UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

DEPARTAMENTO DE GENÉTICA

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

EVOLUÇÃO DA DIETA E DA PEÇONHA EM XENODONTINAE: A IMPORTÂNCIA DAS HISTÓRIAS INDIVIDUAIS

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> Dissertação submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do título de **Mestre em Genética e Biologia Molecular**.

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Porto Alegre, junho de 2022

INSTITUIÇÕES FINANCIADORAS

AGRADECIMENTOS

Primeiramente, gostaria de agradecer à toda a minha família, mas em especial aos meus pais, cujo suporte tornou este mestrado possível. Foram eles que plantaram em mim, desde a infância, o amor pela ciência, pela descoberta e pelas pessoas. Obrigado pelas longas conversas sobre o mundo depois do almoço, mãe, pelo guia ilustrado de serpentes da mata atlântica que você me deu quando criança, pai, ou por levarem eu e meu irmão para ver as estrelas a noite. Momentos como esses ajudaram a me tornar quem eu sou hoje e chegar até aqui.

Também gostaria de agradecer a todos os meus amigos pelo apoio e carinho que me deram, mesmo à distância, durante este período. Foi com a ajuda de vocês que eu consegui seguir com o mestrado durante um período tão difícil como foi a pandemia.

Tenho muito o que agradecer ao Nelson, que me acolheu e incentivou ainda na época da graduação. Obrigado por ter me ensinado tanto e por ser, mais do que um orientador, um grande amigo e parceiro de pesquisa.

Agradeço a todas as pessoas do Instituto Butantan que participaram deste trabalho, por terem confiado esses dados a mim e por toda a ajuda que me deram durante a execução do projeto. A dissertação leva o meu nome, mas este trabalho é de todos nós. Um agradecimento especial ao Pedro Nachtigall, pela ajuda gigantesca, e ao Felipe Grazziotin, por tornar essa colaboração possível.

Gostaria de agradecer a equipe e colegas do PPGBM, em especial ao Elmo, por estar sempre estar disponível para ajudar os alunos, e ao Juliano, por ter aceitado ser relator desta dissertação. Agradeço também a Maria João, por ter aceitado ser minha banca no seminário de dados, pelas valiosas sugestões durante a execução do projeto e por tudo que me ensinou como minha professora.

Finalmente, gostaria de agradecer aos membros da banca examinadora, os Professores André Zelanis, Marcio Borges-Martins e Karen Haag, por aceitarem avaliar este trabalho.

RESUMO

Uma questão central na biologia é como a diversidade e a complexidade são geradas e mantidas no mundo vivo. Parte deste desafio está em entender como a complexidade no nível molecular se relaciona com a diversidade no nível ecológico. Serpentes são ótimos modelos de estudo neste caso, uma vez que são predadores ecologicamente diversos cujas peçonhas possuem uma estrutura molecular subjacente bastante complexa. Um estudo recente testou a relação entre a diversidade (taxonômica e filogenética) da dieta e a complexidade da peçonha de um grupo de víboras norte-americanas. Seus resultados mostram que dietas filogeneticamente mais diversas favorecem venenos mais complexos nesses animais. No presente trabalho, nos testamos a mesma relação, mas em um clado ecologicamente muito mais diverso de serpentes, a subfamília Xenodontinae (Dipsadidae). Utilizando registros de predação na literatura e transcriptomas de glândulas de peçonha, nós caracterizamos a composição, reconstruímos a história evolutiva e estimamos a diversidade/complexidade da dieta e peçonha, respectivamente. Com base nesses dados, nós testamos a relação entre diversidade/composição da dieta e complexidade/composição da peçonha, tanto globalmente quanto para famílias gênicas individuais de toxinas. Nossos resultados indicam que, em geral, essas serpentes possuem dietas especializadas, mas ecológica e evolutivamente versáteis, que, em sua maioria, derivam de uma dieta ancestral baseada em anfíbios. Já suas peçonhas são, em linhas gerais, conservadas, tanto estruturalmente quanto filogeneticamente. Nem a diversidade taxonômica nem diversidade filogenética da dieta foram boas preditoras da complexidade da peçonha. Ao invés disso, histórias individuais de linhagens, hábitos de vida e famílias gênicas parecem ser muito mais importantes do que padrões gerais ao explicar sua complexidade. Serpentes que se alimentam majoritariamente de peixes possuem peçonhas extremamente simplificadas, compostas quase que inteiramente de uma única família de toxinas, as lectinas do tipo c. Serpentes especializadas em moluscos também apresentam peçonhas mais simples, mas sem mudanças marcantes na composição das famílias gênicas. Já serpentes que se alimentam de anelídeos possuem uma representação maior de proteínas com potencial neurotóxico. Finalmente, nós detectamos um caso de convergência na composição da peçonha no qual, quatro vezes independentemente, uma mesma família de toxinas substituiu outra. Com base nesses resultados, nós propomos que: (1) Dietas baseadas em anfíbios podem facilitar

transições de dieta nesses animais. (2) A simplificação da peçonha em serpentes piscívoras pode estar associada a uma maior neurotoxicidade necessária para capturar presas debaixo d'água. (3) A peçonha mais simples encontrada em espécies malacófagas é o resultado de uma menor pressão seletiva por toxicidade associada ao consumo de presas inofensivas. (4) A potencial neurotoxicidade na peçonha de espécies que se alimentam de anelídeos é uma resposta aos mecanismos de defesa destes animais. (5) A recorrente substituição de uma família de toxinas pela outra é uma resposta à aquisição de resistência por parte das presas. O teste dessas hipóteses ajudará a melhorar consideravelmente nosso conhecimento sobre a evolução das relações tróficas em Xenodontinae.

ABSTRACT

A central question in biology is how diversity and complexity are generated and maintained in the living world. Part of this challenge is understanding how complexity at the molecular level relates to diversity at the ecological level. Snakes are excellent study models in this case since they are ecologically diverse predators whose venoms have a very complex underlying molecular structure. A recent study tested the relationship between diet diversity (taxonomic and phylogenetic) and the venom complexity for a group of North American pitvipers. Their results show that phylogenetically more diverse diets favour more complex venoms in those animals. Here, we tested the same relationship, but in a much more ecologically diverse clade of snakes, the Xenodontinae subfamily (Dipsadidae). Using literature prey records and venom gland transcriptomes, we characterized the composition, reconstructed the evolutionary history, and estimated the diversity/complexity of diet and venom, respectively. With this data, we tested the relationship between diet diversity/composition and venom complexity/composition globally and for individual toxin gene families. Our results indicate that, in general, these snakes have specialized, but ecologically and evolutionarily versatile diets, mostly derived from an amphibian-based ancestor diet. On the other hand, their venoms are mostly conserved both structurally and phylogenetically. Neither diet taxonomic nor phylogenetic diversity were good predictors of venom complexity. Instead, individual histories of lineages, life habits, and gene families seem to be far more important than general patterns in explaining complexity. Snakes that feed mostly on fish have extremely simplified venoms, composed almost entirely of a single toxin family, the c-type lectins. Snakes specialized in molluscs also have simpler venoms but without marked changes in the composition of gene families. Snakes that feed on annelids have a greater representation of proteins with neurotoxic potential. Finally, we detected a case of convergence in venom composition in which four times independently the same toxin family replaced another. Based on these results, we propose that: (1) Amphibian-based diets may facilitate diet transitions in these animals. (2) The simplification of venom in piscivorous snakes may be associated with greater neurotoxicity required to capture prey underwater. (3) The simpler venom found in malacophagous species is the result of a lower selective pressure for toxicity associated with the consumption of harmless prey. (4) The potential neurotoxicity in the venom of species that feed on annelids is a response to the defence mechanisms of

these animals. (5) The recurrent substitution of one toxin family by another is a response to resistance acquisition in the prey. Testing of these hypotheses will help to improve considerably our knowledge about the evolution of trophic relations in Xenodontinae.

CAPÍTULO 1: Introdução

A busca por entender os mecanismos que regem o surgimento da diversidade e complexidade na natureza é central para a biologia desde o início da teoria evolutiva (Darwin 1859). Está cada vez mais evidente que as interações entre organismos são um dos principais catalizadores para o aparecimento de ambos os padrões entre os seres vivos (McShea e Brandon, 2010). Com a ajuda de avanços recentes na biologia molecular que tem nos permitido acessar a diversidade molecular em nível genômico (transcriptômico, proteômico etc.), estamos sendo finalmente capazes de entender como a complexidade no nível molecular se traduz em complexidade no nível ecológico, mesmo para caracteres dependentes da interação entre espécies (Holding et al., 2021). Talvez um dos modelos de estudo mais interessantes, nesse contexto, sejam as interações de predação, que envolvem incríveis adaptações que predadores desenvolvem para capturar suas presas, destacadas no próprio artigo de Darwin e Wallace lido perante a Sociedade Lineana de Londres em 1858 (Darwin e Wallace, 1858). Dentre tais adaptações, peçonhas são particularmente fascinantes porque, como são o modo de um organismo interferir nos processos homeostáticos do outro, são ferramentas intrinsecamente ecológicas, mas que possuem estruturas moleculares subjacentes incrivelmente complexas (Fry et al., 2012; Casewell et al., 2013; Jackson e Fry, 2016; Jackson et al., 2019; Casewell et al., 2020). Tais característica tornam peçonhas ótimos modelos para estudar interações predador-presa, seleção natural, evolução molecular, neofuncionalização de proteínas, convergência funcional, entre outros processos evolutivos (Casewell et al., 2013; Sunagar e Moran, 2015).

Muito provavelmente, nenhum outro clado animal tenha atingido o mesmo nível de sucesso no uso de peçonhas para predação que as serpentes (Casewell et al., 2013). Apesar de ainda existir certa controvérsia quanto a origem da peçonha dentro de Squamata (Fry et al., 2006; Fry et al., 2009; Hargreaves et al., 2014; Koludarov et al., 2017) atualmente é consenso que o ancestral comum mais recente de todas as serpentes "colubroides" já possuía algum tipo de sistema de inoculação de peçonha (Vidal, 2002; Fry e Wüster, 2004; Fry et al., 2009). Tal peçonha surgiu pelo recrutamento de diversas proteínas que orginalmente desempenhavam papéis fisiológicos para atuar como verdadeiras armas químicas que auxiliam na captura, abate e mesmo digestão das presas (Thomas e Pough, 1978; Rodriguez-Robles e Thomas, 1992; Fry et al., 2012; Junqueira-de-Azevedo et al., 2016). Em muitas linhagens, tais proteínas se diversificaram a partir de enormes famílias gênicas, resultando

em uma multiplicidade de atividades biológicas e efeitos fisiológicos, incluindo metaloproteases (destruição de tecidos, perturbação da hemóstase), fosfolipases (destruição de tecidos, perturbação da hemóstase, neurotoxicidade), toxinas "*three-finger*" (neurotoxicidade), proteínas secretoras ricas em cisteína (paralisia, neurotoxicidade), lectinas (perturbação da hemóstase), entre outras (Fry et al., 2012; Junqueira-de-Azevedo et al., 2016). Essa diversificação permitiu que altos níveis de complexidade e diversidade acabassem representadas entre as peçonhas das serpentes viventes (Fry et al., 2012; Junqueira-de-Azevedo et al., 2016; Holding et al., 2021).

