. Sequestration and modification of PTX **251D** and DHQ. (A) Species tested by Daly et al. 2003, Jeckel et al. (in prep), and the present study. Hexagons: sequestration and modification, circles: sequestration only, X: no sequestration or modification. (B) Hydroxylation of PTX **251D** to aPTX **267**A. (C) N-methylation of DHQ.

the presence of PTX **251D** might not be as common, we have shown here that at least *R. ventrimaculata* has the ability to hydroxylate PTX **251D**. Conversely, the close related genera, *Andinobates* and *Minyobates*, have both pumiliotoxins alkaloids in different proportions in several species. These two alkaloids in question are taxonomically widespread, and at least one of them has been detected in all the other dendrobatid poison frogs analyzed up to the present: *Phyllobates* and *Ameerega* had PTX**251D** in skin secretions, *Paruwrobates* had aPTX **267A**, and *Epipedobates* species had both alkaloids. The question is if all aPTX **267A** found in those species are formed by complete modification of PTX **251D**. We cannot rule out the hypothesis that part of the aPTX **267A** present in the frogs have been sequestered unchanged as the dietary source of aPTX **267A** is still unknown (Daly et al., 2003).

The *N*-methylation of DHQs can also explain the presence of some *N*-methylated DHQs in the skins extract of many species of *Ameerega*, *Adelphobates*, *Dendrobates*, *Oophaga*, and *Ranitomeya*. DHQ are very common alkaloids in poison frogs and have presumably have an ant source. The presence of *N*-methylated alkaloids could be an indicative of the capacity of methylation, as the source has not yet been identified. In a thorough analysis of several *Ameerega* species, Daly et al. (2009) have found that not all species had *N*-methylated alkaloids, indicating that the ability to modify DHQ might not be present in all species of the genus. Similarly, in the present study, we have shown that even though *D. auratus* was able to modify approximately 50% of the DHQ ingested, *D. tinctorius* did not methylate the DHQ, indicating that this mechanism might not be common to all *Dendrobates* species. DHQs are a common alkaloid in *Dendrobates* species, and account for one of the most diverse alkaloids detected in the members of this genus. In comparison to *D. auratus*, *P. vittatus* sequestered a smaller amount of DHQ and did not methylate it. DHQ are not as common in members of *Phyllobates*, having been detected in *P. aurotaenia*, *P. bicolor*, and *P. vittatus*—none of them *N*-methylated—, and absent in *P. lugubris* and *P. terribilis* (Daly et al 1987, Mebs et al 2014, Protti-Sanchéz et al 2019).

This is the first study to analyze quantitatively alkaloid retention in non-sequestering species. Previous studies (Daly et al. 1994, Sanchez et al. 2019) have done feeding experiences with non-sequestering species, especially *Allobates* species, but the alkaloid feeding procedures did not allow them to quantify the exact amounts of alkaloids that was ingested and retained in the body. By using controlled feeding procedures, we were able to quantify the amount and proportion of alkaloids present in the feeding solution and in the body (Jeckel et al 2020 – in press). Although we were not able to describe the mechanism or the enzymes involved in the detoxification system, we showed that this mechanism exists in

different species of anurans, including sequestering species. Our results bring up new questions about the toxin-resistance, autoresistance, and sequestration evolution among anurans. Is there a conserved mechanism of detoxification of small molecules in all anurans? Has the detoxification mechanism been down regulated or modified in dendrobatid poison frogs to allow sequestration? Also, the presence of enzymes not only for detoxification, but for modification of sequestered alkaloids, has been shown to be more widespread among dendrobatid poison frogs then previously known. Are the modifications of dietary alkaloids an adaptation to increase the diversity of chemical defenses and, ultimately, the capacity of frogs to defend themselves against pathogens and predators? How much of the alkaloids detected in poison frogs are sequestered unchanged and how much is modified? With the advent of "omic" technologies, those questions can be tested in different species and families of sequestering and non-sequestering species, and hopefully, we will be able to understand more about the mechanism that allowed evolution of alkaloid sequestration and modification in the different lineages of dendrobatid poison frogs.

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Supplemental Information

Supplemental Table 1. Average body mass, total quantity of decahydroquinoline (DHQ) and histrionicotoxin **235A** (HTX **235A**) administered, and percentage of alkaloid detected in each organ in all experimental groups. Mean ± sd.

	Average body mass (g)	Alkaloid amount (ug)	Skin (%)	Liver (%)	Kidney (%)	Muscle (%)	Feces (%)	Swab (%)	Total (%)
Adelphobates galactonotus									
DHQ	2.3	140.6	4.5 ± 0.3	1.8 ± 0.2	0	0.1 ± 0.0	0.1 ± 0.0	0.5 ± 0.3	7.0 ± 0.8
N-MeDHQ			2.8 ± 0.0	0.7 ± 0.2	0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.1	3.7 ± 0.2
HTX **235A**	2.3	140.6	48.0 ± 9.8	1.4 ± 0.3	0	0.1 ± 0.0	0	16.2 ± 3.5	65.6 ± 6.4
Dendrobates tinctorius									
DHQ	1.1	67.1	8.1 ± 1.3	5.2 ± 1.6	0.1 ± 0.0	0.3 ± 0.1	0	2.3 ± 0.4	16.0 ± 2.7
HTX **235A**	1.2	71.3	46.1 ± 9.8	1.5 ± 0.4	0	0.1 ± 0.0	0	0.9 ± 0.8	48.5 ± 10.6
Allobates femoralis									
DHQ	1.6	47.5	0.6 ± 0.4	0.3 ± 0.1	0	0	0.2 ± 0.1	1.1 ± 0.6	2.2 ± 0.8
HTX **235A**	1.5	89.2	0	0	0	0	0	0.2 ± 0.2	0.2 ± 0.2
Hyla cinerea									
DHQ	1.9	114.7	0.0 ± 0.1	0	0	0	0	0.5 ± 0.2	0.6 ± 0.2
HTX **235A**	2.9	177.8	0	0.5 ± 0.7	0	0	0	0.6 ± 1.0	1.1 ± 1.7

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Considerações finais

As causas e consequências da variação na composição de alcaloides acumulados por rãs-de-veneno são os principais aspectos investigados para entender a evolução do sequestro de alcaloides e sua importância ecológica na proteção contra predadores e patógenos. Foi testada, principalmente, a influência do mecanismo de sequestro e da sua eficiência, tanto na variação interespecífica, quanto na variação dos tipos de alcaloides presentes em cada espécie. No geral, as hipóteses testadas ajudaram a avançar o limite de conhecimento desta área de estudo, demostrando que (1) existe uma grande variação na eficiência do sequestro por espécie, tipo de alcaloide, concentração de alcaloide e modificação de alcaloide; (2) o sequestro em rãs-de-veneno é um processo pouco eficiente; e (3) parece existir um mecanismo plesiomórfico de detoxificação/degradação de alcaloides em anuros que permite evitar intoxicação.

A variação na eficiência de sequestro entre espécies e tipos de alcaloides era um resultado esperado devido aos estudos anteriores com experimentos de alimentação. Entretanto, os resultados apresentados nesta tese foram os primeiros a demonstrar essa variação de forma quantitativa, permitindo maior refinamento analítico nas comparações entre espécies e tipos de alcaloide. Estudos futuros podem tentar explicar por que existe uma variação na eficiência de sequestro quando a concentração das doses é diferente, e também se existe um efeito do número de doses administrado em cada organismo.

O Capítulo 2, em que foram feitos experimentos com múltiplas doses, mostrou que, para HTX, parece existir um processo endógeno de aumento da eficiência conforme a dose aumenta. Esse resultado pode ser analisado em contraste com o resultado do Capítulo 4, no qual a administração de uma dose única desse mesmo alcaloide, não suscitou uma resposta muito eficiente do mecanismo de sequestro. Em contrapartida, a quantidade de doses ou a concentração da mesma não teve efeito sobre a eficiência de sequestro de DHQ. É possível que as propriedades físico-químicas dos alcaloides tenham efeito direto na eficiência de sequestro, como discutido no Capítulo 2. Entretanto, não podemos descartar a possibilidade de que os mesmos transportadores e outras moléculas envolvidas no mecanismo de sequestro respondam de formas diferentes para distintas classes de alcaloides.

A modificação de alcaloides é outro processo ainda pouco entendido. Provavelmente essa modificação ocorre através da ação de enzimas que promovem a transformação químico-estrutural da molécula e algumas destas podem não estar presentes em todas as espécies de rãs-de-veneno. Até mesmo espécies de um mesmo gênero apresentam capacidades diferentes

de modificação de alcaloide, como demonstrado no caso de *Dendrobates auratus* e *D. tinctorius*, no Capítulo 5. Além da capacidade de modificação, ainda fica em aberto a importância ecológica, se houver, desse processo de modificação na proteção contra predadores e patógenos. Como demonstrado no teste de palatabilidade incluído no Capítulo 1, independentemente da variação observada na composição dos alcaloides entre os indivíduos, todos estavam igualmente protegidos de possíveis predadores quimicamente orientados ao transmitir a mesma mensagem da presença de compostos defensivos. Se a capacidade de modificação existe para aumentar a diversidade de alcaloides presentes na pele, ainda precisamos entender as consequências dessa diversidade. Como demonstrado no Capítulo 5, a capacidade de modificação surgiu em apenas uma das linhagens de Dendrobatidae que sequestram alcaloide, o que significa que não é uma característica fundamental para o sequestro.

