Basil Minder

Hidroxilação do PTX (+)-251D em *Adelphobates galactonotus* (Anura: Dendrobatidae)

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2024

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Thesis presented to the Institute of Biosciences of the University of São Paulo for the attainment of the Master of Science degree in Biology.

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Dr. Norberto Peporine Lopes

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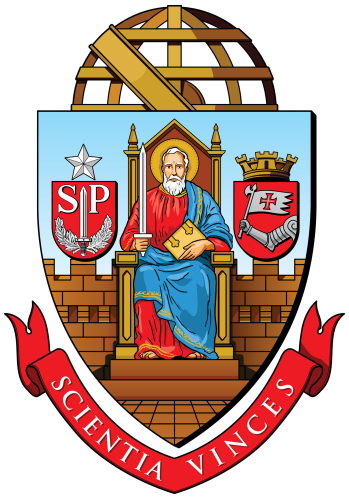

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"It is not the strongest of the species that survive, nor the most intelligent, but the one most responsive to change."

— Charles Darwin

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Overview of Alkaloid Biochemistry in Poison Frogs

The remarkable biochemistry of poison frogs, specifically *Adelphobates galactonotus*, presents an intriguing subject for scientific inquiry. This thesis aims to explore the complex mechanisms of alkaloid sequestration within these amphibians, offering insights into their evolutionary adaptations, ecological interactions, and potential pharmaceutical applications.

Survival and reproduction are fundamental challenges for all organisms, prompting the evolution of various adaptive strategies. One such strategy, chemical defense, involving the production of volatile (Brunetti et al., 2019) and nonvolatile molecules, evolved convergently across many species, serving as an effective means to deter predation (Clark et al., 2005; Hantak et al., 2016), avoid parasitism (Weldon et al., 2006, 2013), and prevent pathogen infection (Hovey, 2018; Saporito et al., 2012; Macfoy et al., 2005; Rivas et al., 2009). This strategy is widespread, spanning across different trophic levels and species, including plants (Dobler, 2001; Heckel, 2018), sea sponges (Webster and Taylor, 2012), invertebrates like butterflies (Malcolm and Brower, 1989), molluscs (Cimino and Ghiselin, 1998), and ants (Löfqvist and Lofqvist, 1977), as well as in vertebrates such as amphibians (Mebs et al., 2005; Daly et al., 1984), snakes (Hutchinson et al., 2013), and birds (Dumbacher et al., 2009).

The ability to sequester alkaloids evolved in multiple times in distinct lineages of five anuran families, comprising more than 300 species (Grant et al., 2017) – namely Bufonidae, (genus *Melanophryniscus* from South America; (Daly et al., 1984)), Eleutherodactylidae (part of the *Eleutherodactylus limbatus* group from Cuba; (Rodríguez et al., 2011)), Mantellidae (*Mantella* from Madacascar; (Garraffo et al., 1993)), Myobatrachidae (*Pseudophryne* from Australia; (Daly et al., 1984)), and in the neotropical family Dendrobatidae, where it occurs in Colostethinae (*Ameerega* , *Epipedobates*, *Silverstoneia* (Grant et al., 2006), in Hyloxalinae (*Paruwrobates*), and Dendrobatinae (*Adelphobates, Andinobates, Dendrobates, Excidobates, Minyobates, Oophaga, Phyllobates*, and *Ranitomeya* inhabit regions stretching from Nicaragua to Bolivia (Grant et al., 2006, 2017; Frost, 2023). Those frogs using alkaloids are commonly known as “poison frogs” (Saporito et al., 2009), while term “dart-poison frogs” traditionally refers to the three species of the genus Phyllobates, used by indigenous people for poisoning darts (Myers, 1978; Saporito et al., 2012).

With the exception of the nocturnal *Aromobates nocturnus*, Dendrobatoidea are diurnal (Myers et al., 1991; Grant et al., 2006) and some express aposematic signaling (Silverstone, 1975, 1976; Myers, 1978; Santos et al., 2003; Vences, 2003), such as bright coloration, to indicate their toxicity to predators. Interestingly, studies suggest that color polymorphisms, specifically in the south Amazonian region living *A. galactonotus* might not differ in alkaloid profiles or palatability (Jeckel et al., 2019) as first studies of *Oophaga pumilio* suggest a correlation between these factors (Hovey, 2018; Bolton et al., 2017; Mebs et al., 2008; Saporito et al., 2007a).