Considerando a homologia presente nas peçonhas das serpentes, a grande diversidade observada entre elas deve ter surgido por meio de "ajustes" ocorridos em diferentes linhagens (Vidal, 2002; Fry et al., 2012; Junqueira-de-Azevedo et al., 2016). Tais "ajustes" se deram através da cooptação de novas famílias genicas, de duplicações gênicas, da evolução de sequências codificantes e de mudanças nos níveis de expressão das diferentes toxinas (Vidal, 2002; Fry et al., 2003; Fry e Wüster, 2004; Li et al., 2005; Juárez et al., 2008; Vonk et al., 2013; Bayona-Serrano et al., 2020). Esses processos permitem que variação na composição de peçonhas seja um fenômeno amplamente distribuído em todos os níveis taxonômicos de serpentes (Chippaux et al., 1991). Considerando que peçonhas são o meio pelo qual esses animais capturam suas presas, faz sentido que, em muitos casos - embora não sempre (Zancolli et al., 2019), sua variação esteja associada à composição da dieta (Daltry et al., 1996a; Daltry et al., 1996b; Daltry et al., 1997; Li et al., 2005; Davies e Arbuckle, 2019). Li et al. (2005), por exemplo, mostraram que a serpente marinha *Aipysurus eydouxii* apresenta uma redução dramática na toxicidade de sua peçonha, muito provavelmente associada ao seu hábito de se alimentar de ovos de peixes, que representam presas indefesas e imóveis. Já Barlow et al. (2009) e Healy et al. (2019) mostraram que a peçonhas geralmente apresentam maior potência nas presas da espécie em questão, enquanto Modahl et al. (2018) encontrou duas toxinas com distintas potências táxon especificas dentro da mesma peçonha. Finalmente, Davies e Arbuckle (2019) e Lyons et al. (2020) mostraram que a diversidade da dieta em serpentes parece estar associada a diversidade de efeitos toxicológicos e a especificidade da peçonha, respectivamente. Tendo isso em mente, e considerando que a serpentes representam um grupo de predadores ecologicamente muito diverso (Alencar et al., 2013; Grundler e Rabosky, 2021), compreender como suas dietas e peçonhas evoluem, bem como de que forma a evolução de um afeta a evolução do outro,

pode nos dar pistas importantes sobre como complexidade e a diversidade são expressas em vários níveis evolutivos (Arbuckle, 2020; Holding et al., 2021).

A maioria das serpentes tem dietas relativamente especializadas, embora muito distintas umas das outras (Grundler e Rabosky, 2021). Queiroz e Rodriguez-Robles (2006) propuseram um modelo de evolução para tais dietas em que as mudanças começam pela incorporação de novas presas eventuais, menos importantes, que ocorrem nos mesmos habitats do que o tipo de presa preferido. Com o tempo, essas novas presas passariam a representar uma parcela cada vez maior da dieta da espécie, que após transitar por um hábito mais generalista, poderia até mesmo se especializar no novo item alimentar. O próprio estudo de Queiroz e Rodriguez-Robles apresenta evidência em favor desse modelo, ao demonstrar que linhagens especializadas em ovos de lagartos e aves surgem, mais frequentemente, de linhagens que se alimentam de lagartos e aves, respectivamente. Já Bellini et al. (2015) demostrou que, depois da filogenia, o habitat é um dos principais fatores que explica a dieta das serpentes de uma comunidade de serpentes sul-americana. Uma relação parecida foi encontrada em serpentes da tribo Pseudoboini (Dipsadidae) por Alencar et al. (2013). Por fim, Grundler e Rabosky (2021), em uma ampla análise cobrindo serpentes em geral, mostrou que determinados itens (normalmente organismos que ocupam os mesmos ambientes) ocorrem com mais frequência nas mesmas dietas.

Para peçonhas, a visão mais tradicional sugere que elas evoluem em um cenário de "corrida armamentista", isto é, de coevolução com a presa (Casewell et al., 2013), onde a resistência da presa exerce pressão seletiva sobre as toxinas e vice-versa, conforme postulado na hipótese da Rainha Vermelha de Van Valen (Van Valen, 1973). Tal visão é suportada por muitos dos achados sobre adaptação da peçonha as presas, acima citados, e outros mostrando a resistência a peçonha desenvolvida por muitos animais (Holding et al., 2016; Smiley-Walters et al., 2017; Robinson et al., 2021; Thiel et al., 2022). Mais recentemente, entretanto, Sunagar e Moran (2015) propuseram que as peçonhas animais, evoluem principalmente em um modo de "duas velocidades", com rápida diversificação nos estágios iniciais de especialização ecológica (mudança de dieta e expansão de área de ocorrência, por exemplo) seguida por longos períodos de seleção purificadora para manter o potencial tóxico.

A hipótese de Sunagar e Moran (2015) parece ser apoiada por resultados de um recente estudo de Holding e colaboradores (Holding et al., 2021). Esses autores compararam a complexidade transcriptômica peçonhas de um clado de víboras norte-americanas (gêneros *Agkistrodon*, *Crotalus* e *Sistrurus*) com as diversidades taxonômica e filogenética de suas dietas. Enquanto a diversidade taxonômica atuaria como um *proxy* para a diversidade de linhagens de presas que estão potencialmente coevoluindo com a serpente, a diversidade filogenética seria um *proxy* para o nível de divergência entre as presas consumidas por ela. Os autores propõem que diferentes cenários evolutivos seriam mais prováveis, dependendo da relação entre essas variáveis. Sendo assim: a) uma relação negativa entre a complexidade da peçonha e a diversidade taxonômica da dieta seria esperada caso uma maior complexidade da peçonha resultasse de uma intensa coevolução par-a-par com a presa, já que a pressão seletiva na coevolução com a presa seria maior quando poucas espécies são consumidas; b) por outro lado, uma associação positiva entre essas variáveis indicaria que uma coevolução difusa com múltiplas espécies de presas favoreceria uma maior complexidade da peçonha, já que a pressão seletiva nesse tipo de relação deve ser maior quando várias espécies de presas são importantes; c) já uma correlação positiva entre a complexidade da peçonha e a diversidade filogenética da dieta indicaria que a divergência evolutiva das presas, ao invés do número de espécies consumidas, exerce a seleção mais forte sobre a complexidade da peçonha; d) finalmente, a falta de qualquer relação entre a diversidade da dieta e a complexidade da peçonha, associada a um forte sinal filogenético, apoiaria um cenário de evolução neutra para a complexidade da peçonha, no qual a contingência histórica sobre a composição da peçonha é preponderante. Os resultados de Holding et al. (2021) indicam que a diversidade filogenética das presas está mais fortemente correlacionada à complexidade da peçonha que a diversidade taxonômica, tanto para a peçonha como um todo, quanto para a maioria das famílias gênicas que compõem as toxinas. Dessa forma, nesse grupo de víboras, a inclusão/exclusão de presas divergentes (oportunidade ecológica) parece ser um catalisador mais importante para a evolução da peçonha do que a coevolução com as presas. Isso está claramente mais de acordo com o modelo de "duas velocidades" de Sunagar e Moran do que com o modelo de "corrida armamentista" em geral.

Devido às suas dietas e peçonhas bem estudadas, o trabalho de Holding se concentrou em um clado restrito de víboras que se alimentavam principalmente de vertebrados terrestres (Holding et al., 2021). Entretanto, a vasta maioria dos estudos sobre evolução de peçonha

em serpentes têm se concentrado em apenas três famílias (Elapidae, Viperidae e Atractaspididae) devido à sua importância médica (Fry e Wüster, 2004; Jackson et al., 2019), deixando as diversas linhagens anteriormente conhecidas como "colubrídeos" (17 famílias, segundo Zaher et al., 2019), pobremente estudadas nesse aspecto, apesar de concentrarem a maior parte da diversidade filogenética e ecológica das serpentes (Saviola et al., 2014; Mackessy e Saviola, 2016; Junqueira-de-Azevedo et al., 2016; Jackson et al., 2019; Zaher et al., 2019). Dessa forma, seria interessante testar a mesma hipótese com um grupo de serpentes mais ecologicamente diverso, pertencente a um destes ditos "clados negligenciados" de colubrídeos (Jackson et al., 2019). Será que os mesmos padrões aparecerão em se tratando de hábitos de vida, dietas e grupos de presas mais diversos?

No presente estudo, tentamos responder a esta questão usando serpentes sul-americanas da subfamília Xenodontinae (Dipsadidae). As serpentes desta subfamília são um ótimo modelo de estudo neste caso, por sua elevada diversidade ecológica (França et al., 2008), e suas peçonhas pouco estudadas (Junqueira-de-Azevedo et al., 2016; Jackson et al. 2019). Com isso em mente, nós também expandimos o trabalho de Holding, com a ajuda de métodos recentemente desenvolvidos (Goolsby et al., 2017; Grundler e Rabosky, 2020), para fazer uma descrição abrangente da composição e história evolutiva da dieta e da peçonha destes animais. Por fim, nós também avaliamos o impacto da composição da dieta na composição e complexidade da peçonha. Até onde sabemos, este é o primeiro estudo capaz de combinar um extenso estudo da composição das dietas e peçonhas de grupo de serpentes com tamanha diversidade ecológica.

CAPÍTULO 2: Objetivos

Objetivo geral

O trabalho tem por objetivo geral elucidar a trajetória evolutiva da peçonha e da dieta, bem como a relação entre ambas, dentro da subfamília Xenodontinae. As serpentes desta subfamília são um ótimo modelo de estudo neste caso, por sua elevada diversidade ecológica (França et al., 2008), e suas peçonhas pouco estudadas (Junqueira-de-Azevedo et al., 2016; Jackson et al. 2019). Para tal, para tal, nós caracterizamos a composição da dieta e da peçonha destes animais, e reconstruímos a história evolutiva de ambas. Nós também testamos a relação entre diversidade e composição da dieta e a complexidade e composição da peçonha.

Objetivos específicos

- Caracterizar, a partir de dados da literatura, a composição da dieta para as espécies de interesse.

- Caracterizar, a partir de dados de transcriptoma, a composição da peçonha para as espécies de interesse.

- Reconstruir, a partir dos dados de transcriptoma e de dados da literatura, uma filogenia datada para as espécies de interesse.

- Reconstruir as dietas e peçonhas ancestrais para as espécies de interesse.

- Testar se as diversidades taxonômica ou filogenética da dieta estão correlacionadas à diversidade da peçonha, tento em nível geral, quanto para cada família de toxina.

- Testar se itens específicos das dietas estão correlacionados à diversidade da peçonha, tento em nível geral, quanto para cada família de toxina.