A dualidade entre sequestro e degradação dos alcaloides parece ser a peça chave da evolução do sequestro. Entretanto, parece contraproducente que um organismo que depende completamente do composto sequestrado para proteção química, degrade quase metade do que é consumido (Capítulos 2, 4 e 5). Pode ser que esse mecanismo ainda esteja presente como resquício de uma condição pleisiomórfica amplamente presente em anuros. Ou ainda, é possível que tal mecanismo tenha a função de evitar a concentração de grandes quantidades de toxina absorvida no corpo em um determinado período de tempo, evitando autointoxicação.

Como perspectivas de pesquisas futuras, surgem diversas perguntas quanto a esse mecanismo contraditório de degradação que pode explicar a evolução dessa habilidade de sequestrar alcaloides da dieta. Pode ser que a diminuição da eficiência de degradação tenha dado oportunidade para os processos envolvidos no sequestro de alcaloides surgirem, como o transporte e o armazenamento. Sob a perspectiva bioquímica, a pergunta é sobre a identidade e o mecanismo de ação das enzimas que aparentemente degradam de forma eficiente os alcaloides ingeridos, tanto pelas rãs-de-veneno, quanto pelos outros anuros que não sequestram (*Allobates* e *Hyla*, por exemplo, estudados no Capítulo 5). Outro aspecto importante é o órgão responsável pela degradação. No Capítulo 4, verificou-se que o fígado pode ter um papel importante na metabolização dos alcaloides, por concentrar parte deles mesmo depois de horas após a sua administração. Mas outros órgãos do sistema digestivo, como o intestino, também podem ser importantes na eficiência de absorção dos alcaloides, e também na metabolização por enzimas locais.

Em uma perspectiva evolutiva, necessitamos responder se todas as linhagens das cinco famílias de anuros, que tem a habilidade de sequestrar alcaloides, possuem mecanismos de sequestro parecidos aos demonstrados para Dendrobatidae. Seria também a capacidade de sequestro dos anuros pertencentes às linhagens ainda não investigadas pouco eficiente devido a degradação dos alcaloides? Caso esses grupos (ou parte deles) apresentem a capacidade de degradação de alcaloides, seria o mecanismo bioquímico envolvido plesiomórfico ou convergente entre linhagens? Através de estudos de expressão gênica e identificação dos possíveis genes candidatos envolvidos na degradação de alcaloides, será possível estabelecer uma hipótese que relacionará expressão gênica à capacidade ou não de sequestro e à taxa de degradação de alcaloides.

O conhecimento sobre os mecanismos de sequestro em rãs-de-veneno vem aumentando pouco a pouco nos últimos anos. Com a emergência das "omics", as possibilidades de investigação sobre transporte, modificação, degradação e armazenamento de alcaloides em rãs-de-veneno são inúmeras. Nesta tese foram investigadas as influências do mecanismo de sequestro sobre a variação de alcaloides presentes em rãs-de-veneno. Os resultados mostram que é um mecanismo ainda mais complexo e multifatorial do que a hipótese original da tese, apontando direções importantes a serem tomadas em pesquisas futuras.

Resumo

Algumas das espécies de animais evoluíram, independentemente, a capacidade de sequestrar esses compostos da dieta. Neste mecanismo, os compostos são ingeridos, absorvidos, transportados e armazenados em tecidos especializados. As espécies que são capazes de sequestrar compostos da dieta desenvolveram mecanismos e estratégias específicas que permitem eles consigam se alimentar e acumular grandes quantidades desses compostos sem se autointoxicar. As rãs-de-veneno são o grupo de vertebrados mais estudado que sequestra compostos defensivos da dieta. Eles sequestram alcaloides principalmente de formigas e ácaros e os acumulam em glândulas de veneno da pele. Devido a origem dos compostos defensivos, uma característica importante desse sistema é a grande variação inter e intraespecífica de tipos, quantidade e composição de alcaloides. Muitas causas ecológicas são conhecidas por interferirem diretamente nessa variação, como a localização geográfica, estação do ano, sexo, idade e disponibilidade de alimentos no ambiente. Entretanto, pouco se sabe sobre o mecanismo de sequestro em si e como ele pode também ser responsável por grande parte da variação de alcaloides em rãs-de-veneno. O objetivo geral da presente tese de doutorado foi avaliar como o mecanismo de sequestro pode interferir na variação de tipos, quantidade e composição geral de alcaloides encontrados em rãs-de-veneno da família Dendrobatidae. A tese se divide em cinco capítulos em formato de manuscrito científico que foram ou serão submetidos a jornais da área. No geral, as hipóteses testadas ajudam a avançar o limite de conhecimento desta área de estudo, demostrando que (1) existe uma grande variação na eficiência do sequestro por espécie, tipo de alcaloide, concentração de alcaloide e modificação de alcaloide; (2) o sequestro em rãs-de-veneno é um processo pouco eficiente; e (3) parece existir um mecanismo plesiomórfico de detoxificação/degradação de alcaloides em anuros que permite evitar intoxicação. Os resultados mostram que o sequestro em rãs-de-veneno é um mecanismo ainda mais complexo e multifatorial do que originalmente hipotetizado, mas apontam direções importantes a serem tomadas em pesquisas futuras.

Abstract

Several species of animals independently evolved the ability to sequester compounds from their diet. In this mechanism, compounds are ingested, absorbed, transported and stored in specialized tissues. These species have developed mechanisms and strategies to eat and accumulate high quantities of these chemical compounds avoiding self-intoxication. Poison frogs are the most widely studied group of vertebrates that sequesters defense compounds from dietary sources. They primarily sequester alkaloids from ants and mites and accumulate them in skin poison glands. Due to the origin of defense compounds, an important characteristic of this system is the high inter- and intraspecific variation of types, quantity and composition of alkaloids. Ecological factors such as geographic location, season, sex, age and life stage and body size are known to directly interfere in the composition and amount of alkaloids present in each individual and population. However, little is known about the mechanism of sequestration itself and how it can also be responsible for the variation of alkaloids in poison frogs. The general objective of this doctoral thesis was to evaluate how the sequestration mechanism affects the variation of types, quantity and general composition of alkaloids found in poison frogs of the family Dendrobatidae. The chapters follow a manuscript format to be submitted to their respective journals. In general, the hypotheses tested provided more information about this area of study, demonstrating that (1) there is wide variation in sequestration efficiency per species, alkaloid type, alkaloid concentration, and alkaloid modification; (2) sequestration in poison frogs is an inefficient process; and (3) there seems to be a plesiomorphic mechanism of detoxification/degradation of alkaloids in anurans that prevents intoxication. Those results support that this mechanism is even more complex and multifactorial than originally hypothesized, as well as point to important directions for future research.

Apêndice 1 – English versions of Introduction and Final Considerations

Introduction

Chemical defenses are an interspecific interaction mediated by chemical compounds that negatively influence molecular targets of predators or pathogens, thus protecting the organisms who use this defense (Wink, 2003). Structures of defense compounds are similar to those of innate endogenous molecules in animal metabolism and present both agonist effects by binding to cellular receptors, with similar action as innate compounds, as well as antagonistic effects by blocking cell receptors (e.g. Wink et al., 1998; Badio & Daly, 1994).

The presence of defense compounds has evolved independently in different lineages, from sea sponges (Taylor et al., 2007) and large invertebrate lineages (*e.g.* Cimino & Ghiselin; 1998, Laurent et al., 2003) to vertebrates (*e.g.* Noguchi et al., 2006; Ligabue-Braun et al., 2012; Ligabue-Braun & Carli, 2015). Currently, thousands of different types of compounds are known, with broad structural variations between species (Wink, 1993; Daly et al., 2005). In addition to variation between species, the defense compounds of a single individual can also be highly diverse, containing more than one substance type (*e.g.* Jeckel et al., 2015), which protect and act against different threats and molecules, respectively (Wink, 2003). Moreover, this variety may be functionally important as it can interfere with the viscosity of the substance (Blum et al., 1973); or a synergistic role in which the sum of two or more substances is more effective than the action of one single substance (Pasteels, 1983), or even as a form to avoid predator adaptations (Barnett et al., 2014). Some compounds are unique to certain taxonomic groups and can be used to infer hypotheses about the evolutionary relationships between groups, and therefore as evidence in taxonomy and systematics as well (*e.g.* Pasteels, 1993; Cei et al., 1967; Grant et al., 2017).

Although chemical defenses are usually related to lethal toxicity, the vast majority of chemical compounds present in these organisms are not lethal to predators in the amounts presented. One consequence of this defense strategy is that predators survive and learn to avoid attacking such organisms in future encounters (Brower et al., 1968; Servedio, 1999). For this, defense compounds usually cause some kind of discomfort, e.g., bad taste (e.g. Skelhorn & Rowe, 2006; Bolton et al., 2017), change in blood pressure or heart rate (e.g. Clarke 1997), gastrointestinal indigestibility and discomfort (e.g. Brower et al., 1968; Wouters et al., 2016) or even temporary changes in behavior (e.g. Jumar et al., 2014; Zou et al., 2016). One hypothesis that explains the importance of this learning by potential predators is the emergence of bright and disruptive colors to warn and reinforce learning for visually

oriented predators, a strategy known as aposematism (review in Mappes et al., 2005). This hypothesis has been empirically tested for many groups of organisms (*e.g.* Schmidt & Blum, 1977; Rubino & McCarthy, 2004; Saporito et al., 2007). However, aposematism and chemical defense do not always protect organisms from attacks, predation, and/or infection by their natural enemies, as many predators can become resistant to compounds and overcome their chemical arsenals (Geffeney et al., 2005; Despres et al., 2007; Pittendrich et al., 2013; Ujvari et al., 2015).