Currently more than 1200 lipophilic alkaloids from 28 structural classes have been identified (Saporito et al., 2009, 2012) in variable alkaloid profiles in these animals (Mebs et al., 2008; Daly et al., 1992; Myers et al., 1995; Daly et al., 2009, 1994; Saporito et al., 2006). Among the most commonly found alkaloids among frogs are indolizidine and quinolizidine based alkaloids (Fig. 1A; Saporito et al., 2012) The indolizidine based pumiliotoxins are known for their cardiotonic and myotonic effects (Gusovsky et al., 1988, 1992; Daly et al., 1990), representing the primary alkaloids found in these *A. galactonotus* (Fig. 1A; Jeckel et al., 2019). The defensive compound composition varies significantly between species, populations, and individ-

uals, influenced by factors like geographical location, season, age, life stage, and body size (Saporito et al., 2006, 2007b; Daly et al., 2002, 2007; Stynoski et al., 2014; Jeckel et al., 2015; Saporito et al., 2010a,b).

For a significant period, it was hypothesized that all defensive chemicals in frogs were produced via internal biosynthetic pathways (Daly, 1995). However, captive bred poison frogs lack lipophilic alkaloids, and experiments have shown that they sequester lipophilic alkaloids from their diet (Daly et al., 1994, 1997, 2003; Daly, 1998; Saporito et al., 2009, 2012; Mebs, 2001), primarily ants and mites, but also beetles, and millipedes (Daly et al., 2002; Saporito et al., 2007b, 2009), while oribatid mites and formicine ants are significant contributors (Saporito et al., 2004, 2007b, 2011; Takada et al., 2005), especially of the so called pumiliotoxins (Saporito et al., 2007b, 2011; Takada et al., 2005). The indolizidine-based alkaloids, named after their discovery in *Oophaga pumilio* by Daly and Myers (1967), include Pumiliotoxin A (PTX 307A) and B (PTX 323A)(Fig. 1B). Pumiliotoxin C, later reclassified as homopumiliotoxin due to its quinolizidine structure, differs chemically from the indolizidine-based pumiliotoxins. As of now, approximately 50 pumiliotoxins and allopumiliotoxins have been identified, along with about 20 deoxy and desmethyl analogs (Fig. 1C; Michael, 2016).

Pumiliotoxins (PTX), specifically the naturally occurring enantionmer PTX (+)-251D, and their derivatives, such as allopumiliotoxins (aPTX) (+)-267A (Fig. 1C), are prevalent across a diverse range of poison frog species within the clade that includes Dendrobatidae (Daly et al., 2003) and Mantellidae (Daly et al., 1997), exemplifying convergent evolution in anuran chemical defenses (Daly et al., 1997, 2003; Smith et al., 2002). Notably, laboratory feeding experiments reveal that certain dendrobatid frogs, like *A. galactonotus*, or *A. cataneoticus* can metabolize PTX 251D up to 70% into the more toxic aPTX 267A, suggesting the presence of an enzyme responsible for the 7'-hydroxylation process, although this enzymatic mechanism remains unidentified and is probably not universally present across all species (Daly et al., 2003; Alvarez-Buylla et al., 2022), as for example in *Phyllobates bicolor* and *Epipedobates tricolor* only PTX 251D was found (Daly et al., 2003).