- Testar se as diversidades taxonômica ou filogenética da dieta, ou se itens específicos dela, estão correlacionadas a proporção de determinadas famílias de toxinas na peçonha.

CAPÍTULO 3: Diet and venom evolution in Xenodontinae: the importance of individual histories

Manuscrito em preparação para a revista *Proceedings of the Royal Society B: Biological Sciences*. Foram tomadas algumas liberdades em relação ao formato de submissão da revista para facilitar a leitura por parte da banca e a posterior inclusão de sugestões por feitas ela.

Diet and venom evolution in Xenodontinae: the importance of individual histories

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Abstract

Snake venoms are excellent models to understand how complexity at the molecular level translates into diversity at the ecological level. A recent study shows that phylogenetically diverse diets favour more complex venoms in North American pitvipers, but it is unknown if this holds for more diverse snake taxa. In this work, we tested this relationship in the much more ecologically and phylogenetically diverse group of Xenodontinae snakes using transcriptome data of venom glands and dietary data collected from the literature. We present a novel phylogenetic tree for Xenodontinae including the ancestral estimates of their diets and venoms. Our results indicate that most xenodontine snakes have specialized, but versatile diets, with relatively conserved venoms overall. Differently from North American pitvipers, diet diversity was not a good predictor of venom complexity. Instead, particular life histories and changes in specific toxin gene families seem

to be much more important in explaining venom complexity. For example, we found an extreme venom streamlining linked to piscivory and potential coevolutionary patterns associated with the evolution of snake venom metalloproteases. Finally, we propose a series of hypotheses that, if tested, would improve our knowledge about snake venom and diet coevolution.

1. Introduction

A central question in biology, probably as old as the theory of evolution itself, is how diversity and complexity are generated and maintained in the living world (Darwin 1859). It is now clear that both diversity and complexity depend on the interactions among organisms (McShea and Brandon, 2010). Recent advances in molecular biology have allowed us to understand how phenotypic complexity at the molecular level translates into functional complexity at the ecological level (Holding et al., 2021).

Antagonistic interactions in trophic chains, especially those involving the complex adaptations that predators develop to seize their prey, are among the most interesting to model the emergence of biological diversity (Darwin and Wallace, 1858). Among such adaptations, predatory venoms are particularly fascinating because they are intrinsically ecological traits, but with an incredibly complex underlying molecular architecture (Fry et al., 2012; Casewell et al., 2013; Jackson and Fry, 2016; Jackson et al., 2019; Casewell et al., 2020). Indeed, predatory venoms proved to be great models to study predator-prey interactions, natural selection, molecular evolution, protein neofunctionalization, and functional convergence, among other evolutionary processes (Casewell et al., 2013; Sunagar and Moran, 2015).

Probably no other animal clade has reached the same level of success in using predatory venoms as snakes (Casewell et al., 2013). Snakes have incredibly complex venoms (Fry et al., 2012; Junqueira-de-Azevedo et al., 2016) and represent a highly ecologically diverse group of predators (Alencar et al., 2013; Grundler and Rabosky, 2021). Understanding how snakes' diets and venoms (co)evolve can reveal important insights into the ways by which complexity and diversity are expressed in multiple levels of evolution (Arbuckle, 2020; Holding et al., 2021). Most snakes have relatively specialized, although

highly divergent, diets (Grundler and Rabosky, 2021), which have been proposed to evolve under a model where diet shifts start by the incorporation of eventual, less important, prey items that occur in the same habitats than the preferred prey type (Queiroz and Rodriguez-Robles, 2006). This model has been supported by the correlation between co-occurrent prey types for egg-eating snakes (Queiroz and Rodriguez-Robles, 2006) and snakes in general (Grundler and Rabosky, 2021), as well as by the association between diet and habitat use for both South American snakes' clades (Alencar et al., 2013) and communities (Bellini et al., 2015). Snakes' venoms, on the other hand, are formed by multiple gene families which have been independently recruited many times from proteins with physiological roles to be used as predatory tools (Fry et al., 2012; Junqueira-de-Azevedo et al., 2016). Traditionally an "arms race" scenario of coevolution has been proposed between venoms and prey (Casewell et al., 2013), where prey resistance exerts selective pressure upon the toxins and vice versa, as postulated in Van Valen's Red Queen hypothesis (Van Valen, 1973). More recently, however, Sunagar and Moran (2015) proposed that, in general, animal venoms evolve mostly in a "two-speed" mode, with rapid diversification in the early stages of ecological specialization (e.g., diet and range expansion) followed by long periods of purifying selection to maintain the toxic potential.

The hypothesis of Sunagar and Moran (2015) is supported by the results of a recent study by Holding and colleagues (Holding et al., 2021). They compared the venom transcriptomic complexity for a clade of North American pitvipers (genera *Agkistrodon*, *Crotalus*, and *Sistrurus*) with the diversity (both taxonomic and phylogenetic) of their diets. These authors propose distinct evolutionary scenarios for venom complexity depending on the relationship between these variables: a) a negative relationship between venom complexity and diet diversity is expected if intense pairwise coevolution favoured trait complexity, as coevolutionary selective pressure should be the highest when few species are consumed; b) on the other hand, a positive association between these variables would indicate that diffuse coevolution with multiple species exerts the strongest selection on trait complexity, as the coevolutionary selective pressure should be the strongest when several prey species are important; c) a positive correlation with the diet phylogenetic diversity would indicate that prey evolutionary divergence, rather than number of consumed species, exerts the strongest selection on trait complexity; d) finally, the lack of any relationship between diet diversity and venom complexity, associated with strong phylogenetic signal,

would support neutral evolution of venom complexity. They found that prey phylogenetic diversity was more strongly correlated to venom complexity than prey taxonomic diversity for both the venom as a whole and most of the toxin gene families, indicating that in this group of pitvipers the inclusion/exclusion of divergent prey (ecological opportunity) seems to be a more important catalyser for venom evolution than coevolution with the prey. This is, of course, in better agreement with Sunagar and Moran's "two-speed" model than with the traditional "arms race" model.

While Holding's work focused on a restricted clade of pitvipers that fed mostly on terrestrial vertebrates (Holding et al., 2021), it would be interesting to test the same hypothesis with a more ecologically diverse group of snakes. Would the same patterns appear when dealing with more diverse habits, diets, and prey groups? In the present study, we attempted to answer this question using South American snakes from the Xenodontinae subfamily (Dipsadidae). Xenodontine snakes are great models in this case, not only because of their high ecological diversity (França et al., 2008) but also because, like most "colubrids", they represent a neglected clade with poorly studied venoms (Jackson et al. 2019). Besides testing Holding's hypothesis, we also make a comprehensive description of their diets, venoms' compositions, and evolutionary histories. We then evaluate the impact of diet composition on both venom composition and diversity. To our knowledge, this is the first work to be able to combine an extensive study of both diet and venom composition for a group of highly ecologically diverse snakes.

2. Materials and methods

(a) Collection of samples and transcriptome production

124 snake specimens from 72 species comprising eight Dipsadidae tribes (seven Xenodontinae: Echinantherini, Elapomorphini, Hydropsini, Philodryadini, Pseudoboini, Tachymenini, and Xenodontini - one Dipsadinae: Imantodini) and one outgroup taxa (*Mastigodryas boddaerti*) were collected during multiple field trips across several localities and deposited at the Herpetology Collection of Instituto Butantan. Their venom glands were dissected four days after the last venom collection and stored in RNAlater at -80ºC. The

transcriptome sequencing was performed following the protocol described in Bayona-Serrano et al. (2020). The produced reads were trimmed using Trim Galore v.0.4.4 (https://github.com/FelixKrueger/TrimGalore), to remove reads with phred scores less than five and a length less than 75 bp. Paired-end reads were merged using the PEAR software (Zhang et al., 2013). Finally, reads were assembled using various de novo assemblers: Trinity (kmer parameter set to 31) (Haas et al., 2013), Extender (overlap parameter set to 120 and 150) (Rokyta et al., 2012), NGen (using the default parameters; Lasergene DNAStar software package) (https://www.dnastar.com/t-nextgen-seqman-ngen.aspx), Bridger (kmer parameter set to 30) (Chang et al., 2015), and rnaSPAdes (kmer parameter set to 31, 75, and 127) (Bushmanova et al., 2019).

(b) Phylotranscriptomic analysis

We used the assembled transcriptomes to retrieve non-toxin coding regions to be used in the inference of the phylogenetic tree. We used the "aves_odb10" set from BUSCO v.5.2.2; (Waterhouse et al., 2018) to search for these conserved loci in all samples. These genes in the set are known to be present at a single copy in most species within Aves, which represents the set most closely related to snakes in the BUSCO databases and allowed to recover the higher number of loci to be used in the tree inference (i.e., 5983 from 8338 genes in the BUSCO set). Then, we filtered these recovered loci to keep only loci containing at least 51 samples to avoid bias related to missing data, which resulted in a final set containing 1496 loci. This final set was used in further alignments and tree inference.

We produced alignments of loci in the final set using MAFFT v.7.310 (Katoh and Standley, 2013) with the parameters "–auto" and "–adjustdirectionaccurately". The alignments were cleaned using CIAlign v.1.0.14 (Tumescheit et al., 2022), with the following parameters "–remove_divergent –remove_divergent_minperc 0.80 –remove_insertions –crop_ends –remove_short" and trimmed using trimAl v.1.2 (Capella-Gutiérrez et al., 2009) with the "-automated1" parameter. These trimmed alignments were used to infer the phylogenetic trees for each loci using Iqtree v.2.0.3 (Nguyen et al., 2014). Then, branches with Bootstrap values lower than or equal to 10 were removed and Astral v.5.15.4 (Zhang et al., 2018) was used to generate the final consensus tree. The consensus

tree was then dated in the treePL software (Smith and O'Meara, 2012) using 8 calibration points (Supplementary File 1) extracted from the Dipsadidae phylogeny of Zaher et al., 2018 and then pruned using the R package ape 5.6-2 (Paradis and Schliep, 2018) for future analyses.

(c) Toxin Transcriptome

(i) Toxin reads mapping and venom composition

Toxin reads were identified through ToxCodAn (Nachtigall et al., 2021) to generate tribe-specific toxin databases against which the cleaned reads of each sample were aligned using the *mem* algorithm within *bwa* (Li and Durbin, 2009). These databases were composed of 13 gene families known to integrate Dipsadidae venoms: three-finger toxins (3FTx), C-type natriuretic peptides (CNP), cysteine-rich secretory proteins (CRISP), C-type lectins (CTL), Kunitz type proteins (KUNITZ), L-amino acid oxidases (LAAO), phospholipases A2 (PLA2), phospholipases B (PLB), snake venom acid lipases (svLIPA), snake venom matrix metalloproteinases (svMMP), snake venom metalloproteinases (SVMP), snake venom serine proteases (SVSP) and wap domain containing proteins (WAP). To have insights into the snake's venom compositions, we calculated, for each sample, the fraction of the total number of toxins reads mapped belonging to each toxin gene family (Supplementary Dataset 1). We use the venom gland toxin transcriptome composition to infer the venom composition, since seem to be a reasonable correlation between them (Bayona-Serrano et al., 2020; Holding et al., 2021). For simplicity, hereafter we will refer to them interchangeably. Only samples with at least 1 million identified toxin reads were kept for posterior analyses (107 samples). We focused our study on the Xenodontinae subfamily, since this was the best-sampled group, and included two Dipsadinae species as outgroups (*Leptodeira annulata* and *Imantodes cenchoa*), resulting in a final count of 59 snake species (Figure 1).