Resistance to chemical compounds can be achieved through chemical detoxification by general or specific enzymes (Hartmann & Ober, 2000; Heidel-Fischer & Vogel, 2015). Predation almost always involves ingesting prey and, consequently, defensive compounds are exposed to the metabolism of the predator's digestive system. The metabolism of active compounds has been widely studied in mammals, mainly for pharmaceutical and economic interests. For example, in humans, the total concentration of xenobiotics (chemical compounds foreign to an organism, such as medicines and pesticides) decreases considerably in the liver before reaching the systemic circulation (Pond & Tozer, 1984). The enzymes of the cytochrome P450 system (CYP450) are primarily responsible for modifying toxic compounds into more stable and hydrophilic compounds, facilitating excretion by the kidney. Herbivorous mammals, such as sheep and hamsters, and insects that feed on chemically defended plants have an arsenal of CYP450 enzymes, as well as enzymes from the flavin-dependent monooxygenase family, which allows them to feed on these plants by stabilizing and excreting the toxins (Ehmke et al., 1990; Miranda et al., 1991; Huan et al., 1998; Hartmann et al., 1999).

Another way to oppose the chemical defenses of prey is through insensitivity of target molecules, usually ion channels, using substitutions at binding sites in amino acid sequences (Wang & Wang, 1999; Geffeney et al., 2002; Tarvin et al., 2016). However, this substitution can be costly, which makes this form of resistance less common and, when it does occur, the substituted amino acids are quite conserved and convergent between different species (Feldman et al., 2012; Ujvari et al., 2015). A classic example of this is the snake *Thamnophis sirtalis* (Linnaeus, 1758) that is resistant to tetrodotoxin (TTX) present in the salamander *Taricha granulosa* (Skilton, 1849). This toxin is one of the most potent known toxins and acts by blocking sodium channels (Mosher et al., 1964). These snakes can feed on these salamanders due to a variety of mutations in the gene that encodes the NaV1.4 region of TTX affinity with sodium channels (Geffeney et al., 2002). The variation in types and amounts of amino acid mutations in this region gives snakes different degrees of resistance, allowing

them to adapt to toxin concentrations of specific salamander populations (Hanifin et al., 2008; Williams et al., 2010). For example, in locations where the salamander presents lower TTX concentrations, snake mutations are different from those where salamanders have higher TTX concentrations (Hanifin et al., 2008). This occurs because these mutations are very costly, interfering with the snake's ability to move and react after ingesting the toxin (Feldman et al., 2012). Such relationship is a classic example of a coevolutionary arms race between two species (Brodie III et al., 2005). Adaptations to counteract prey toxins seem to be an important strategy, especially for animals that have developed specialized diets.

Origin of compounds in chemically defended organisms

Defensive compounds can be (1) synthesized by the organism itself (biosynthesized), (2) acquired by symbiosis or (3) acquired from the environment. The sources of these compounds are not mutually exclusive. For example, an individual can both synthesize substances and acquire them from the environment (*e.g.* Jeckel et al., 2015). Among the biosynthesized compounds, some are products of secondary metabolism, using steroids or amino acids as substrate (Erspamer, 1954; Pasteels, 1983), while others are proteins and peptides, which are genetically coded (König et al., 2015). These compounds are produced in specific tissues and may or may not be stored in glands. Biosynthesis, either by direct translation of proteins or by *de novo* synthesis (i.e. synthesis of complex molecules from smaller molecules) of molecules, seems to be the most common form of chemical defense in animals, and is very common in terrestrial arthropods (Whitman et al., 1990) and anurans (Erspamer, 1994).

The other source of defense compounds is the symbiotic association with microorganisms (Flórez et al., 2015). For example, toxins found in the sponges *Theonella swinhoei* (Gray, 1868) from the Philippines are produced by filamentous and single-celled symbiotic bacteria (Bewley et al., 1996). However, the most famous and controversial case is that of TTX-producing bacteria found in several species of pufferfish (review by Noguchi et al., 2006) and other marine invertebrates (Chau et al., 2011). Although several studies have shown that TTX-producing bacteria live in symbiosis with numerous species containing TTX, the biosynthetic route or TTX coding genes have not yet been described for any of these bacteria. In addition, this type of bacteria has not been found in any terrestrial vertebrate that has TTX, as is the case for some amphibian species (Mosher et al., 1964). In salamanders, it is believed that TTX is produced endogenously, without symbiotic interaction

for synthesis (Lehman et al., 2004). However, there is still no empirical evidence to support this hypothesis.

Finally, defense compounds can be acquired through the environment. For example, some locusts feed on plants with chemical defense and obtain some protection while harmful compounds are in their digestive tracts. However, as soon as their digestive tracts are emptied and/or their food source changes, locusts lose their chemical protection (Sword, 1999). Alternatively, compounds can be sequestered from their diet, that is, compounds are ingested, absorbed, transported and stored in specialized tissues.

Herbivore insects have evolved the ability to sequester as a way to overcome the arsenal of secondary metabolites produced by plants in response to herbivory (Pasteels, 1983; Wink, 1993). Many insect species can tolerate toxicity and use the compounds for both defense and other physiological and ecological functions (Dussourd et al., 1989; Brückmann et al., 2000; Erb & Robert, 2016). For example, the sequestration of cardenolides, a sterol from the cardiac glycosides group, is known from several insect orders as Lepidoptera (Brower et al., 1984; Black, 1976; Nishio, 1980; Cohen & Brower, 1982), Coleoptera (Dobler et al., 1998; Isman et al., 1977b; Duffey & Scudder, 1972; Nishio et al., 1983), Hemiptera (Rothschild et al., 1970; Duffey & Scudder, 1972; Duffey et al., 1978) and Orthoptera (von Euw et al., 1967). A classic example is cardenolides sequestration by monarch butterfly larvae from plants of the *Asclepsias* genus (Malcom & Brower, 1989). Compounds sequestered as larvae are stored in a way that protects them after metamorphosis and into adulthood (Brower et al., 1968). Another widely studied sequestering system in insects is that of the pyrrolizidine alkaloids (PA). Several species of Lepidoptera (Nickisch-Rosenegk & Wink, 1993) and Coleoptera (e.g., species of the genus *Oreina*, Rowell-Rahier, et al 1991) feed on PA-producing plants and sequester the alkaloid, transporting it to specialized glands or simply accumulating it in the hemolymph (Hartmann & Ober, 2000).

Among vertebrates, some lineages of birds, snakes and amphibians have also acquired the ability to sequester compounds from their diets. For example, the snake *Rhabdophis tigrinus* (Boie, 1826) feeds on frogs of the Bufonidae family (Hutchinson et al., 2007). These frogs have high concentrations of a cardiac glycoside, bufodienolide, in their cutaneous glands, which has high affinity with Na^+/K^+-ATPase, a molecule that maintains the membrane potential of cells (Agrawal et al., 2012). *R. tigrinus* is able to sequester these sterols and store them in specialized glands in its head region (Hutchinson et al., 2007). Another example is the species of New Guinea birds (*Pitohui* and *Ifrita* genera) that sequester batrachotoxin (BTX) from *Choresine* beetles (Melyridae; Dumbacher et al., 1992, 2009;

Ligabue-Braun & Carli, 2015). BTX is one of the most toxic alkaloids found in nature as it presents high affinity with and blocks sodium channels of muscle and nerve cell membranes (Daly et al., 1965). The high toxicity of BTX has already been recorded in other vertebrates that sequester alkaloids: poison frogs. Poison frogs are the most widely studied group of vertebrates that sequesters defense compounds from dietary sources. They primarily sequester alkaloids from ants and mites and accumulate them in skin poison glands (Daly et al., 1994, 2005; Smith et al., 2002; Hantak et al., 2013).

Sequestration of chemical compounds from dietary sources adds another level of trophic interaction between organisms (Fig. 1). The chemically defended organism that needs to sequester defense compounds is the predator of another chemically defended animal, therefore, it must also have developed ways to prevent not only the action of a toxin, but also the deliberate accumulation of these compounds in its body. For example, ants that are a source of alkaloids for poison frogs produce a wide variety of these compounds (Edcoubas & Blum, 1990). Due to toxicity and repellent function, these alkaloids are readily used by ants during aggressive and competitive interactions (Adams & Traniello, 1981; Adams et al., 2013; Obin & Vander Meer, 1985). In turn, poison frogs seem to have developed mechanisms that allow them to feed on and accumulate these compounds without any adversity. On the contrary, they have gained advantages by sequestering these compounds, which has helped them defend themselves from their own predators. The same system occurs in insects that sequester secondary metabolites from plants. These compounds repel the vast majority of generalist herbivores, thus protecting leaves and other plant organs (e.g., Pasteels,

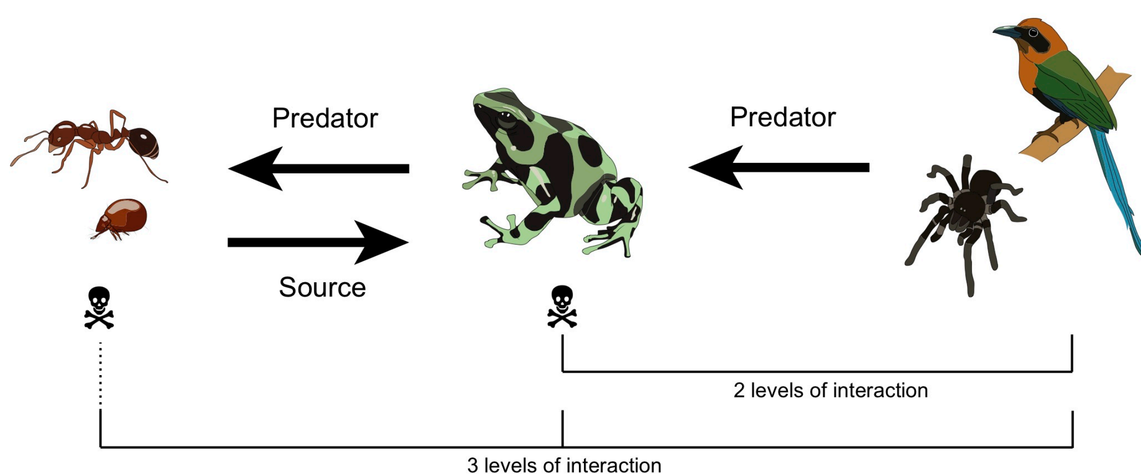

Figure 1. Ecological interactions in a predator-prey system of chemically defended animals. In a system where there is sequestration of defense compounds, one of the preys is the toxin source for the sequestering organism.