The Myobatrachidae family, particularly *Pseudophryne* species, displays a remarkable ability not only to sequester pumiliotoxins from their diet but also to synthesize pseudophrynamine alkaloids, with some species capable of further enzymatic modifications like reductions and hydroxylations (Daly et al., 1994, 1997, 2003; Saporito et al., 2012; Smith et al., 2002), or recently discovered methylation of decahydroquinoline (DHQ) (Jeckel et al., 2022). Such biochemical transformations are intriguing because toxins acquired through diet are typically stored unaltered. Modifications can potentially reduce their toxicity, yet in this instance of PTX 251D, result in a compound five times more toxic (Saporito et al., 2012; Alvarez-Buylla et al., 2022; Daly et al., 2003). PTX 251D is a potent alkaloid with pronounced toxic effects, including pain, hyperactivity, convulsions, and mortality in both mice and insects (Daly et al., 1997; Bargar et al., 1995; Weldon et al., 2006). In murine models, PTX 251D primarily acts as a cardiac depressant, with overdosage leading to cardiac failure (Daly et al., 1985, 1988). Research into its electrophysiological impact reveals that PTX 251D disrupts the function of voltage-gated sodium and potassium channels in both mammalian and insect systems (Vandendriessche et al., 2008), which is consistent with its mosquito-repellent properties due to the inhibition of insect sodium channels (Weldon et al., 2006). The adverse effects of PTX 251D on mice can be mitigated by the preemptive administration of anticonvulsants like phenobarbital and carba-

mazepine, indicating its interaction with calcium and sodium channels (Daly et al., 2003).

Further research is necessary to elucidate the detailed concentration-dependent responses of PTX 251D and to identify additional molecular targets, such as G-protein coupled receptors (Alvarez-Buylla et al., 2022). The derivative aPTX 267A is even more lethal, with an injection of 2mg/kg causing hyperactivity, convulsions, and death in mice — a lethal dose fivefold lower than PTX 251D (Daly et al., 2003). This heightened toxicity suggests that the biosynthesis and sequestration of aPTX 267A could be an evolutionary adaptation in certain poison frog species, enhancing their chemical defense mechanism, or their detoxification process of the pumiliotoxin.

The process of alkaloid sequestration in poison frogs is a sophisticated, enigmatic biological phenomenon, one that is selective and potentially occurs through both active and passive mechanisms (Mebs, 2001; Opitz and Müller, 2009; Williams et al., 2001; Saporito et al., 2012). Laboratory research has observed rapid accumulation of these compounds in various tissues, such as skin, liver, muscles and kidney suggesting efficient transport systems and a necessity for mechanisms that prevent self-intoxication (O'Connell et al., 2020; Jeckel et al., 2022). While sequestering ready-made toxins appears to be less physiologically demanding than biosynthesizing them (Zvereva and Kozlov, 2015), it demands intricate physiological adaptations for the safe handling of these potent molecules. A critical aspect of these adaptations is the modification of target molecules, including amino acid substitutions in ion channels, to allow for resistance to the sequestered toxins while balancing the sensitivity to endogenous substances (Wang and Wang, 1999; Tarvin et al., 2016). Moreover, the sequestration process can involve oxidative biochemical reactions that may modify the alkaloids through oxidation, reduction, or hydrolysis, maintaining their core structure for functional utility (Hartmann et al., 1999; Naumann et al., 2002). It raises the question of how and why certain poison frogs can perform such biomodifications and the effect on its organs regarding toxicity (Mebs, 2001; Opitz and Müller, 2009; Williams et al., 2012). Research by Sanchez et al. (2019) suggests that upregulation of genes related to muscle and mitochondrial processes is likely due to the lack of mutations for alkaloid resistance, indicating alkaloid degradation as a potential resistance mechanism. The capability of various poison frog species to metabolize PTX 251D into aPTX 267A, as well as the specific molecular processes involved in this transformation, remains a mystery (Saporito et al., 2012; Daly et al., 2003). Similarly, the underlying mechanisms governing the sequestration of alkaloids are not yet understood (Santos et al., 2016; Caty et al., 2019).

In our study, we aim to map the metabolic fate of PTX 251D within *A. galactonotus*. The primary objective of this study is to shade light into the metabolic pathways involved in the sequestration of alkaloids within *A. galactonotus*, with a particular emphasis on the hydroxylation of PTX 251D to aPTX 267A. A core component of this thesis is to ascertain which specific organ systems partake in the hydroxylation process, the proportion of ingested PTX 251D that is bioconverted as opposed to being metabolically discarded, and the subsequent transport pathways of the resultant aPTX 267A within the organism. By measuring the metabolic conversion and tracing the transportation of aPTX 267A, we aim to illuminate the functional dynamics of toxin deployment in these frogs, providing key insights into their detoxification and sequestration process. This study seeks to advance our comprehension of the frog's detoxification strategy and the ecological role of these potent alkaloids.