(ii) Toxin Transcriptome complexity

Following Holding et al. (2021), we used a kmer-based approach to measure the complexity of the expressed venom genes. From the set of reads mapped as toxin genes, we sampled randomly 1 million reads for each sample using seqtk (https://github.com/lh3/seqtk) and then counted the number of unique 60 bp sequences (kmers) using Jellyfish v.2.2.1 (Marçais and Kingsford, 2011). We excluded poly-A kmers, as well as kmers with less than 10 counts, as they were probably artefacts (transcriptional noise or sequencing/mapping errors). Using the kmer counts, we calculated the Shannon Diversity Index (H) to measure the kmer diversity of each sample and expressed it as exp(H), the effective number of kmers, for downstream analyses (Jost, 2006). We also did the same for each toxin gene family individually. In this study, we started from the assumption that the intraspecific variation in venom complexity and composition on these snake species is lower than the interspecific variation. Therefore, the value for each species, for every venom metric, was taken to be the average between samples for that species, with sample sizes ranging from 1 to 4 samples per species (average=1.8). The diversity values can be found in the Supplementary Dataset 1.

(d) Snake diet diversity

We retrieved predation records from the literature (see Supplementary Material) to represent the diet of the studied species. Only gut contents and direct observations were included, resulting in a data set with 2,796 recorded prey items (Supplementary Dataset 2). The prey counts were tabulated to the species level and, following Holding et al. (2021), prey records identified only at higher taxonomic levels were distributed proportionally to the lower taxonomic levels of recorded prey consumed by that snake species until the species level. If no prey of that taxon was identified to the species level in that snake species, we considered the identification for the closest snake species in the dataset for which there was identification to the species level of prey belonging to that taxon. We used two metrics to quantify the diversity in the snakes' diets: the taxonomic species diversity and the phylogenetic diversity. The taxonomic species diversity was estimated using the Shannon Diversity Index (H), expressed as the normalised version of the effective number of species (exp(H)), ranging from 0 to 1 (Jost, 2006). The phylogenetic diversity was estimated through the mean pairwise phylogenetic distance (MPD) using the ses.mpd function from picante

(Kembel et al., 2010). To access the phylogenetic distance between consumed prey, we generated a phylogeny of all listed prey species (Supplementary File 2) using the phylogeny available on www.timetree.org (Kumar et al., 2017) combined with published phylogenies (see Supplementary Material). When a prey species was not present in the available phylogenies, its counts were assigned to the nearest species present. For both metrics, to control for differences in sample size, we generated 10,000 random subsamples of 10 prey items for each species and took the average between them. We excluded species with less than 10 prey items reported in the literature, resulting in a final dataset of 44 lineages for the comparative analysis involving the diet diversity indexes.

(e) Statistical Analyses

(i) Venom and diet: composition and evolution

To better understand the snake's venom transcriptomes/diets composition and evolution, we applied the Bayesian analysis developed by Grundler and Rabosky (2020) and implemented in the package macroevolution (https://github.com/blueraleigh/macroevolution). This method estimates dietary niches (diet classes, in terms of composition) from prey counts and phylogenetic data, taking into account differences in sample size and sampling error, for living species, and then use them to reconstruct the ancestral states as well. In the case of venom transcriptome, we adapted the method to recover venom classes by multiplying the fraction of each toxin gene family in the sample's transcriptome by 100, rounding to the nearest integer, to create pseudocounts that could be inputted into the algorithm. For the diet data, we grouped the prey counts into nine reasonable eco-functional prey categories, according to similar diet studies (Alencar et al., 2013; Gaiarsa et al., 2013; Bellini et al., 2015; Grundler and Rabosky, 2020; Grundler and Rabosky, 2021): amphibians, annelids, arthropods, birds, fishes, lizards, mammals, mollusks and snakes. In both cases, the K parameter was set to be 100, a reasonable value for a parsimonious evolutionary inference, given the number of species in question (Grundler and Rabosky, 2020; Grundler and Rabosky, 2021). We ran the Markov chains through 1 million states, sampling every 100th state, for a total of 10,000 posterior samples. All analyses were performed in R v.4.1.3 (R Core Team, 2022). Chain convergence was

evaluated in Tracer v.1.7.1 (Rambaut et al., 2018) and the burnin was set at 10%. We then calculated the mode value in the number of inferred venom/diet classes, after the burnin, and took, from the samples with the number of inferred venom/diet classes equal to the mode, the one with the highest posterior probability value as representative of the real states.

(ii) Phylogenetic generalised least squares (PGLS)

The correlation between venom and diet variables was verified through phylogenetic generalised least squares (PGLS) models. The venom variables (response variables) were the total toxin transcriptomic diversity and the diversity and fraction of each toxin gene family that represented 10% or more of any transcriptome from 44 species for which we had diet data (CTL, CRISP, PLA2, svMMP, SVMP), totalizing 11 response variables. Some of these variables (CTL and PLA2 diversity and fraction, CRISP diversity, and svMMP fraction) were log10-transformed to better fit in the PGLS, as determined by the analysis of residuals and histograms. In those cases, zero values were substituted by one-tenth of the lowest value found in the respective dataset (diversity or fraction). The diet variables (explanatory variables) were the diet taxonomic diversity, the diet phylogenetic diversity, and the fraction of prey items in each one of the previously described eco-functional prey categories. The PGLS models were constructed with the packages nlme (Pinheiro and Bates, 2000) and ape (Paradis and Schliep, 2018). In the phylogenetic corrections, we used the best evolutionary model for each response variable (Supplementary Dataset 3), as determined in Rphylopars (Goolsby et al., 2017). For illustrative purposes, we also used this package to reconstruct and plot the evolutionary history of the transcriptome and diet diversities found in Figure 3 and Supplementary Material, Figure S1.

We divided the PGLS investigation into two steps: one a priori hypothesis testing and one exploratory analysis. For the a priori hypothesis testing we compared the fit of three models for each venom variable: one with the diet phylogenetic diversity as the explanatory variable, one with the diet taxonomic diversity as the explanatory variable, and one null model (~1). In the exploratory analysis, we created global models, for each venom variable, including all 11 diet variables. We then used an exhaustive search to find the subset of explanatory variables that generate the model with the best fit, using the function dredge of

the package MuMIn (Barton, 2022). In all cases, the fit of the models was evaluated through Akaike's second-order corrected Information Criterion (AICc).

3. Results

(a) Phylogenetic relationships

The recovered phylogeny (Figure 1) supports the monophyly of all sampled xenodontine tribes and is composed of two major clades: one having the Hydropsini and Tachymenini as the sister clade of Pseudoboini, and Xenodontini as a more external group, and the other having Echinantherini and Elapomorphini as the sister group of Philodryadini. This tree was used in the reconstruction of diet and venom evolution, and for the comparative analyses of the relationship between diet diversity and venom complexity.

(b) Venom and diet composition

We recovered 13 diet and six venom classes in our analysis (Figures 1 and 2). In general, the venom seems to be much more conserved and to have a stronger phylogenetic signal than the diet. The diet classes recovered in our analysis indicate that xenodontine snakes are mostly specialized predators (Figure 2 - Bottom Left). Of the 13 diet classes, 10 have one type of prey representing over 70% of its composition, such as amphibians (D1 and D2), fishes (D4), mammals (D7), lizards (D8), snakes (D9), birds (D10), arthropods (D11), mollusks (D12), and annelids (D13). When we consider the two most consumed types of prey of each diet, they represent almost 90% of the diet's composition for 12 diet classes (D3 is mostly composed of fishes and amphibians, and D6 of lizards and mammals). The only exception is D5, which represents a generalist diet that relies on similar proportions of the terrestrial vertebrates, and that is found in most Philodryadini, except *Philodryas agassizii* and *P. nattereri*. On the other hand, many specialized diets included small proportions of other prey types that may occupy the same environments in which the preferred prey type can be found, for instance, lizards (D1, D7, D9, and D11), fishes (D2), amphibians (D4), snakes/mammals (D8), etc. The ancestral diet D1 is the most widespread one (Figure 1) and the one from which most diet types are derived. There seems to be certain

trait conservation along the clades, especially when it comes to the preferred type of prey. Echinantherini, Tachymenini, and Xenodontini snakes feed mostly on amphibians (diets D1 and D2), Hydropsini on fishes (D3 and D4), Pseudoboini on lizards (D8), and the Philodryadini snakes were generalists (D5). However, in contrast to what we found for venom evolution, exceptions are common. In Tachymenini, *Tomodon* and *Gomesophis* feed on mollusks (D12) and annelids (D13), respectively. In Pseudoboini, *Oxyrhopus* relies considerably on mammals (D6 and D7), *Boiruna* on snakes (D9), and *Rhachidelus* on birds (D10). In Xenodontini, *Erythrolamprus aesculapii* is a snake specialist (D9) and in Philodryadini *P. agassizii* and *P. nattereri* feed mostly on arthropods (D11) and lizards/mammals (D6), respectively. Another difference in regard to venom evolution is the occurrence of multiple second, and even third-order transitions (Figure 2 - Bottom Right). Of the 18 transitions between distinct diet classes, 7 occurred between diets specialized in different items (D1 to D8, D9, D12, and D13; D8 to D9 and D10), 5 were cases of diet broadening (D1 to D2, D3, and D5; D8 to D6), 5 represented diet narrowing (D3 to D4; D5 to D6; D6 to D7), and one was a specialization in an item not consumed by the ancestral (generalist) diet (D5 to D11). Convergence also appears to be much more common, with six instances, all involving different diet classes. In most cases of convergence (4), the diets arise from the same ancestral diet (D2 and D8 from D1, D4 from D3, and D7 from D6), but in two instances the same diet came from two different ancestral diets (D9 from D1 or D8, and D6 from D5 or D8).