1983; Detzel & Wink, 1995; Wouters et al., 2016). As a way to overcome the toxicity of metabolites and adapt against predators, insects specialized in certain plant species have mechanisms that allow such compounds to be absorbed and transported by the body in a non-toxic structural form. Each species that sequesters a certain type of chemical compound has developed specific mechanisms and strategies that allow this system to work efficiently.

Physiological and molecular adaptations of sequestration

Sequestration capacity has evolved independently in several animal groups (Daly et al., 1994; Duffey, 1980; Dumbacher et al., 1992; Hutchinson et al., 2007) and although the mechanism itself varies between species and types of chemical compounds, they all have had to adapt to similar circumstances in order for the defense strategy to be efficient. This convergence in different groups could be the result of lower physiological cost when sequestering an existing compound instead of biosynthesizing (Zvereva et al., 2016). Thus, several metabolic routes would not need to exist. However, when physiological and ecological parameters are observed, sequestration is not necessarily less costly or simpler (Duffey, 1980; Zvereva et al., 2016). In fact, several adaptations are necessary, including physiological mechanisms to absorb and transport compounds while avoiding atuointoxication (Duffey, 1980) and behavioral mechanisms, such as foraging, since they depend on diet to acquire chemical protection (Termonia et al., 2001; Darst et al., 2005; Agrawal et al., 2012). Adaptations to avoid autointoxication are similar or even equal to previously mentioned mechanisms about predators adapted to chemically defended organisms. The difference for animals that sequester is that their resistance must be efficient enough to transport and store large quantities of the defense compound.

One of the strategies to avoid autointoxication is through insensitivity of target molecules via substitutions in amino acid sequences at binding sites (Wang & Wang, 1999; Tarvin et al., 2016). However, toxin insensitivity can affect the sensitivity of binding sites to its endogenous ligand (Tarvin et al., 2017). Therefore, in proteins that are as conserved as ion channels, possible substitutions are restricted, resulting in substitutions in the same amino acid position (Ujvari et al., 2016). This is true for resistance to cardiac glycosides, which has arisen several times independently among invertebrates and vertebrates (Dobler et al., 2012; Bramer et al., 2015; Ujvari et al., 2016; Mohammadi et al., 2016; Holzinger & Wink, 1996; Petschenka et al., 2012). The two previously mentioned types of cardiac glycosides, cardenolides and bufodienolides, interact with the ubiquitous membrane enzyme Na^+/K^+-ATPase, blocking ion transport (Agrawal et al., 2012). The most important region for the

steroid binding with the enzyme is in the extracellular loop between the first two transmembrane segments (H1-H2) of the ten in the alpha subunit (Agrawal et al., 2012). Studies have found that substitutions in two amino acids at positions 111 and 122 of the H1-H2 loop are enough to confer low enzyme affinity to steroids and consequent resistance to toxins in several insect species (Dobler et al., 2012), especially Lepidoptera (Holzinger & Wink, 1996; Petschenka et al., 2012; Bramer et al., 2015), and snakes with bufanoid specialized diets (Ujvari et al., 2015; Mohammadi et al., 2016).

Regarding poison frogs, *Phyllobates* species have an insensitivity to the BTX alkaloid present in their skin glands, while other species of the same family do not. This insensitivity is due to certain substitutions in segment 6 of domain I and IV of the alpha subunit of voltage-dependent sodium channels (Wang & Wang, 1999; Tarvin et al., 2016). Substitutions in these segments are also presumed to confer insensitivity to pumiliotoxin and histrionicotoxin, two alkaloid classes that are commonly found in amphibians that sequester alkaloids (Fig. 2; Tarvin et al., 2016). Insensitivity to epibatidine, another alkaloid found in poison frogs, has also recently been elucidated (Tarvin et al., 2017). This alkaloid interacts with nicotinic acetylcholine receptors and the alkaloid binding site is in the exact same place as acetylcholine. Only one amino acid substitution has been found at this site, which was enough to decrease the site's sensitivity to epibatidine, but also to acetylcholine. Therefore, other substitutions in different regions of the receptor have been found repairing acetylcholine's binding ability, maintaining insensitivity to the alkaloid (Tarvin et al., 2017).

Although amino acid substitutions to the molecular targets of these compounds provide insensitivity allowing resistance and sequestration, they may not be sufficient for some taxa due to the complex variety of compounds that one individual can possess (Pasteels, 1983; Jeckel et al., 2015a). Poison frogs present this wide variation, with dozens of different alkaloid types on their skin that can act on different target molecules in a variety of ways (review in Daly et al., 2005). Therefore, in addition to insensitivity, physiological pathways of resistance and metabolization of compounds can play an important role in preventing autointoxication.

Some metabolization strategies have already been described for herbivorous insects. These strategies help store compounds in a less harmful way or facilitate sequestration by modifying the polarity of compounds, since transmembrane transport of nonpolar compounds is easier, and maintenance of polar compounds in the cytosol is less costly (Duffey, 1980; Lindigkeit et al., 1997). One well-known example is that of the larvae of certain moth species. Plants have pyrrolizidine alkaloids (PA) in the N-oxidized form (hydrophilic), which

is the non-toxic form. When ingested by these larvae, PA is reduced to its tertiary alkaloid form (lipophilic) in the digestive tract, becoming susceptible to the action of P450 monooxygenase enzymes. P450 enzymes help metabolize lipophilic xenobiotics by converting them into excretable metabolites. However, in the case of PA's, they convert the alkaloid to an unstable form, bioactivating the compound and making it toxic. Insects that can sequester PA have flavoprotein enzymes that quickly N-oxidize the alkaloid back to its non-toxic form, allowing accumulation and storage (Hartmann & Ober 2000). This form of compound stabilization for sequestration also occurs in the western corn rootworm

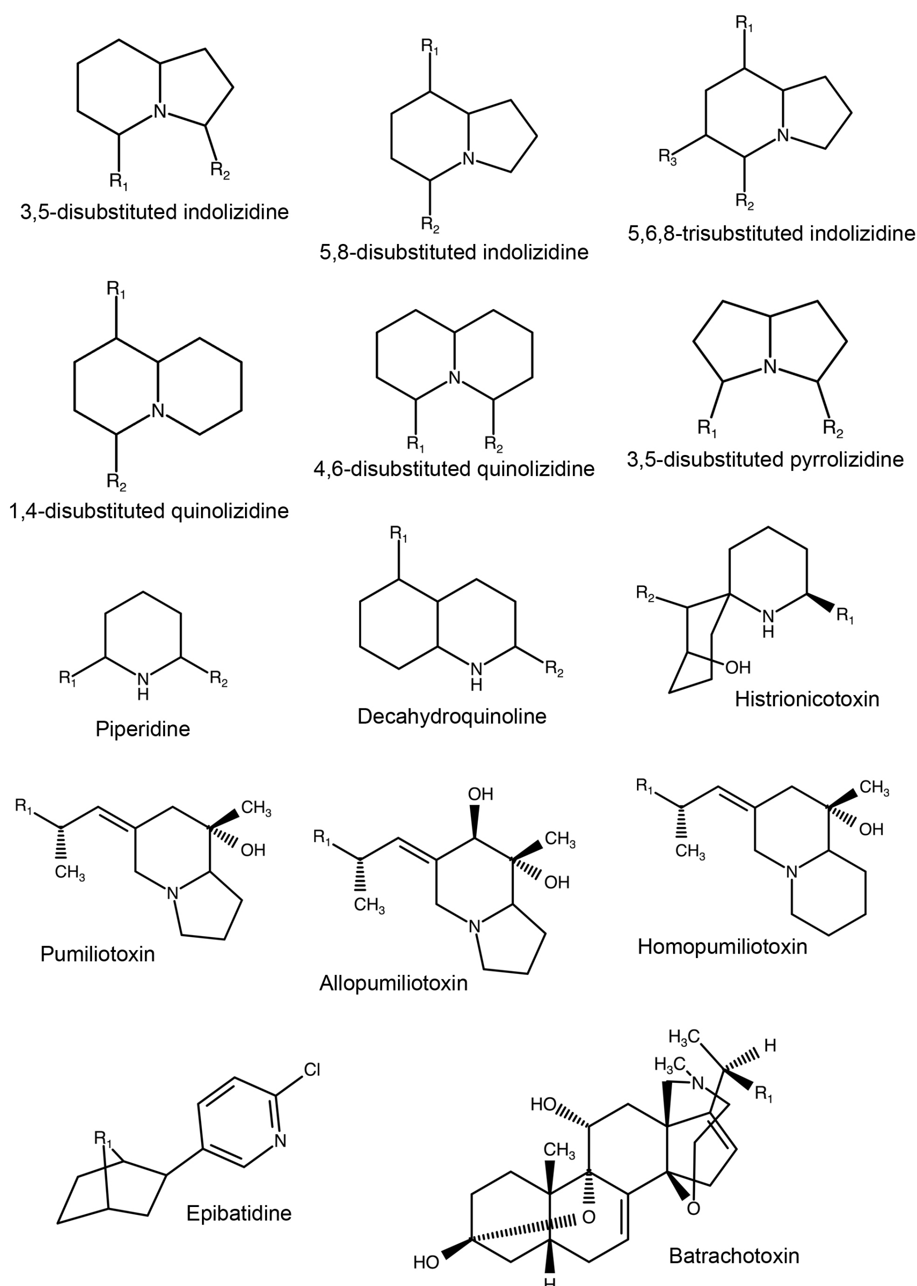

Figure 2. Chemical structure of alkaloids commonly found in poison frogs.