The first chapter provides an in-depth examination of various research methodologies, especially on the analytical techniques like gas chromatography and matrix assisted laser imaging. Additionally, it discusses innovative approaches to parasite control and fungal prevention in *Drosophila* breeding, highlighting practical challenges and solutions in maintaining fly colonies and frog populations for research.

Chapter two presents a detailed study on the gas-phase fragmentation reactions of pumiliotoxin and allopumiliotoxin using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Focusing on the fragmentation mechanism and pathways, the research applies different collision energies to provide a robust map of fragmentation. This approach not only elucidates the structural determination and fragmentation pathways of these frog toxins but also opens new perspectives for the study of polar analogues, marking significant advancements in the field of mass spectrometry.

The extensive study in chapter three delves into the metabolic processing of PTX 251D to aPTX 267A in *A. galactonotus*, exploring the intricacies of toxin hydroxylation and sequestration in various organs of this species. It underscores the rapid absorption and transformation of toxins in the liver and skin, alongside examining the unique roles of the digestive tract, kidneys, fat bodies, and gallbladder in toxin storage and excretion. This research on *A. galactonotus* offers critical insights into the amphibian's metabolic pathways, the organs and tissues involved in the PTX (+)-251D sequestration, and conversion to aPTX (+)-267A and enable us to estimate the efficiency of both processes. Our findings illuminate the complex interplay of various organ systems in the detoxification and sequestration of these toxins, emphasizing the liver and skin's central roles in rapid absorption and long-term storage, respectively. The ability to modify the structures of the sequestered toxins could play a key role in greater understanding of the evolution of sequestering in poison frogs, the dynamic detoxification process, and its implications for the frog's chemical defense strategy, survival, and evolutionary adaptations.

Recent studies started examinig the physiological processes coupled to sequestration and conversion (Alvarez-Buylla et al., 2022, 2020; Jeckel et al., 2022; Salgado Costa et al., 2023) which makes the present thesis a significant addition to the field. Moreover, this research casts light on the potential pharmacological applications of these metabolic processes, opening new opportunities for drug discovery. The cytochrome P450 enzymes play a vital role in the hydroxylation of xenobiotics, including drugs, aiding detoxification, and sometimes activating prodrugs or increasing their biological activity and toxicity. This understanding is key in drug discovery and development, as it shapes the pharmacokinetic profiles of drugs and ensures their safety. Our research on *A. galactonotus* may thus reveal unique aspects of amphibian xenobiotic metabolism with potential applications to human medicine.

We achieved that by administering PTX (+)-251D, to toxin-free, captive-bred, *A. galactonotus*, ensuring a clean baseline for tracking the metabolic transformation to aPTX 267A. We have designed two dose- and time-dependent experiments, were we mapped this metabolic journey of PTX 251D, focusing on key organs like the skin, liver, spleen, digestive tract, gallbladder, fat bodies, and kidneys. We're pioneering the use of solid-phase extraction (SPE) for sample purification, improving efficiency and minimizing alkaloid loss. Liquid mass spectrometry techniques (LC), including high-resolution electrospray ionization (ESI), were used to quantify conversion rates to aPTX 267A and track its subsequent transport within the frogs, as well to the fragmentation mechanisms of these alkaloids in electrospray ionization systems.