For venom, the ancestral type V1, from which all other venom classes, except V5, are derived (Figure 2 - Top Right), is present in the outgroup and, at least partially, in 4 of the 7 xenodontine families. This venom class is composed mostly of SVMP transcripts, with smaller fractions, in decreasing order, of CRISPs, CTLs, and svMMPs (Figure 2 - Top Left). The next venom type, V2, appears only in a subclade of Pseudoboini, comprising four genera: *Boiruna*, *Clelia*, *Pseudoboa*, and *Rhachidelus*. V2 is very similar to its ancestor, V1, from which it differs for having a considerable proportion of PLA2 transcripts, absent in V1, making PLA2 take the place of CRISP as the second most abundant gene family in this venom, only behind SVMP. Venom type V3 is characteristic of Elapomorphini. Besides still having SVMPs as the most abundant transcripts, this venom class is the most diverse in terms of gene families. V3 presents significant proportions of LAAOs and svLIPAs, not found in other xenodontine venoms, and an especially large proportion of KUNITZs, only

found in small quantities in V6. It also has larger and smaller amounts, respectively, of CTLs and CRISPs, compared to similar venoms. Venom class V4 is the only case of convergence (at least on a higher level) on venom composition found in our research (Figure 1), arising independently four times in three xenodontine tribes: once in the common ancestor of Tachymenini, twice in Xenodontini (in the common ancestor of the genus *Erythrolamprus* and in *Lygophis flavifrenatus*), and once in Philodryadini (in the common ancestor of *P. viridissima* and *P. laticeps*). This venom class evolved multiple times from V1 by a rise in the proportion of svMMPs, taking the place of SVMP as the most abundant toxin gene family. Venom type V5 is a derivation from V4 present only in *Gomesophis brasiliensis*, characterized by a much larger proportion of CRISP transcripts. Finally, V6 is the characteristic venom of Hydropsini. This was the most strikingly divergent venom class recovered by our analysis. V6 is almost entirely composed of CTLs (over 90%), with only small amounts of CRISPs, KUNITZs, and SVMPs.
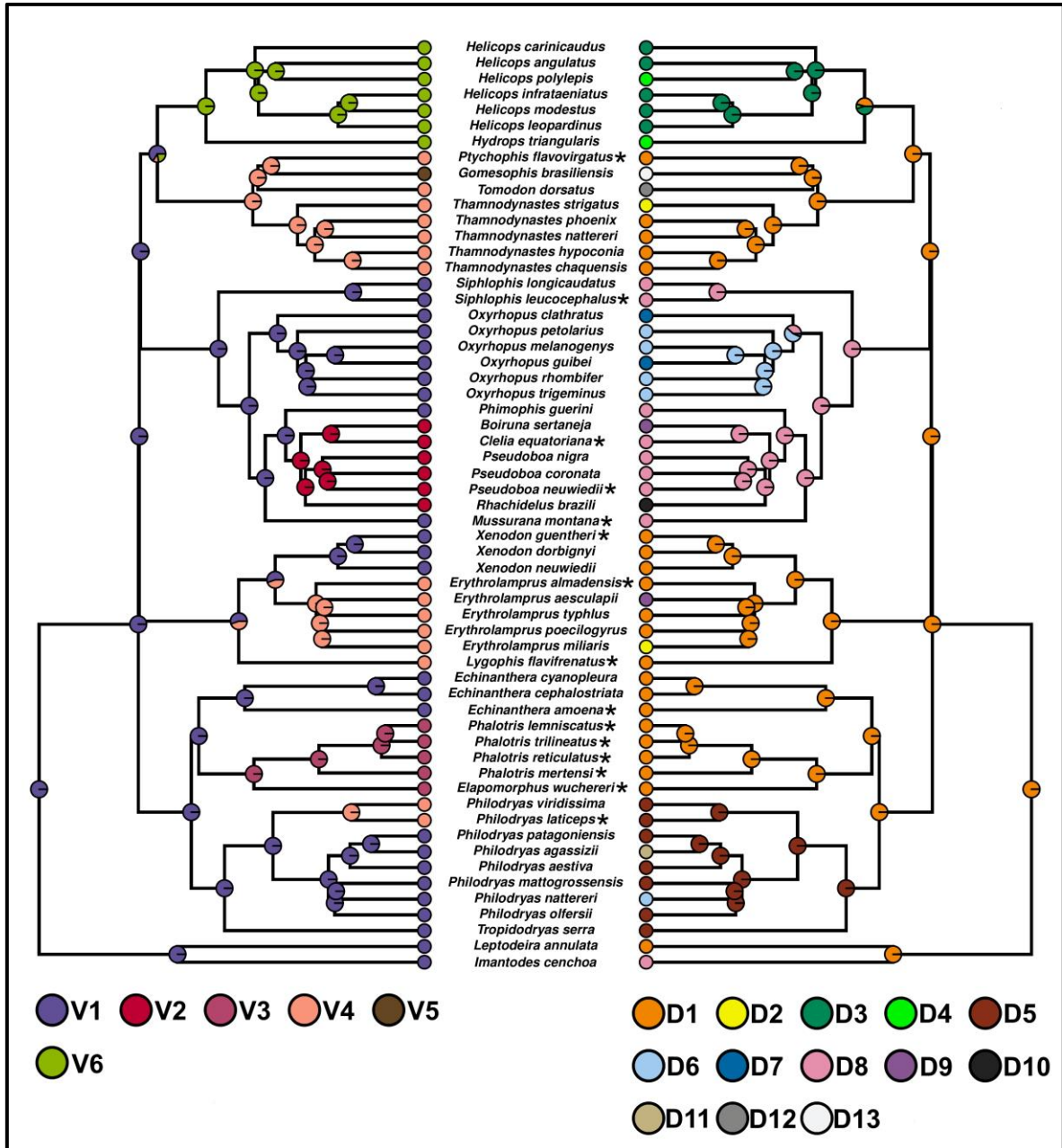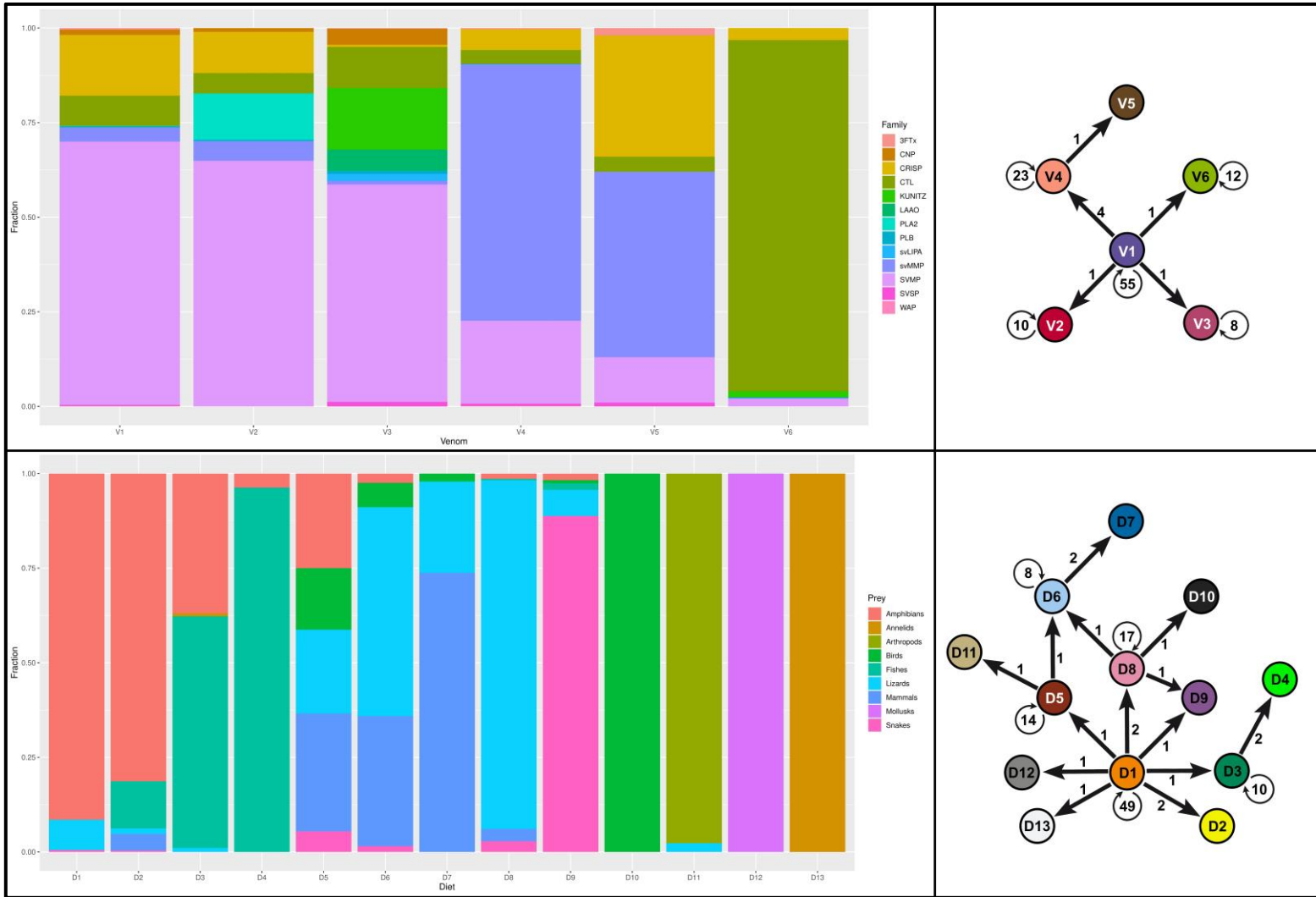
Figure 1. Venom (left) and diet (right) classes for the 59 species studied. Species marked with an asterisk had less than 10 prey items reported in the literature (diet results mostly reconstructed from the phylogeny).

Figure 2. Top Left – Composition of the 6 venom classes found in the 59 studied snake species. **Top Right** – Graph showing the transitions between venom classes. **Bottom Left** - Composition of the 13 diet classes found in the 59 studied snake species. **Bottom Right** - Graph showing the transitions between diet classes.