(*Diabrotica virgifera virgifera* [LeConte, 1868]). They sequester benzoxazinoides (BXD) present in the corn plant and accumulate them in the hemolymph and chitin of the exoskeleton (Robert et al., 2017). Plants store BXD in its glycosylated form, i.e. with a coupled glucose molecule. When attacked by an herbivore, BXD is deglycolated in the plant's own cytoplasm, becoming toxic and causing the herbivore to stop feeding (Wouters et al., 2016). These larvae, however, feed on these compounds and glycosylate BXD in their own digestive tract, preventing toxic action and accumulating the compounds for defense against their own entomopathogens (Robert et al., 2017).

Unlike the detoxification of harmful compounds, the sequestration system requires that compounds maintain their basic chemical structure to retain their functionality. For this, there are many systems in which each species presents unique ways of dealing with specific compounds, allowing absorption, transport, and storage to prevent autointoxication, but maintaining the defensive nature of the sequestered compound. Thus, the focus of this study is the alkaloid sequestering system of a poison frog lineage from the Dendrobatidae family.

Poison frogs

Approximately 160 anuran species are able to sequester alkaloids from their diet, forming a polyphyletic group with wide geographical distribution called "poison frogs" (Fig. 3A), including members of the families Bufonidae (*Melanophryniscus*; Daly et al., 1984), Dendrobatidae (genera *Ameerega*, *Epipedobates* and subfamily Dendrobatinae; Myers et al., 1978), Eleutherodactylidae (part of the species group of *Eleutherodactylus limbatus*; Rodriguez et al., 2010), Mantellidae (*Mantella*; Daly et al., 1984) and Myobatrachidae (*Pseudophryne*; Daly et al., 1984). All these non-related genera share several characteristics that might be related to the ability to sequester alkaloids. Poison frogs are diurnal (Santos & Grant, 2010); mainly myrmecophagous (Toft, 1995; Bonanseira & Vaira, 2007; Moskowitz et al., 2018); have similar snout-vent sizes (15–45mm); normally inhabit the ground leaf litter; and generally present aposematic coloration, i.e. a coloration that signals the presence of chemical defensives to visually oriented predators (Saporito et al., 2007b; Noonan & Comeault, 2008; Bordignon et al., 2018).

Among dendrobatid frogs, the ability to sequester alkaloids seems to have evolved at least four times within its phylogeny (Grant et al., 2017), including 10 of the 16 described genera (Adelphobates, Andinobates, Ameerega, Dendrobates, Epipedobates, Minyobates, Oophaga, Paruwrobates, Phyllobates and Ranitomeya; Fig. 4), and almost half of the 200 species of the family (Frost, 2020). Although the monofily of this group is well established

(Grant et al., 2017), its position in the amphibian tree of life is still very variable (e.g. Pyron 2014; Feng et al. 2017; Jetz and Pyron 2018). These species have a ample geographic distribution, from Nicaragua, to the Amazon basin of Bolivia and southeastern Brazil, to the Guianas (Frost, 2020).

This group is known for its bright coloration (Fig. 3B) and its diverse and complex modes of reproduction and parental care. Unlike the classic mode of reproduction of amphibian involving big bodies of water, dendrobatid frogs lay eggs in the leaflitter of the forest floor, or in phytotelma of bromeliads or other natural cavities. Also, some species present elaborate parental care behavior, that involves tadpole transportation on the dorsum to the phytotelma, and oocytes provision as the only source of larvae nutrition. Another characteristic of this group that is also related to reproduction is the wide variety of polymorphism in some species. Research have shown that females base their choice in the visual cues of the males, and that the preference may be result of imprinting during larval stage, when the larvae was carried by its progenitors (Yang et al., 2019). All the frogs of this family are diurnal and active foragers, and the sequestering species are specialized in ants and mites (Santos et al., 2003; Darst et al., 2005), their main source of defensive alkaloids.

More than 1,200 alkaloids from 28 different structural classes have already been reported for these poison frogs (Fig. 2; Daly et al., 2005; Jeckel et al., 2019). The main sources of these alkaloids are mites and ants, which provide most of the alkaloids found on the skin of these amphibians (Saporito et al., 2004, 2007). Due to the origin of defense compounds, an important characteristic of this system is the high inter- and intraspecific variation of types, quantity and composition of alkaloids. Factors such as geographic location (Saporito et al., 2006, 2007), season (Saporito et al., 2010a), age and life stage (Daly et al., 2002; Stynoski et al., 2014; Jeckel et al., 2015) and body size (Saporito et al., 2010b) directly interfere in the composition and amount of alkaloids present in each individual and population. Along with the causes of this great variation, it is also important to understand the consequences and how, to what extent, and if this characteristic acts as a defense mechanism against the frogs' natural enemies. For visually oriented predators, the relationship between alkaloids and coloration is signaled by the aposematic coloring of these frogs, especially those of the Dendrobatidae family (Saporito et al., 2007b; Noonan & Comeault, 2008). However, the direct relationship between visual parameters and alkaloid diversity is still controversial. Some studies have shown that individuals from populations with higher brightness or hue parameters tend to be more "toxic" than individuals with duller color skin (Summers & Clough, 2001; Maan & Cummings, 2012). However, other studies have found

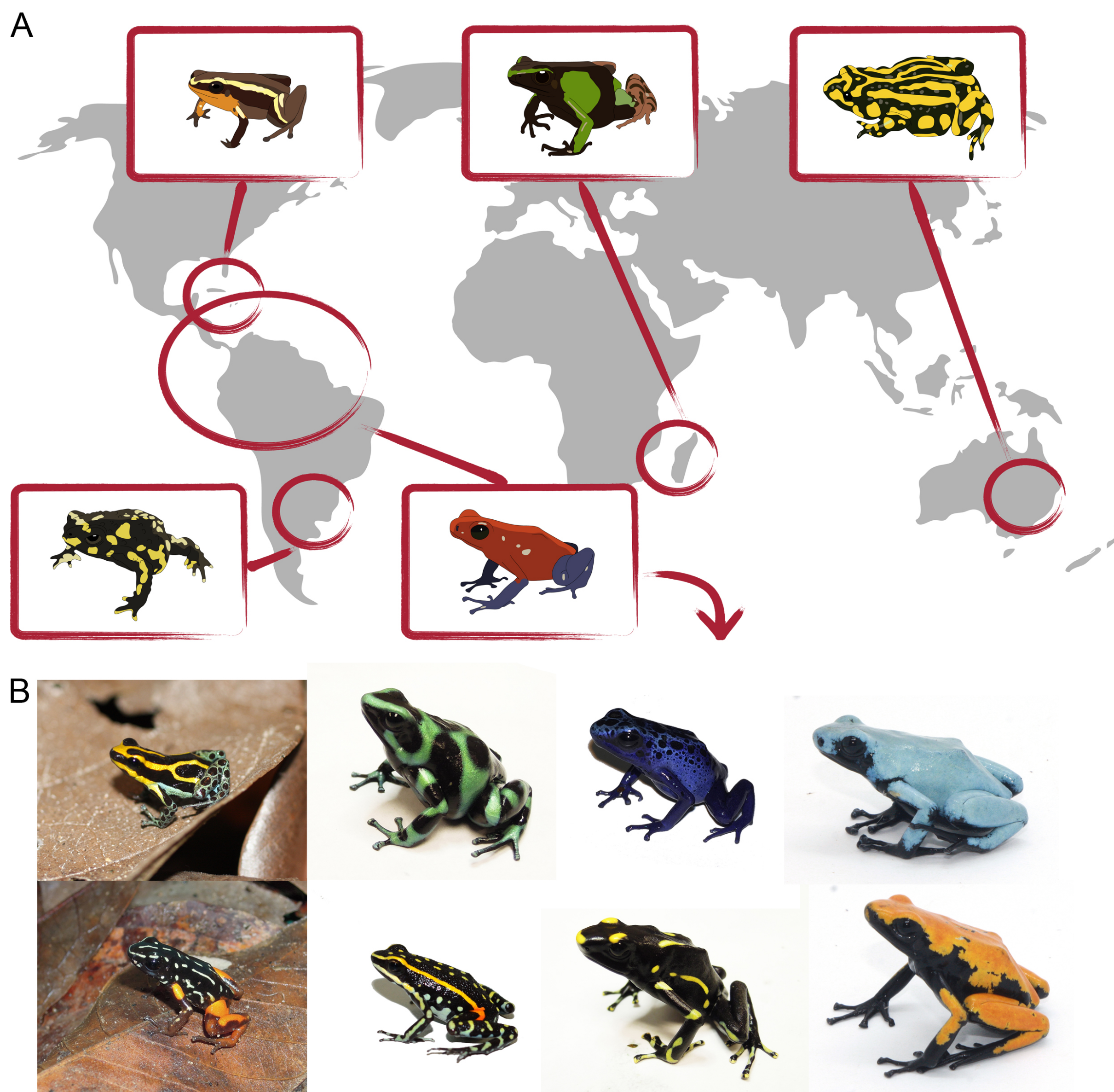

Figure 3. A) Geographical distribution of the five anuran families that sequester alkaloids: Eleutherodactylidae (part of the group *Eleutherodactylus limbatus*) from Cuba, Mantellidae from Madagascar, Myobatrachidae from Australia, Bufonidae from the southern South American region, and Dendrobatidae form the neotropical region of Central and South America. B) Poison frogs of the Dendrobatidae family. From left to right, from top to bottom: *Ranitomeya amazônica, Dendrobates auratus, Dendrobates tinctorius, Adelphobates galactonotus, Adelphobates castaneoticus, Ameerega flavopicta* (photo by M. Anganoy-Criollo), *Dendrobates tinctorius, Adelphobates galactonotus.*

an inverse or even no relationship between these factors (Daly & Myers, 1967; Wang, 2011; Lawrence et al., 2019). Regardless of the values of visual parameters, such coloration is an accurate sign of the presence of harmful compounds for visually oriented predators (Stuckert et al., 2014, 2018).