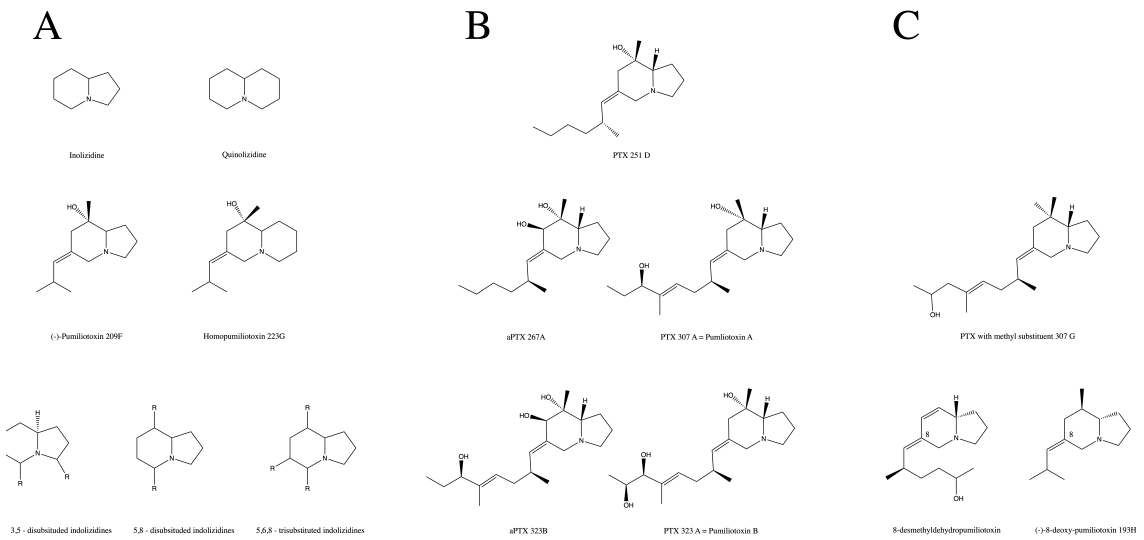


Figure 1: A: Indolizidine is characterized by a structure comprising a 6-carbon (6C) ring connected to a 5-carbon (5C) ring, whereas quinolizidine features two 6C rings as its base, marking them as chemically distinct molecules. Below, we identify the smallest known pumiliotoxin, PTX 209F, which exhibits a minimal structure with a hydroxy and methyl group at the 8th position, as well as a butyl group (C4H9) as a 2-methylpropenyl substituent at the 6th position. This configuration can vary in length and the number of substituted hydroxy groups. To the right is the corresponding quinolizidine based homopumiliotoxin. In the third line are the three primary alkaloid classes found in poison frogs based on the indolizidine structure.
B: PTX 251D is the alkaloid examined in this study. Below it is its metabolite, which features a hydroxy group at the 7th position, aPTX2678A. While PTX 307A (Pumiliotoxin A) contains a single hydroxyl group in its side chain, PTX 323 (Pumiliotoxin B) serves as an example of a pumiliotoxin with multiple hydroxylations in the side chain. Since "Pumiliotoxin" is not an IUPAC name, these names were arbitrarily assigned following their discovery in *Oophaga pumilio*
C: Additionally identified are derivatives such as desmethyl, dehydro, and deoxy pumiliotoxin, which are as chemically distinct from indolizidine based pumiliotoxins as homopumiliotoxin is. From a chemical standpoint, these trivial name based classifications are not ideal. Naming these compounds as 6,8-substituted indolizines would be more logical and clearer. Moving forward, a consistent naming convention should be established to distinguish when a compound is considered a pumiliotoxin derivative.

This study uncovered a multifaceted metabolic processing of toxins in *A. galactonotus*, revealing alkaloid storage in fat bodies and the presence of aPTX in urine and kidney after a detox period of four days. Intriguingly, a secondary hydroxylation site was identified in the skin, suggesting a complex regulation of toxin levels. Additionally, the findings suggest that toxins may be bound to proteins in the liver, offering insights into their transport and sequestration mechanisms. These discoveries not only contribute to our understanding of amphibian biology but also open new avenues for drug discovery, considering the relevance of these processes in the metabolism of xenobiotics.

Bibliography

Alvarez-Buylla, A., Payne, C. Y., Vidoudez, C., Trauger, S. A., and O'Connell, L. A. (2020). Pumiliotoxin metabolism and molecular physiology in a poison frog. Preprint, Physiology.

Alvarez-Buylla, A., Payne, C. Y., Vidoudez, C., Trauger, S. A., and O'Connell, L. A. (2022). Molecular physiology of pumiliotoxin sequestration in a poison frog. *PLOS ONE*, 17(3):e0264540.