(c) Venom complexity and diet diversity

Venom complexity was medium to high in most xenodontine species, with the exceptions being Hydropsini, the clade formed by *Ptychophis*, *Tomodon*, and *Gomesophis*, in Tachymenini, and *Phalotris trilineatus* (Figure 3 - Left). On the other hand, the diet phylogenetic diversity was medium to low for most species, with Hydropsini and Philodryadini as the standout exceptions (Figure 3 - Right). The diet taxonomic diversity varied wildly between species and did not present much evidence of phylogenetic signal (Supplementary Material, Figure S1). When we look at the results of the a priori hypothesis

testing (Table 1), neither the diet phylogenetic nor taxonomic diversities could explain most venom variables better than the null model, including venom complexity. The exceptions were the CTL fraction and complexity, which were positively correlated with the diet phylogenetic diversity and the svMMP complexity, which was positively correlated with the diet taxonomic diversity. When we turn to the results of the exploratory analysis (Table 2), however, in which the models are corrected for diet composition, the CTL signal disappears in both cases. The CTL fraction and complexity became positively correlated with the diet taxonomic diversity instead, when we take into account diet composition. On the other hand, the positive correlation with diet taxonomic diversity is not only maintained for the svMMP complexity but also appears for the svMMP fraction. Here, both variables also became slightly negatively correlated with the diet phylogenetic diversity, as seems to happen to the CRISP fraction. All venom variables had at least one diet variable included in its best model and all diet variables were included in at least one of the best models. The diet variable included in more models was the fraction of fish. In fact, together with the fraction of molluscs, this was the only diet variable included in the best model for the overall venom complexity, both negatively correlated with it, indicating that species that prey more on these groups have simpler venoms.
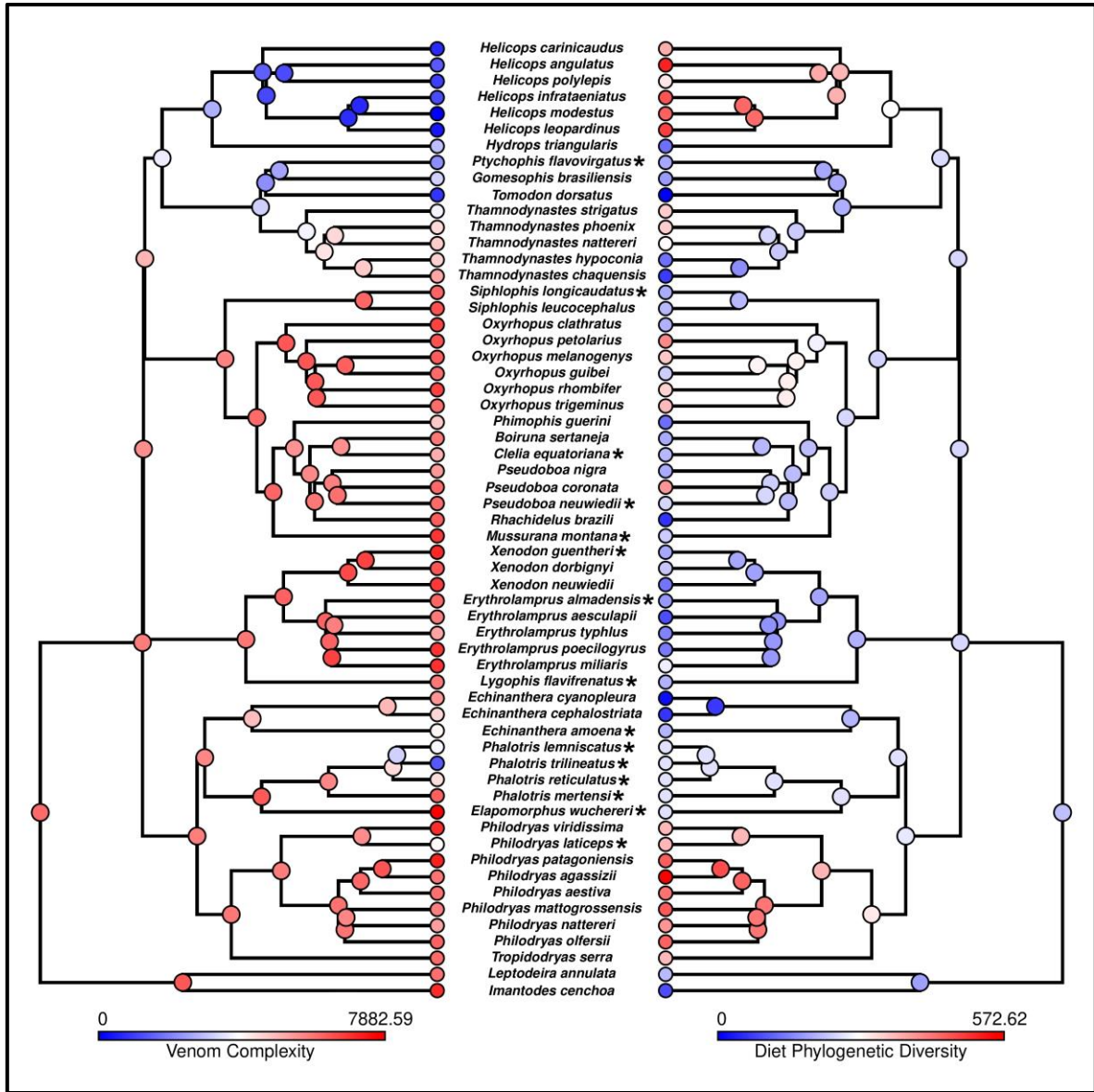
Figure 3. Venom complexity (left) and diet phylogenetic diversity (right) for the 59 studied snake species.

Table 1. AICc values of the three a priori models for the venom variables: one with each diet diversity measure as explanatory variables and the null model. The lowest AICc value for each response variable is in bold.

	PHYLOGENETIC DIVERSITY	TAXONOMIC DIVERSITY	NULL
VENOM COMPLEXITY	730.71	729.28	**728.43**
CRISP COMPLEXITY	-48.77	-49.00	**-51.03**
CRISP FRACTION	-99.47	-99.98	**-100.93**
CTL COMPLEXITY	**-16.79**	-14.37	-15.76
CTL FRACTION	**42.21**	45.74	43.51
PLA2 COMPLEXITY	96.57	96.59	**94.29**
PLA2 FRACTION	127.66	128.21	**126.45**
SVMMP COMPLEXITY	747.36	**742.54**	745.29
SVMMP FRACTION	110.72	109.67	**109.30**
SVMP COMPLEXITY	750.35	750.94	**748.71**
SVMP FRACTION	-43.07	-42.88	**-44.72**

Table 2. Standardized coefficients of the variables included in the best model to predict each venom variable. The explanatory variables are diet diversity measures and the fraction of each prey type in the diet. Values in bold have P<0.05. AICc values are shown in the last column.

	Phylogenetic diversity	Taxonomic diversity	Amphibians	Annelids	Arthropods	Birds	Fishes	Lizards	Mammals	Molluscs	Snakes	AICc
Venom complexity							**-0.30**			**-0.21**		716.00
CRISP complexity						**0.28**	-0.41		0.26		**-0.29**	-61.35
CRISP fraction	-0.25		**-0.42**	**0.30**		0.17	**-0.49**			**-0.28**	**-0.42**	-124.71
CTL complexity		0.21			**0.34**		**0.65**	0.29	**0.44**			-21.78
CTL fraction		0.13	**-0.31**		0.17		**0.49**			-0.14		25.53
PLA2 complexity									0.24			93.33
PLA2 fraction					**0.16**							123.44
SVMMP complexity	-0.27	**0.31**					-0.21					739.32
SVMMP fraction	**-0.17**	**0.14**				-0.14	**-0.58**					92.52
SVMP complexity				-0.20			**-0.49**		0.14	**-0.20**		739.83
SVMP fraction							**-0.28**					-46.99

4. Discussion

(a) Specialized but versatile

Our results shown that xenodontine snakes are, generally, diet specialists. Most species rely on one or two types of preferred prey, with the single truly generalist diet (D5, found only in Philodryadini) been composed exclusively of terrestrial vertebrates. This is well in accord with the pattern previously described for snakes in general (Grundler and Rabosky, 2021). Paradoxically, however, our results also indicate an opportunistic character in the diet of these snakes. Although almost all diet classes where centred one or two preferred prey types, most of them also included, sporadically, other types of prey (less 10% of the diet). The exceptions, diets D10, D12 and D13, for which only the preferred prey type was recorded, are the ones with the smallest sample sizes, with n=30 or less, where all other diet classes had at least 47 (but generally much more) recorded prey items (Supplementary Dataset 2). It is possible that, as more data is gathered for species having these diets, eventual prey items could be recorded on them as well. This apparent capacity of consume other types of prey indicates that specialization does not necessarily impairs an organism's ability to explore other resources (Futuyma and Moreno, 1988). This, in combination with the fact that most diet transitions here included specialized ancestors, supports the notion that diet specialists are not less evolutionary versatile than diet generalists (Futuyma and Moreno, 1988).

The diet results also seem to agree with the model of diet transitions between specialists proposed by Queiroz and Rodriguez-Robles (2006). Here, most diet transitions came from ancestors who fed, at least occasionally, on the new preferred type of prey, and prey items living in the same habitats appeared in the same diets more frequently (e.g., amphibians/fishes, mammals/lizards, etc). With this model of diet evolution in mind, is interesting to note that the ancestral diet in Xenodontinae (D1), from which most of the other diets were derived, is based on amphibians. Amphibians, more specifically frogs, which are the vast majority of amphibian prey listed here (Supplementary Dataset 2), are the only animals consumed by the xenodontines that are very common in all habitats occupied by the other prey classes. South America presents a high richness of terrestrial, aquatic, and arboreal frogs (Duellman, 1999). Therefore, it is possible that for xenodontines an

amphibian-base diet facilitates changes habitat use without the need of a drastic and immediate shift in diet composition. Once in the new habitat, the snake would be able to encounter and include in its diet new types of prey and even become, eventually, a specialist on them. For instance, in most South American ecosystems a frog-eater linage could shift between terrestrial, aquatic, or arboreal habits while keeping the frog-based diet. In this new environment, the linage would have the opportunity to prey upon groups that are much more common (depending on the ecosystem) or even exclusive of this environment, such as lizards (D1 to D8), fishes (D1 to D2, D1 to D3) or birds (D1 to D5). We hypothesize that an amphibian-based diet may have act as "gateway" for xenodontine linages to access new habitats and, as consequence, specialize in consume new types of prey.

(b) Same venom, different diets

While xenodontine diets present a high degree of diversification and numerous cases of convergence, xenodontine venoms seem to be much more conserved both phylogenetically and structurally. Phylogenetically, not only because the ancestral venom is the most widespread one, but also because once a new type of venom is acquired by an ancestor, all its descendants tend to keep that venom type (Figure 1). Structurally because, except for Hydropsini's venom V6, they maintain the same overall structure, in terms of toxin family's composition. Venoms V1 to V5 are all composed mostly of metalloproteinases (SVMPs or svMMPs) with varying amounts of CRISPs and CTLs (Figure 2 – Bottom Left). This apparent conservation may be in accord with Sunagar and Moran (2015)'s "two-speed" model where purifying selection play a major in venom maintenance through most of the linage's history. Combined with the fact that most diet transitions were not followed by venom shifts, it suggests that the same general venom makeup is effective in neutralizing most kinds of prey. A major decoupling between venom variation and diet composition, although surprising, would not be unprecedented (Zancolli et al., 2019). It is possible that most of venom evolution related to prey acquisition occurs at the sequence level, by "recycling" the main proteins already in the venom, or by changes in the levels of expression of less abundant toxin gene families. Both explanations are supported by the results in Table 2, where the proportion of many prey items in the diets was

positively correlated with the complexity (sequence level) and/or fraction (expression level) of some toxin gene families.