Part of the variation found among individuals, populations and species could also be a consequence of variations in the sequestration mechanism. Although it is believed that the mechanism should be similar, if not equal, for species of Dendrobatidae that sequester compounds, studies involving controlled alkaloid feeding show that the mechanism does not always respond equally to all types of alkaloids. In the first alkaloid feeding experiments, differences in sequestration between alkaloid types were evidenced. For example, *Dendrobates auratus* (Girard, 1855) promptly sequestered the decahydroquinolines, pyrrolilines, indolizidines, quinolizidines and histrionicotoxins offered in their diet, but did not sequester 2,5-disubstituted pyrrolidine or 2,6-disubstituted piperidine (Daly et al., 1994). Alkaloid modifications also show that not all alkaloid diversity is a direct result of availability in the diet. For example, *Adelphobates castaneoticus* (Caldwell & Myers, 1990), *A. galactonotus* (Steindachner, 1864) and *D. auratus* sequester and hydroxylate the PTX **251D** alkaloid into a compound that is five times more toxic, allopumilliotoxin (aPTX) (+)-**267A**, whereas *Epipedobates anthony* and *Phyllobates bicolor* sequester PTX **251D** without modification (Daly et al., 2003). In addition, the same species that modify the PTX **251D** do not seem to efficiently sequester the alkaloid decahydroquinoline (DHQ) **233F** (Daly et al., 2003). The variation and specificity of the system become even more complex when alkaloids that are not naturally found in certain species are offered to poison frogs. For example, *Oophaga pumilio* (Schmidt, 1857) sequesters an unnatural form of DHQ (only the skeleton of the basic alkaloid structure, without natural substitutions in positions 2 and 5), storing the alkaloid in its skin glands and even transferring the alkaloid to feeding oocytes for its tadpoles (Saporito et al., 2019). Another example is epibatidine, an alkaloid only found in species of *Ameerega* and *Epipedobates* (Spande et al., 1992). When offered to two species of *Dendrobates*, only *D. auratus* was able to sequester this alkaloid, while *D. tinctorius* (Cuvier, 1797) was not (Sanchez et al., 2019). Such examples demonstrate that all alkaloids present in the skin of these animals come from dietary sources, but not all dietary alkaloids are sequestered and stored in the skin glands.

In this context, the general objective of this doctoral thesis was to evaluate how the sequestration mechanism affects the variation of types, quantity and general composition of alkaloids found in poison frogs of the family Dendrobatidae.

Presentation of the thesis chapters

Due to their wide variety of bright colors and daytime activity behaviors, these animals are sought after by zoos and aquariums around the world, with large pet markets for

these amphibians in European and North American countries. Regardless of ethical and legal issues, such as the incentive of biopiracy, this *ex situ* breeding of poison frogs has produced much information about the maintenance and reproduction of these animals in captivity (Lötters et al., 2010), which has facilitated their breeding for scientific purposes. Since poison frogs acquire toxins from food, animals born in captivity are completely devoid of any type of alkaloid in their skin. This allows controlled alkaloid feeding experiments to be carried out in the laboratory to test several hypotheses about sequestration and how alkaloids are related to other factors such as skin coloration and behavior.

For the first part of this study, we used the species *Adelphobates galactonotus* as a model, as it is a Brazilian species that metabolizes one type of alkaloid (Daly et al., 2003). This species is distributed in the southern lowland forest region of the Amazon River, east of the Tapajós River to the mouth of the Amazon River (Frost, 2020), and presents a wide variety of dorsal colorations, with populations ranging from yellow, orange and red to light blue, all of which contrasting with the black ventral coloration (Hoogmoed & Ávila-Pires, 2012). In January 2017, we collected adult individuals of *A. galactonotus* from the Caxiuanã National Forest, Pará, Brazil. In the Caxiuanã Bay region, there are two contrasting color morphotypes on opposite sides of the bay: orange and light blue (Hoogmoed & Ávila-Pires, 2012). In order to identify the alkaloids naturally present in the individuals of these two populations and guarantee successful reproduction in captivity regardless of morphotype, we collected individuals from both populations and transported them to the Vivarium of the Cell Biology Laboratory at the Butantan Institute, in collaboration with the Professor Carlos Jared and Professor Marta Maria Antoniazzi. After identifying all individuals through photo-identification and sexing by dilation of terminal portion of fingers (Lötters et al., 2010), we established reproductive couples that were kept in terrariums (Lötters et al., 2010). Ten individuals (5 of each morphotype) were used to evaluate the variation in alkaloid composition and palatability between the two populations. This part was important for determining the types of alkaloids sequestered in these populations and to establish possible differences in the amount or predominance of any given alkaloid. This study resulted in **chapter 1** of this thesis, entitled: "*Geographically separated orange and blue populations of the Amazonian poison frog* Adelphobates galactonotus *(Anura, Dendrobatidae) do not differ in alkaloid composition or palatability*", published in Chemoecology in November 2019.

For the second part of this study, we experimentally investigated whether alkaloid sequestration, both type and quantity, is limited by the alkaloid availability in the diet or by the sequestration mechanism itself. To answer this question, we fed each experimental group

two different alkaloids at three different concentrations. We hypothesized that the sequestration mechanism would be as efficient as possible, sequestering all the available alkaloids, regardless of quantity. This is because these frogs depend on the alkaloids available in food for sequestration, thus the absorption, transportation and storage systems would be almost 100% efficient. However, before we could answer that question, we developed a new method of alkaloid feeding that would allow us to quantify the alkaloids offered to and sequestered by each experimental individual.

Although ecological aspects related to integumentary alkaloids of poison frogs have been studied since the 1960s, sequestration was only confirmed in the mid-1990s. Since then, the method used for feeding experiments is to induce alkaloid ingestion through a mixture of powdered alkaloids with the dietary vitamin sprayed on drosophila flies (Daly et al., 1994; Hantak et al., 2013). Small parts of alkaloids are attached to the flies, which frogs eat, therefore ingesting alkaloids. After a few days or weeks of feeding, sequestration is evidenced by analyzing the skin of these animals, using compound separation and identification methods, such as liquid or gas chromatography accompanied by mass spectrometry analysis. This method of alkaloid feeding was and still is widely used in sequestration experiments with various lineages of poison frogs (Hantak et al., 2013; Saporito et al., 2019; Sanchez et al., 2019). The disadvantage of this method is that it does not quantify the ingested alkaloids, and only indirectly compares the efficiency of sequestration by comparing the proportion of two or more sequestered alkaloids in relation to the proportion offered (Hantak et al., 2013).

In **chapter 2,** we describe a new method of administering alkaloids to poison frogs that involves using a micropipette to inject an alkaloid solution dissolved in 50% alcohol directly into the back of the frog's mouth. Since this method administers exact amounts of an alkaloid into the body, we describe an additional method to determine the alkaloid concentration to be administered at each dose. As the purpose of this experiment was to test sequestration efficiency, we wanted the amount of alkaloid administered per day to be biologically relevant. We did not want to exceed the amount administered per day, as we did not know if this would trigger another type of reaction in the animal, e.g., detoxification reaction. Once the feeding protocols were established, we demonstrated that the sequestration mechanism responded differently to each alkaloid tested and that efficiency differed depending on the concentration administered per dose. The chapter, entitled *"Dose dependent sequestration efficiency in poison frogs"*, will be submitted to the *Physiological and Biochemistry Zoology* journal.

The third aspect we investigated was the spatio-temporal distribution of the alkaloid in the body after ingestion. By assessing which organs and tissues come into contact with the alkaloid before it is stored in the skin, we can infer what type of transporters may be involved in sequestration and where mechanisms such as alkaloid modification occur. The anatomical distributions of alkaloids in animals collected from natural environments have been reported for a few species, demonstrating the presence of alkaloids in liver, muscle and ovary tissues, among others (Grant et al., 2012; Stynoski et al., 2014). This type of evaluation does not suggest that any of these tissues are involved with sequestration itself, because we did not know when and what the animals collected from nature last ate. In order to determine the distribution of alkaloids at certain times after administration, we used the Mass Spectrometry Imaging (MSI) method. This method separates, ionizes and detects the substances directly from the specimen, producing a set of mass spectra representing the molecular composition of a pixel in a section of a tissue (McDonnell & Heeren, 2007). Using specialized software, pixels are combined to assemble the final image of the analyzed section. The advantage of this method over methods such as histochemistry or immunohistochemistry is that, in addition to detecting more than one type of compound at a time, it does not require markers with specific affinity to certain molecules. Depending on the mass spectrometry imaging technique, any treatment is dispensed to the target tissue, minimizing contamination or washing of the molecule (Cooks et al., 2006). We used one of these methods called mass spectrometry imaging with electrospray desorption ionization (DESI-MSI; Wiseman et al., 2008). For training and obtaining data with this technique, I received the International Research Internship Scholarship (BEPE) from FAPESP and joined Dr. Demian Ifa's laboratory at York University, in Toronto, Canada form July 2018 to January 2019. During this time, we developed a protocol for cryosection and detection of alkaloids in frog's whole-body sections, which resulted in a publication describing the method in the April 2020 edition of *Journal of Mass Spectrometry* entitled: "*Use of whole-body cryosectioning and desorption electrospray ionization mass spectrometry imaging to visualize alkaloid distribution in poison frogs*" (**Chapter 3**).