Bargar, T. M., Lett, R. M., Johnson, P. L., Hunter, J. E., Chang, C. P., Pernich, D. J., Sabol, M. R., and Dick, M. R. (1995). Toxicity of Pumiliotoxin 251D and Synthetic Analogs to the Cotton Pest Heliothis virescens. *Journal of Agricultural and Food Chemistry*, 43(4):1044–1051.

Bolton, S. K., Dickerson, K., and Saporito, R. A. (2017). Variable Alkaloid Defenses in the Dendrobatid Poison Frog Oophaga pumilio are Perceived as Differences in Palatability to Arthropods. *Journal of Chemical Ecology*, 43(3):273–289.

Brunetti, A. E., Lyra, M. L., Melo, W. G. P., Andrade, L. E., Palacios-Rodríguez, P., Prado, B. M., Haddad, C. F. B., Pupo, M. T., and Lopes, N. P. (2019). Symbiotic skin bacteria as a source for sex-specific scents in frogs. *Proceedings of the National Academy of Sciences*, 116(6):2124–2129.

Caty, S. N., Alvarez-Buylla, A., Byrd, G. D., Vidoudez, C., Roland, A. B., Tapia, E. E., Budnik, B., Trauger, S. A., Coloma, L. A., and O'Connell, L. A. (2019). Molecular physiology of chemical defenses in a poison frog. *The Journal of Experimental Biology*, 222(12):jeb204149.

Cimino, G. and Ghiselin, M. T. (1998). Chemical defense and evolution in the Sacoglossa (Mollusca: Gastropoda: Opisthobranchia):. *Chemoecology*, 8(2):51–60.

Clark, V. C., Raxworthy, C. J., Rakotomalala, V., Sierwald, P., and Fisher, B. L. (2005). Convergent evolution of chemical defense in poison frogs and arthropod prey between Madagascar and the Neotropics. *Proceedings of the National Academy of Sciences*, 102(33):11617–11622.

Daly, J. W. (1995). The chemistry of poisons in amphibian skin. *Proceedings of the National Academy of Sciences*, 92(1):9–13.

Daly, J. W. (1998). Thirty Years of Discovering Arthropod Alkaloids in Amphibian Skin †. *Journal of Natural Products*, 61(1):162–172.

Daly, J. W., Garraffo, H. M., Spande, T. F., Clark, V. C., Ma, J., Ziffer, H., and Cover, J. F. (2003). Evidence for an enantioselective pumiliotoxin 7-hydroxylase in dendrobatid poison frogs of the genus Dendrobates. *Proceedings of the National Academy of Sciences*, 100(19):11092–11097.

Daly, J. W., Gusovsky, F., McNeal, E. T., Secunda, S., Bell, M., Creveling, C. R., Nishizawa, Y., Overman, L. E., Sharp, M. J., and Rossignol, D. P. (1990). Pumiliotoxin alkaloids: A new class of sodium channel agents. *Biochemical Pharmacology*, 40(2):315–326.

Daly, J. W., Gusovsky, F., Myers, C. W., Yotsu-Yamashita, M., and Yasumoto, T. (1994). First occurrence of tetrodotoxin in a dendrobatid frog (Colostethus inguinalis), with further reports for the bufonid genus Atelopus. *Toxicon*, 32(3):279–285.

Daly, J. W., Highet, R. J., and Myers, C. W. (1984). Occurrence of skin alkaloids in non-dendrobatid frogs from Brazil (Bufonidae), Australia (Myobatrachidae) and Madagascar (Mantellinae). *Toxicon*, 22(6):905–919.

Daly, J. W., Kaneko, T., Wilham, J., Garraffo, H. M., Spande, T. F., Espinosa, A., and Donnelly, M. A. (2002). Bioactive alkaloids of frog skin: Combinatorial bioprospecting reveals that pumiliotoxins have an arthropod source. *Proceedings of the National Academy of Sciences*, 99(22):13996–14001.

Daly, J. W., McNeal, E., Gusovsky, F., Ito, F., and Overman, L. E. (1988). Pumiliotoxin alkaloids: Relationship of cardiotonic activity to sodium channel activity and phosphatidylinositol turnover. *Journal of Medicinal Chemistry*, 31:477–480.