(c) When diet and venom change together

Although xenodontine venoms are generally conserved, there were at least two occasions when the diet and venom changed simultaneously. First, the most striking case happened in the common ancestor of Hydropsini, where the venom became almost entirely composed of one gene family, CTL (V1 to V6), while was a shift to mostly fish-based diets (D1 to D3). This relationship was so strong that the proportion of fish in the snakes' diets was positively correlated with the CTL fraction and complexity in the venom, as well as negatively correlated with the overall venom complexity (Table 2), even with the appropriated phylogenetic correction. This would not be the first time that simpler, so-called streamlined, venoms are associated with a fish-based diet (Mackessy and Tu, 1993; Fry et al., 2003; Durban et al., 2018). Marine elapids acquired twice independently a venom composed of only a few highly neurotoxic 3FTx proteins, as they shift to an aquatic teleost-based diet (Fry et al., 2003). It has been suggested that since tracking prey underwater may be difficult for snakes, this highly neurotoxic venom is necessary to rapidly paralyze the target, preventing it to scape or injure the predator while seized in its mouth (Mackessy and Tu, 1993). In fact, studying the feeding behaviour of *Helicops modestus*, Oliveira et al. (2016) suggested that its venom's main function is to induce prey quiescence/immobilization, since most fishes were swallowed alive but clearly immobilized. Laboratory tests show that the venom of *H. modestus* induces in fish around 30 minutes of paralysis/immobilization before death, while killing mice in approximately 10 minutes (Albolea et al, 2000). The venom of *H. angulatus* does the same to mice in 6-8 minutes (Estrella et al., 2011). Estrella et al. (2011) showed that the main effect of the venom of *H. angulatus* on mice is neurotoxicity, and the same research group isolated a CRISP neurotoxin from this snake called helicopsin (Estrella et al., 2010). CRISPs were the second most abundant toxins in hydropsine venoms in our study. CTLs, however, are not associated with neurotoxicity in general (Fry et al., 2012), functioning as multimeric heterodimers that disrupt haemostasis by binding to platelet receptors such as the von Willebrand factor (vWF) or GP1b (Clemetson et al., 2001; Eble, 2019). Nevertheless, Xie et al. (2022) discovered that

the venom gland transcriptome of *H. leopardinus* contains a unique, highly derived, type of lectin, which may have a different function than those already described. If future studies show that CTLs from hydropsine snakes have neurotoxic activity, this will represent an incredible case of convergent evolution, where, using completely different molecular mechanisms, two distantly related snake groups acquired a streamlined neurotoxic venom after transitioning to a fish-based diet.

The second case in which venom and diet change simultaneously happened in the branch leading to *Gomesophis brasiliensis*, which has its own unique diet (D13) and venom (V5) (Figure 1). *G. brasiliensis'* diet is composed of annelids (Figure 2 – Bottom left), all of them earthworms, whose proportion in the diet was positively correlated with the fraction of CRISPs in the snakes' venoms (Table 2). Its venom also contained a larger amount of 3FTxs than similar venoms (Figure 2 – Top left). Both CRISPs and 3FTxs are known to act as neurotoxins (see above). CRISPs also cause paralysis of peripheral smooth muscle through blockage of ion channels (Fry et al., 2012). One of the main ways by which earthworms defend themselves against snake predation, besides burying, is by wrapping around the snake's head and rubbing their body's mucus on it (Cunningham and Burghardt, 1999). In addition to causing apparent discomfort (Cunningham and Burghardt, 1999), this mucus also contains an alarm pheromone (Ressler et al., 1968; Ratner and Boice, 1971) that makes it aversive to other earthworms (Ratner and Boice, 1971; Halpern et al., 1987). After preying on earthworms, snakes try to clean their heads, but consuming paralyzed animals, as they are not able to effectively deposit mucus, considerably reduces this behaviour (Cunningham and Burghardt, 1999). Therefore, we hypothesize that a more neurotoxic venom may help *G. brasiliensis* both to avoid prey fleeing and to reduce mucus rubbing. This would be corroborated if future studies show that *G. brasiliensis'* venom do immobilize earthworms and reduce mucus deposition, and if predation success is reduced in the absence of venom.

A third possible case of simultaneous shift in diet and venom may have occurred in the common ancestor of Elapomorphini. The results indicate a unique and more diverse (in terms of gene families) venom (V3) for this tribe, in accord with what has been previously reported for *Phalotris mertensii* (Campos et al., 2016). Although our analysis reconstructed an amphibian-based diet for the members of this tribe (Figure 1), this result may have been affected by the low number of prey records for its species. The little evidence available

suggests that they feed primarily on amphisbaenians and other elongated vertebrates (Sawaya et al., 2008). Currently, we have more knowledge about the molecular characteristics of these snakes' venoms than about their diets (Sawaya et al., 2008; Campos et al., 2016). Until more life history data is gathered for these species, any attempt to discuss the patterns and causes behind their venom's evolution will be futile.

(d) When venom changes, but diet does not

There were five instances where snakes' venoms shifted without respective change in diet. One of them happened in a clade within Pseudoboini (Figure 1), where there was an increase in the proportion of PLA2s on the transition between venoms V1 and V2 (Figure 2 - Top). Torres-Bonilla et al. (2018) showed that the venom of *Pseudoboa neuwiedii* has similar PLA2 activity to the venom of *Bothrops* snakes, more specifically "similar to *B. atrox*, greater than *B. neuwiedii* but less than *B. jararacussu*". Even though this shift occurred in association with a lizard-based diet, the PLA2 complexity is correlated with the proportion of preyed mammals (which were also present in diet D8), while its fraction with the proportion of arthropods (Table 2). Mammals are an important part of *Bothrops'* diets (Martins et al., 2002), although the only snake that specialized in mammals in Torres-Bonilla's study, *B. neuwiedii,* had the lowest PLA2 activity in its venom. On the other hand, unlike *B. neuwiedii*, *B. atrox* and *B. jararacussu* are generalists that include arthropods in their diets (Martins et al., 2002). Further studies will be necessary to clarify the possible relationship between these prey items and the PLA2 activity on snakes' venoms.

The other instances of venom change with diet maintenance represent the same shift that happened four times independently: the shift from V1 to V4 (Figure 2 – Top Right). This is shift characterized by an increase in the proportion of svMMPs and a respective reduction in the fraction of SVMPs. Our study is not the first to show this tendency of substitution of SVMPs by svMMPs in xenodontine venoms (Junqueira-de-Azevedo et al., 2016; Bayona-Serrano et al., 2020). Bayona-Serrano et al. (2020) previously identified three of the four shifts reported here (those happening in Tachymenini and Xenodontini) and one additional in the tribe Conophiini (not included in this study). We show for the first time the same transition happening in the common ancestor of *P. viridissima* and *P. laticeps*. In fact,

these two species represent a divergent linage inside *Philodryas*, to the point where Arredondo et al. (2020) proposed to resurrect the genus *Chlorosoma* to cover them. Bayona-Serrano et al. (2020) also demonstrate that, although recruited from as different ancestral metalloproteinase, svMMPs likely execute the same functions that SVMPs in the venom. Animals preyed by snakes with SVMP-rich venoms frequently evolve SVMP resistance (Holding et al., 2016; Robinson et al., 2021; Thiel et al., 2022). This happens by the presence in the blood of SVMP inhibitors (SVMPIs) that bind to SVMPs, preventing their protease activity (Valente et al., 2001). SVMPIs evolved from different gene families independently across multiple distinct mammal and snake lineages (Thiel et al., 2022). With that in mind, considering that svMMPs play the same role as SVMPs, and that its increased representation in venoms is not associated with changes in diet composition, we propose that the substitution of SVMPs by svMMPs is a mechanism to avoid SVMP resistance in the prey. Most of the transitions between V1 and V4 happened in clades with a frog-based diet. Despite no SVMPI having been identified in frogs yet (Thiel et al., 2022), there is at least one case of resistance to an SVMP-rich venom in these animals (Gibbs et al., 2011; Smiley-Walters et al., 2017). We hypothesize that SVMPIs will be unable to, or at least less effective in, neutralizing svMMPs and that the ratio between svMMP and SVMP toxicity will be much higher for species preyed by snakes with venom type V4 than for other similar animals.

(e) Overall patterns of diversity

In contrast to what was found by Holding et al. (2021) for pitvipers, diet phylogenetic diversity was not able to predict venom complexity in xenodontine snakes (Table 1). In their work, Holding and colleagues argued that this relationship is the result of subtle variation in conserved toxin targets that appeared during prey species' divergence. Albeit having diverse diets, pitvipers terrestrial snakes that feed almost entirely on terrestrial vertebrates, generally using the same "bite and release" strategy (Hayes et al., 2002; Holding et al., 2021). We propose that, as ecological diversity increases, in terms of life habits and groups preyed upon, idiosyncrasies in predation behaviour and prey physiology become more important than molecular divergence in toxin targets. These idiosyncrasies may reflect, for instance, the need for rapid prey immobilization under water or to avoid aversive defence strategies (see above), as well as physiological differences between vertebrates and invertebrate groups, for

which divergence time will not be a good proxy for ecological relevant traits, as the evolution of these characters is not necessarily clocklike (Koonin et al., 2011). The best model for venom complexity in Xenodontinae (Table 2) supports this notion, as it only included negative correlations with the fractions of fish and molluscs in the diet. For fishes, this is probably the result of a streamlined neurotoxic venom to prey upon these animals, as previously discussed. Molluscs, however, were only consumed by one species, *Tomodon dorsatus*, a slug-eater snake (Bizerra et al., 2005). Slugs are very slow animals for which the main form of defence is mucus secretion that hamper the ingestion by the predator (Bizerra et al., 2005). *T. dorsatus* has specialized dentition and preying behaviour that allows it to swallow live slugs (Bailey, 1966; Bizerra et al., 2005). These traits are common in other snakes that feed on slugs/snails, as well as atrophied venom glands and non-venomous secretions that may help to dissolve the slug's mucus (Bizerra et al., 2005; Zaher et al., 2014). For these animals, mucus control and prey transport may more important than prey envenomation (Zaher et al., 2014). Therefore, we suggest that for *T. dorsatus*, the decrease in venom complexity is due to a reduction in the selective pressure to maintain venom toxicity, which leads to loss of expression of multiple proteins uniformly across toxin gene families. This is supported by the fact that a mollusc-rich diet is not associated with the increase in the portion or complexity of any toxin gene family in our results (Table 2). This hypothesis implies that the toxin genes in *T. dorsatus'* venom are under low purifying selection and/or that the venom itself has little to no impact on the predation success of this species. It is interesting to compare the case of *T. dorsatus*, which feeds on slugs, with the case of *G. brasiliensis*, which feeds on earthworms. In snake diet studies, both kinds of prey are sometimes grouped in the same category of "soft-bodied invertebrates", with their snake predators being the so-called "goo-eaters" (Cadle and Greene, 1993; Bizerra et al., 2005; Zaher et al., 2014; Bellini et al., 2015). However, from a diet specialization, predation/defence behaviour, and venom composition/evolution standpoint, these groups actually represent two very distinct kinds of prey.