Combining the MSI method with the GC-MS method already used by our research group, we mapped and quantified the alkaloids in the frogs that were administered a single dose of alkaloids, at different times after administration. Thus, the objective of this chapter was to determine the spatio-temporal distribution of alkaloids after ingestion. With these two methods, we were able to evaluate the speed of sequestration, along with the efficiency of a single alkaloid dose. In this third stage of the project, developed entirely at York University

(DESI-MSI) and John Carroll University (GC-MS), we used *Dendrobates tinctorius* as a model species, which has previously been used in alkaloid feeding studies and is easily acquired in the United States pet market. **Chapter 4,** entitled "*Spatiotemporal distribution of two alkaloids in a dendrobatid poison frog*", suggests for the first time that the sequestration mechanism is even faster than previously mentioned and that the anatomical distribution of the alkaloid does not vary between the two alkaloids tested. This chapter will be submitted to the *Journal of Experimental Biology*.

The main aspect interfering with the presence of alkaloids is the organism's ability or inability to sequester the alkaloids from dietary sources. In the Aromobatidae family, the sister group of Dendrobatidae (Grant et al., 2017), there are species that are sympatric with several species of poison frogs, but no analyses have detected alkaloid accumulation in these species. In addition, within the Dendrobatidae family, there are several genera that present no alkaloids in their skin (Fig. 4). In one study (Daly et al., 1994), *Allobates talamancae* (Cope, 1875; Aromobatidae) and *Colostethus panamansis* (Dunn, 1933; Dendrobatidae) were fed a diet containing alkaloids for five weeks, using the same treatment used for the poison frogs *Dendrobates auratus* and *Phyllobates bicolor* Bibron, 1840 (Dendrobatidae). The two poison frog species efficiently sequestered the alkaloid from the diet, while *Allobates* and *Colostethus* species did not accumulate any of the alkaloid in their skin. Nevertheless, no adverse effects were reported in these two species. Since alkaloids come from dietary sources, these compounds are exposed to pharmacokinetic pathways of any xenobiotic, suffering the necessary mechanisms for excretion. Regardless of the resistance mechanism for autointoxication, resistance is assumed to have arisen prior to sequestration capacity *(Darst et al., 2005;* Mohammadi et al., 2016). For animals that sequester, it is likely that this metabolic pathway is modified to enable compound sequestration and storage.

However, this metabolic pathway could be also modifying certain alkaloids, as previously stated. Specific enzymes modify sequestered alkaloids into other alkaloids (Daly et al., 2003), and may play a role in the diversity of alkaloids found in the skin of different species. The final stage of the project was to test whether resistance to alkaloids is plesiomorphic for poison frogs and their relatives who do not sequester, e.g., members of the Aromobatidae family. In addition, we compared sequestration efficiency and alkaloid modification capacity in different species of poison frogs in different alkaloid feeding

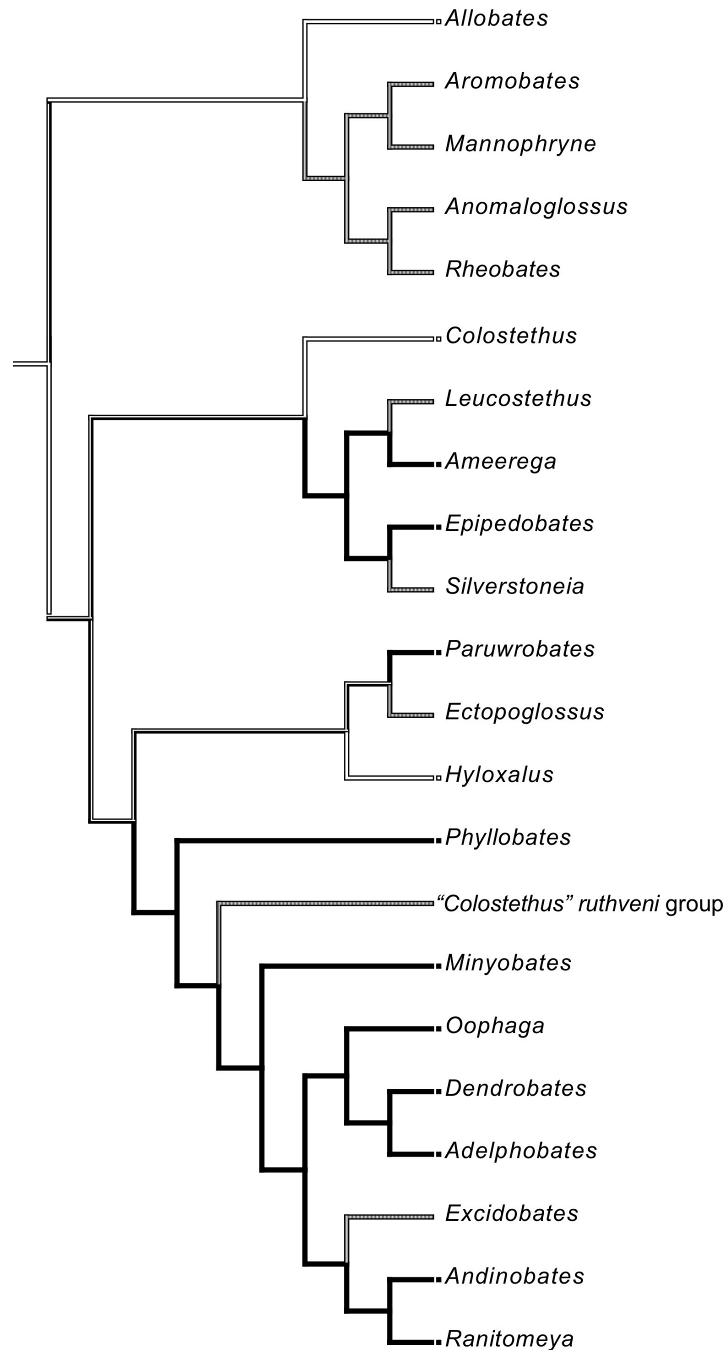

Figure 4. Phylogenetic distribution of the presence and absence of alkaloids in the skin. In black, the genera in which alkaloids were tested and detected; in white, the genera that were tested and no alkaloids were found; and in gray, the genera without information about the presence of alkaloids. Topology taken from Grant et al 2017.

experiments. In this chapter, we support a plesiomorphic mechanism in anurans that deals with the harmful effects of alkaloids, which has been reduced in poison frogs, thus allowing the evolution of the sequestration capacity. **Chapter 5** is entitled "*On the evolution of*

sequestration of alkaloids in poison frogs" and will be submitted to the *Proceeding of the National Academy of Sciences*.

All chapters will follow a manuscript format to be submitted to their respective journals. For aesthetic purposes, the headings, format of margins, and line spacing follows the pattern recommended in the dissertation model of the Zoology Department of the Biosciences Institute at the University of São Paulo.

Final Considerations

In order to understand the evolution of alkaloid sequestration and its ecological importance in protection against predators and pathogens, the causes and consequences of the presence and variation of alkaloid composition accumulated by poison frogs were investigated. I tested the influence of the sequestration mechanism and its efficiency in interspecific variation and the variation of alkaloid types present in each species. In general, the hypotheses tested provided more information about this area of study, demonstrating that (1) there is wide variation in sequestration efficiency per species, alkaloid type, alkaloid concentration, and alkaloid modification; (2) sequestration in poison frogs is an inefficient process; and (3) there seems to be a plesiomorphic mechanism of detoxification/degradation of alkaloids in anurans that prevents intoxication.

The variation in sequestration efficiency between species and alkaloid types was expected based on previous studies with feeding experiments. However, the studies presented in this thesis were the first to quantitively determine this variation, allowing greater analytical refinement in comparisons between species and alkaloid types. Future studies could try to understand why there is variation when the dose concentration is different and also if the number of doses administered affects each organism. In Chapter 2, in which multiple dose experiments were performed, it appears that there is an endogenous process for HTX sequestration, with increasing efficiency as the dose increases. Such result directly contrasts that of Chapter 4, in which administering a single dose of this same alkaloid did not elicit an efficient response from the sequestration mechanism. Conversely, the quantity of doses or their concentration did not affect the efficiency of DHQ sequestration. It is possible that the physical and chemical properties of alkaloids have a direct effect on sequestration efficiency, as discussed in Chapter 2. However, we cannot rule out the possibility that the transporters and other molecules involved in the sequestration mechanism respond differently to different alkaloid classes.

The alkaloid modification is another process that is still poorly understood. It is likely that there are enzymes that promote the chemical-structural transformation of the molecule and some of them may not be present in all poison frog species. Even species of the same genus have different alkaloid modification capabilities, as demonstrated for *Dendrobates auratus* and *D. tinctorius* in Chapter 5. In addition to the modification ability, the ecological importance of this modification process in the protection against predators and pathogens is still unknown. As demonstrated by the palatability test in Chapter 1, despite the variation in alkaloid composition between individuals, all were equally protected from chemically oriented predators by transmitting the same message regarding the presence of defense compounds. If the modification capacity exists to increase the diversity of alkaloids present in the skin, we still need to understand the consequences of this diversity. As demonstrated in Chapter 5, the ability to modify emerged in only one of the Dendrobatidae lineages that sequester alkaloids, which means that it is not a fundamental trait for sequestration.