Daly, J. W., McNeal, E. T., Overman, L. E., and Ellison, D. H. (1985). A new class of cardiotonic agents: Structure-activity correlations for natural and synthetic analogs of the alkaloid pumiliotoxin B (8-hydroxy-8-methyl-6-alkylidene-1-azabicyclo[4.3.0]nonanes). *Journal of Medicinal Chemistry*, 28(4):482–486.

Daly, J. W. and Myers, C. W. (1967). Toxicity of Panamanian Poison Frogs (Dendrobates): Some Biological and Chemical Aspects. *Science*, 156(3777):970–973.

Daly, J. W., Padgett, W. L., Saunders, R. L., and Cover, J. F. (1997). Absence of tetrodotoxins in a captive-raised riparian frog, Atelopus varius. *Toxicon*, 35(5):705–709.

Daly, J. W., Secunda, S. I., Garraffo, H., Spande, T. F., Wisnieski, A., Nishihira, C., and Cover, J. F. (1992). Variability in alkaloid profiles in neotropical poison frogs (Dendrobatidae): Genetic versus environmental determinants. *Toxicon*, 30(8):887–898.

Daly, J. W., Ware, N., Saporito, R. A., Spande, T. F., and Garraffo, H. M. (2009). *N* -Methyldecahydroquinolines: An Unexpected Class of Alkaloids from Amazonian Poison Frogs (Dendrobatidae). *Journal of Natural Products*, 72(6):1110–1114.

Daly, J. W., Wilham, J. M., Spande, T. F., Garraffo, H. M., Gil, R. R., Silva, G. L., and Vaira, M. (2007). Alkaloids in Bufonid Toads (Melanophryniscus): Temporal and Geographic Determinants for Two Argentinian Species. *Journal of Chemical Ecology*, 33(4):871–887.

Dobler, S. (2001). Evolutionary aspects of defense by recycled plant compounds in herbivorous insects. *Basic and Applied Ecology*, 2(1):15–26.

Dumbacher, J. P., Menon, G. K., and Daly, J. W. (2009). Skin as a Toxin Storage Organ in the Endemic New Guinean Genus *Pitohui*. *The Auk*, 126(3):520–530.

Frost, D. R. (2023). Amphibian Species of the World: An Online Reference.

Garraffo, H. M., Caceres, J., Daly, J. W., Spande, T. F., Andriamaharavo, N. R., and Andriantsiferana, M. (1993). Alkaloids in

Madagascan Frogs (Mantella): Pumiliotoxins, Indolizidines, Quinolizidines, and Pyrrolizidines. *Journal of Natural Products*, 56(7):1016–1038.

Grant, T., Frost, D. R., Caldwell, J. P., Gagliardo, R., Haddad, C. F., Kok, P. J., Means, D. B., Noonan, B. P., Schargel, W. E., and Wheeler, W. C. (2006). Phylogenetic Systematics of Dart-poison frogs and their relatives (Amphibia: Athesphatanura: Dendrobatidae. *Bulletin of the American Museum of Natural History*, 299:1–262.

Grant, T., Rada, M., Anganoy-Criollo, M., Batista, A., Dias, P. H., Jeckel, A. M., Machado, D. J., and Rueda-Almonacid, J. V. (2017). Phylogenetic Systematics of Dart-Poison Frogs and Their Relatives Revisited (Anura: Dendrobatoidea). *South American Journal of Herpetology*, 12(s1):S1–S90.

Gusovsky, F., Padgett, W. L., Creveling, C., and Daly, J. W. (1992). Interaction of pumiliotoxin B with an "alkaloid-binding domain" on the voltage-dependent sodium channel. *Molecular Pharmacology*, 42(6).

Gusovsky, F., Rossignol, D. P., McNeal, E. T., and Daly, J. W. (1988). Pumiliotoxin B binds to a site on the voltage-dependent sodium channel that is allosterically coupled to other binding sites. *Proceedings of the National Academy of Sciences*, 85(4):1272–1276.