Although none of the patterns described by Holding et al. (2021) were found for the whole venom here, individual toxin families have their own histories. CTL's complexity and fraction were better explained by the diet phylogenetic diversity (Table 1), but this was clearly caused by its association with the fish-rich diets since once we correct the model for diet composition, this relationship disappeared (Table 2). Fishes generally have deeper

divergence times than the tetrapod prey consumed in other diets (Supplementary File 2). On the other hand, after correction, CTL's complexity and fraction became positively correlated with the diet taxonomic diversity. This may indicate a pattern of diffuse coevolution with multiple species (Goüy De Bellocq et al., 2008; Salazar et al., 2018; Holding et al., 2021). Curiously, CTL was the only gene family in Holding's work for which the complexity was not well predicted by the diet phylogenetic diversity. They suggest that this may be due to coevolution with opossum (Didelphidae) predators, which prey on pitvipers and coevolved CTL resistance by the rapid evolution of vWF 's binding sites (Jansa and Voss, 2011). In our results, the proportion of consumed mammals, many of which were opossums (Supplementary Dataset 2), was positively correlated with the complexity of CLTs (Table 2). This may implicate different patterns of coevolution when the same trait is employed as a predatory or as a defensive tool. Another toxin gene family that presented the same pattern of diffuse coevolution, in both the a priori and exploratory analyses (Tables 1 and 2), was svMMP. This gives additional support to our hypothesis that the substitution of SVMPs by svMMPs is likely a response to SVMP resistance in the prey. svMMPs are also associated with diets that have lower phylogenetic diversity, i.e., more specialized diets, probably because the svMMP-rich venom V4 is present mostly in snakes specialized in amphibians (Figure 1). The same seems to happen for CRISPs, which are abundant in venom V5 that occurs in association with the annelid specialized diet D13.

5. Conclusion

Our work shows that xenodontine snakes mostly have specialized, but ecologically and evolutionarily versatile, diets, as well as phylogenetically and structurally conserved venoms. We also demonstrate that neither diet phylogenetic nor taxonomic diversity are good predictors for venom complexity in these animals. Instead, as ecological diversity increases, individual histories of lineages, diets, and gene families become more important than general patterns in explaining complexity. These individual histories, in which both ecological opportunity and coevolution play a part, are marked by idiosyncrasies associated with life habits, predation behaviour, prey physiology, mechanisms of action, and prey resistance. We also suggest a series of hypotheses that, if tested, will improve our knowledge

about xenodontine ecology and evolution. Such hypotheses are: (1) A frog-based diet acted as a "gateway" allowing xenodontine snakes to shift between habitats without the need for drastic changes in diet; (2) Hydropsini's highly derived CLTs acquired neurotoxic activities to help immobilize fish prey underwater. (3) *G. brasiliensis* venom helps this species to reduce the scaping and defensive mucus deposition by its annelid prey. (4) The tendency SVMPs substitution by svMMPs in xenodontine venoms is a mechanism to avoid coevolved SVMP resistance in the prey. (5) The venom of *T. dorsatus* is under low purifying selective pressure and has little impact on this species' predatory capacity. There is still much work to be done before we are able to fully understand Xenodontinae's evolution and the intricate patterns that govern complexity in the living world.

Acknowledgments

Supplementary Information

Supplementary Material. Figure S1, representing the diet taxonomic diversities. References for the prey records data, and the phylogenetic relationship among species reported as prey.

Supplementary Dataset 1. Estimates of venom complexity per sample, number of reads mapped to each toxin family, and percentual of reads mapped to each toxin family. Estimates of venom complexity per species for each toxin family.

Supplementary Dataset 2. Distribution of prey records at the species level.

Supplementary Dataset 3. AICc values for the evolutionary models. Unstandardized coefficients and *P*-values for the best models in Table 2.

Supplementary File 1. Calibration points used for the dating the phylogeny of Xenodontinae snakes.

Supplementary File 2. Phylogenetic relationship among species reported as prey.

The Supplementary Information can be accessed through the link:

https://drive.google.com/drive/folders/1jSq7IOLZlGYhhfFAuqOi9p8eJAscuU05?usp=sharing

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CAPÍTULO 4: Considerações finais

Embora um estudo recente tenha proposto que serpentes com dietas mais amplas teriam maior facilidade em consumir novos tipos de presa, pois suas peçonhas teriam menor especificidade – e, portanto, maior versatilidade (Lyons et al., 2020), nossos resultados indicam claramente que serpentes especialistas não são menos versáteis. Aliás, essa versatilidade se manifesta tanto do ponto de vista ecológico quanto evolutivo. Ecológico porque a maioria das dietas especializadas aqui descritas inclui o consumo eventual de itens alimentares bastante distintos da presa preferencial. Evolutivo, por outro lado, porque a maioria das transições entre dietas se originou a partir de dietas especializadas, mostrando a capacidade destas linhagens de se adaptar para consumir novos tipos de presas. Apesar da ideia de que especialistas não são necessariamente menos versáteis que generalistas ser relativamente antiga (Futuyma e Moreno, 1988), apenas mais recentemente foi proposto um modelo explicando como, em serpentes, podem ocorrer transições a partir de dietas especializadas (Queiroz e Rodriguez-Robles, 2006). Tal modelo foi suportado por achados posteriores (Alencar et al., 2013; Bellini et al., 2015; Grundler e Rabosky, 2021) e sugere que o uso do habitat pode ser o principal fator limitante no consumo de novas presas. Com base nisso, nós hipotetizamos que a dieta ancestral aqui reconstruída para Xenodontinae, baseada majoritariamente em anfíbios, serviu como um facilitador na transição para novas dietas, uma vez que anuros terrestres, aquáticos e arbóreos são muito abundantes em ecossistemas sul-americanos (Duellman, 1999). Tal fato permitiria que serpentes com esse tipo de dieta consigam se adaptar mais facilmente a novos habitats sem a necessidade de uma mudança imediata na composição da dieta.

Uma das principais diferenças entre o nosso trabalho e aquele que detectou menor especificidade na peçonha de serpentes generalistas é o fato de que o banco de dados no qual ele foi baseado tinha uma super-representação das famílias Elapidae e Viperidae (Healy et al., 2019). Devido a sua dentição inoculadora frontal, os animais dessas famílias apresentam comportamentos de predação distintos daqueles observados nos xenodontíneos proteróglifos, que podem muito bem muito tornar a peçonha um fator de maior importância na transição entre dietas (Deufel e Cundall, 2006). Uma evidência em favor da proposição de que a composição da peçonha talvez não seja tão restritiva em xenodontíneos foi o fato de que, nesses animais, ela parece ser, do ponto de vista de representação de famílias gênicas, filogenética e estruturalmente conservada. Transições de dieta desacompanhadas de

transições entre tipos de peçonha foram frequentes, e a maioria das peçonhas nesse grupo é composta majoritariamente de metaloproteases, proteínas secretórias ricas em cisteína e lectinas do tipo c.

Isso não é o mesmo que dizer que mudanças na composição das peçonhas não estão associadas à aquisição de novas presas ou mesmo à manutenção da capacidade de captura da presa preferencial. Xenodontíneos piscívoros, por exemplo, apresentam uma marcada redução na complexidade da peçonha, que passou ser composta quase que inteiramente por uma única família de toxinas, as lectinas do tipo c. Tal simplificação parece ser um caso de convergência com elapídeos marinhos, cuja peçonha é composta por apenas um pequeno conjunto de toxinas *three-finger* (Mackessy e Tu, 1993; Fry et al., 2003; Durban et al., 2018). Nós propomos que tal simplificação é o resultado da seleção por uma peçonha mais neurotóxica, necessária à captura de presas embaixo d'agua (Mackessy e Tu, 1993; Albolea et al, 2000; Estrella et al., 2010; Oliveira et al., 2016). Já *Gomesophis brasiliensis*, uma espécie especializada em anelídeos, tem em sua peçonha maior porcentagem de toxinas com potencial neurotóxico/paralisante (proteínas secretórias ricas em cisteína e toxinas *three-finger*). Nós sugerimos que tal composição atua para reduzir a possibilidade de fuga da presa e a deposição do muco aversivo utilizado como mecanismo de defesa por anelídeos (Ressler et al., 1968; Ratner e Boice, 1971; Halpern et al., 1987; Cunningham e Burghardt, 1999).

Para outras serpentes desse grupo, no entanto, a peçonha parece ser menos importante na captura de presas. É o que nós hipotetizamos ser o caso para *Tomodon dorsatus*, uma espécie malacófaga que apresenta considerável redução na complexidade da peçonha desassociada de qualquer mudança drástica na composição de famílias gênicas. Nossa argumentação é que isso deve ser o resultado de uma redução na seleção purificadora para a manutenção de toxidade, levando a uma perda relativamente uniforme da expressão de diversas toxinas. Isso se deve ao fato de que, para espécies com dieta similar, muitas das quais apresentam glândulas de peçonha atrofiadas, adaptações ligadas a movimentação da presa e ao controle de muco, tais como dentição, comportamento e secreções especializados, parecem muito mais importantes do que a inoculação da peçonha (Bailey, 1966; Bizerra et al., 2005; Zaher et al., 2014).

Mudanças na composição das peçonhas também podem estar associadas a manutenção da capacidade de consumir a mesma presa, em um cenário de "corrida

armamentista". É o que nós sugerimos estar por trás das massivas substituições, já previamente reportadas por Bayona-Serrano et al. (2020), de metaloproteases da peçonha de serpentes (SVMP) por metaloproteases de matriz da peçonha de serpentes (SVMMP), o que, segundo nossos resultados, teriam ocorrido quatro vezes independentemente em três tribos de Xenodontinae (Philodryadini, Tachymenini e Xenodontini). Nossa hipótese é que tais substituições são uma resposta à resistência a SVMPs frequentemente desenvolvida por diversos grupos de presas (Valente et al., 2001; Holding et al., 2016; Robinson et al., 2021; Thiel et al., 2022). Essa hipótese é reforçada pela correlação positiva encontrada entre a complexidade das metaloproteases de matriz da peçonha de serpentes e a diversidade taxonômica da dieta, que sugere um padrão que coevolução difusa associado a essa família de toxinas (Goüy De Bellocq et al., 2008; Salazar et al., 2018; Holding et al., 2021).

Nós não fomos capazes de replicar os resultados obtidos por Holding et al. (2021). Nenhuma medida de diversidade da dieta foi uma boa preditora da complexidade da peçonha como um todo. Com base nisso e no acima exposto, fica claro que, ao invés disso, à medida que a diversidade ecológica aumenta, as histórias individuais de linhagens, dietas e famílias gênicas tornam-se mais importantes do que padrões gerais para explicar a complexidade da peçonha. Essas histórias individuais, nas quais tanto oportunidade ecológica quanto coevolução têm seu papel, são marcadas por idiossincrasias associadas a hábitos de vida, comportamentos de predação, fisiologia da presa, mecanismos de ação e resistência à peçonha.

Perspectivas futuras para este trabalho incluem datar a árvore utilizada nas análises com base em métodos mais sofisticados e testar novas estratégias para a análise estatística dos dados de composição, uma vez que lidar com dados composicionais pode ser estatisticamente desafiador (Aitchison, 1982). Para futuros trabalhos, nós consideraríamos testar as inúmeras hipóteses aqui apresentadas, o que certamente ajudaria a melhorar nosso conhecimento sobre a ecologia e evolução de serpentes da subfamília Xenodontinae.

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