The duality between sequestration and degradation of alkaloids seems to be the key to the evolution of sequestration. However, it seems counterproductive that an organism that depends completely on the sequestered compound for chemical protection, degrades almost half of what it consumes (Chapters 2, 4 and 5). This mechanism could be a remnant of a pleisiomorphic mechanism that is widely present in anurans or it could be preventing the build-up of large amounts of toxins in the body in a certain time period, thus preventing autointoxication.

Regarding future research, several questions have arisen regarding this contradictory degradation mechanism that may explain the evolution of the ability to sequester alkaloids from dietary sources. The decrease in degradation efficiency could have provided an opportunity for the alkaloid sequestration processes, such as transport and storage, to emerge. From a biochemical perspective, the question is about the identity and mechanism of action of enzymes that efficiently break down the ingested alkaloids, both in poison frogs and other frogs that do not sequester (e.g., *Allobates* and *Hyla*, studied in Chapter 5). Another important aspect is the organ responsible for the degradation. In Chapter 4, we found that the liver can play an important role in alkaloid metabolism by concentrating a portion of them, even hours after administration. However, other organs of the digestive system, such as the intestine, can also be important for alkaloid absorption efficiency, as well as for metabolism by local enzymes.

From an evolutionary perspective, we need to determine if all lineages of the five anuran families that sequester alkaloids have sequestration mechanisms that are similar to those

found in Dendrobatidae. Does the sequestration ability of anurans belonging to the other lineages that have not yet been investigated also inefficient due to alkaloid degradation? If these groups (or part of them) have the ability to degrade alkaloids, is the biochemical mechanism involved plesiomorphic or convergent between lineages? Through studies of gene expression and identification of possible candidate genes involved in alkaloid degradation, it will be possible to establish a hypothesis that relates gene expression to the ability or inability to sequester alkaloids and their degradation rates.

Information about the sequestration mechanisms in poison frogs has been slowly increasing. Along with the emergence of “omics” comes numerous possibilities for research about transport, modification, degradation, and storage of alkaloids in poison frogs. In this thesis, I investigated the influences of the sequestration mechanism on the variation of alkaloids present in poison frogs. My results support that this mechanism is even more complex and multifactorial than originally hypothesized, as well as point to important directions for future research.

Biografia

Adriana Moriguchi Jeckel se formou bacharel em Ciências Biológicas no ano de 2011 e licenciada em Ciências Biológicas no ano de 2012 pela Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). De janeiro de 2008 a dezembro de 2009, foi bolsista do Programa de Educação Tutorial - SeSU/MEC. Durante a graduação, fez parte como iniciação científica do Laboratório de Sistemática de Vertebrados sob orientação do prof. Taran Grant (junho/2007–junho/2008; junho/2010–dezembro/2012) e do Laboratório de Plasticidade no Sistema Nervoso sob orientação da profa. Monica Ryff Moreira Vianna (julho/2008–dezembro/2009). O primeiro semestre da graduação de 2010 foi cursado na University of Regina, em Regina, Canadá, como participante do programa de Mobilidade Acadêmica da PUCRS, onde cursou as seguintes disciplinas: Vertebrate Animal Biology, Vascular Plants, Biogeochemistry e Evolutionary Biology of Reproduction. Em fevereiro de 2011, estagiou no Laboratório de Biologia Celular do Instituto Butantan, sob orientação do prof. Carlos Jared e da profa. Marta Antoniazzi.

Adriana é mestra em Ciências Biológicas (Área Zoologia) pelo Programa de Pós-Graduação em Zoologia do Instituto de Biociências da Universidade de São Paulo (PPG-Zoo/IBUSP; janeiro/2013–junho/2015). Durante o mestrado, fez parte da sua pesquisa na John Carroll University (JCU), nos Estados Unidos, sob orientação do prof. Ralph Saporito, financiado pela Bolsa de Estágio em Pesquisa no Exterior (BEPE), da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), a qual financiou também parte do seu mestrado. Os primeiros seis meses de mestrado foram financiados pelo Conselho Nacional de Pesquisa do Brasil (CNPq).

Durante o doutorado pelo PPG-Zoo/IBUSP, Adriana liderou a montagem e a manutenção da criação de rãs-de-veneno da espécie *Adelphobates galactonotus* no Biotério do Laboratório de Biologica Celular do Instituto Butantan, em colaboração com os prof. Carlos Jared e profa. Marta Antoniazzi, e da colônia de moscas-da-fruta, *Drosophila melanogaster*, em colaboração com técnicos do IBUSP e colegas do Laboratório de Anfíbios USP. Além disso, fez pelo menos seis visitas oficiais ao laboratório do prof. Ralph Saporito, co-orientador desta tese, na JCU, para fazer análises químicas e experimentos de sequestro. Financiada pela BEPE-FAPESP, fez parte da sua pesquisa em mapeamento de compostos químicos através de espectrometria de massas na York University (YorkU), no Canadá, no laboratório do prof. Demian Ifa. Adriana também teve a oportunidade de orientar quatro alunos de graduação, na USP e também como pesquisadora visitante na JCU e na YorkU, em

projetos de ecologia química e de otimização de protocolos de alimentação de animais e de imageamento por espectrômetro de massas.

Ao longo da sua vida acadêmica na USP, de 2014 a 2019, fez parte da comissão organizadora do Curso de Verão em Zoologia, o qual organizou e lecionou aulas na área de zoologia. Além disso, foi representante discente na Comissão de Coordenação do PPG-Zoo/IBUSP (2017) e no Conselho do Departamento de Zoologia (2019–2020). Fez parte também de promoção das mulheres na ciência e na Herpetologia, resultando em publicações e capítulos de livro no tópico.

<u>Durante o doutorado, publicou os seguintes artigos:</u>

Soares, K.D.A., **Jeckel, A.M.**, Silva, G.M., Giovannetti, V., Mathubara, K. 2020. University extension and teacher training in Brazil: The Zoology Summer Course. Revista Brasileira de Extensão Universitária, *in press*.

Jeckel, A.M., Matsumura, K., Nishikawa, K., Morimoto, Y., Saporito, R.A, Grant, T., Ifa, D. 2020. Use of Whole-Body Cryosectioning and Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI- MSI) to Visualize Alkaloid Distribution in Poison Frogs. Journal of Mass Spectrometry, v. 55, e4520. DOI: doi.org/10.1002/jms.4520

Werneck, F.P; **Jeckel, A.M.**; Friol, N.R.; Toledo, D.G.P.; Targino, M.; Montesinos, R.; Nascimento, L.B.; Silvano, D.L.; França, D.P.F; Pereira, J.A.; Pinto, R.R.; Costa-Rodrigues, A.P.V.; Pereira, E.G.; Mângia, S.; Canedo, C. 2019. Diagnóstico e Propostas para Ampliar a Representatividade de Pesquisadoras em Herpetologia no Brasil. Herpetologia Brasileira, v. 8, p. 36–48.

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Jeckel, A.M.; Caorsi, V.Z.; Grant, T; Borges-Martins, M. 2019. The Nuptial Pads of *Melanophryniscus* (Anura: Bufonidae), with the Unexpected Occurrence of Nuptial-Pad-Like Structures in Females of Two Species. Journal of Herpetology, v. 53, p. 53–61. DOI: 10.1670/18-104

Grant, T.; Rada, M.; Anganoy-Criollo, M.; Batista, A.; Dias, P.H.; **Jeckel, A.M.**; Machado, D.J.; Rueda-Almonacid, J.V. 2017. Phylogenetic Systematics of Dart-Poison Frogs

and Their Relatives Revisited (Anura: Dendrobatoidea). South American Journal of Herpetology, v. 12, p. S1–S90. DOI: 10.2994/SAJH-D-17-00017.1

Rada, M.; **Jeckel, A.M.**; Caorsi, V.Z.; Barrientos, L.S.; Rivera-Correa, M.; Grant, T. 2017. A Remarkable New White-Eyed Glassfrog Species of *Sachatamia* from Colombia (Anura: Centrolenidae), with Comments on the Systematics of the Genus. South American Journal of Herpetology, v. 12, p. 157–173. DOI: 10.2994/SAJH-D-16-00041.1

Capítulo de livro:

Jeckel, A.M.; Henrique, R.S. 2017. A Zoologia e seu Papel na Sociedade. In: Beneti, J.S.; Montesinos, R.; Giovannetti, V. (Org.). Tópicos de pesquisa em Zoologia. 1 ed., p. 177-184.

Publicações anteriores ao doutorado:

Jeckel, A.M.; Saporito, R.A.; Grant, T. 2015. The Relationship Between Poison Frog Chemical Defenses and Age, Body Size, and Sex. Frontiers in Zoology, v. 12, p. 1-8. DOI: 10.1186/s12983-015-0120-2

Jeckel, A.M.; Grant, T.; Saporito, R.A. 2015. Sequestered and Synthesized Chemical Defenses in the Poison Frog *Melanophryniscus moreirae*. Journal of Chemical Ecology, v. 41, p. 505–512. DOI: 10.1007/s10886-015-0578-6

Carvajalino-Fernandez, J.M.; **Jeckel, A.M.**; Indicatti, R.P. 2013. *Melanophryniscus moreirae* (Amphibia, Anura, Bufonidae): Dormancy and Hibernacula Use During Cold Season. Herpetologia Brasileira, v. 2, p. 61.