Hantak, M. M., Paluh, D. J., and Saporito, R. A. (2016). Bufadienolide and alkaloid-based chemical defences in two different species of neotropical anurans are equally effective against the same arthropod predators. *Journal of Tropical Ecology*, 32(2):165–169.

Hartmann, T., Theuring, C., Schmidt, J., Rahier, M., and Pasteels, J. M. (1999). Biochemical strategy of sequestration of pyrrolizidine alkaloids by adults and larvae of chrysomelid leaf beetles. *Journal of Insect Physiology*, 45(12):1085–1095.

Heckel, D. G. (2018). Insect Detoxification and Sequestration Strategies. In Roberts, J. A., editor, *Annual Plant Reviews Online*, pages 77–114. John Wiley & Sons, Ltd, Chichester, UK.

Hovey, K. J. (2018). Sequestered Alkaloid Defenses in the Dendrobatid Poison Frog Oophaga pumilio Provide Variable Protection from Microbial Pathogens. *J Chem Ecol*, page 14.

Hutchinson, D. A., Savitzky, A. H., Burghardt, G. M., Nguyen, C., Meinwald, J., Schroeder, F. C., and Mori, A. (2013). Chemical defense of an A sian snake reflects local availability of toxic prey and hatchling diet. *Journal of Zoology*, 289(4):270–278.

Jeckel, A. M., Bolton, S. K., Waters, K. R., Antoniazzi, M. M., Jared, C., Matsumura, K., Nishikawa, K., Morimoto, Y., Grant, T., and Saporito, R. A. (2022). Dose-dependent alkaloid sequestration and *N* -methylation of decahydroquinoline in poison frogs. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 337(5):537–546.

Jeckel, A. M., Grant, T., and Saporito, R. A. (2015). Sequestered and Synthesized Chemical Defenses in the Poison Frog Melanophryniscus moreirae. *Journal of Chemical Ecology*, 41(5):505–512.

Jeckel, A. M., Kocheff, S., Saporito, R. A., and Grant, T. (2019). Geographically separated orange and blue populations of the Amazonian poison frog Adelphobates galactonotus (Anura, Dendrobatidae) do not differ in alkaloid composition or palatability. *Chemoecology*, 29(5-6):225–234.

Löfqvist, J. and Lofqvist, J. (1977). Toxic Properties of the Chemical Defence Systems in the Competitive Ants Formica rufa and F. sanguinea. *Oikos*, 28(1):137.

Macfoy, C., Danosus, D., Sandit, R., Jones, T. H., Garraffo, H. M., Spande, T. F., and Daly, J. W. (2005). Alkaloids of Anuran Skin: Antimicrobial Function? *Zeitschrift für Naturforschung C*, 60(11-12):932–937.

Malcolm, S. B. and Brower, L. P. (1989). Evolutionary and ecological implications of cardenolide sequestration in the monarch butterfly. *Experientia*, 45(3):284–295.

Mebs, D. (2001). Toxicity in animals. Trends in evolution? *Toxicon*, 39(1):87–96.

Mebs, D., Pogoda, W., Batista, A., Ponce, M., Köhler, G., and Kauert, G. (2008). Variability of alkaloid profiles in Oophaga pumilio (Amphibia: Anura: Dendrobatidae) from western Panama and southern Nicaragua. *Salamandra*, 44(4):7.

Mebs, D., Pogoda, W., Maneyro, R., and Kwet, A. (2005). Studies on the poisonous skin secretion of individual red bellied toads, Melanophryniscus montevidensis (Anura, Bufonidae), from Uruguay. *Toxicon*, 46(6):641–650.

Michael, J. P. (2016). Simple Indolizidine and Quinolizidine Alkaloids. In *The Alkaloids: Chemistry and Biology*, volume 75, pages 1–498. Elsevier.

Myers, C. W. (1978). A dangerously toxic new Frog (Phyllobates) used by Embera Indians of Western Colombia, with Discussion of Blowgun Fabrication and Dart Poisoning. *Bulletin of the American Museum of Natural History*, 161:72.

Myers, C. W., Daly, J. W., Garraffo, H. M., Wisnieski, A., and Cover, J. F. (1995). Discovery of the Costa Rican Poison Frog Dendrobates granuliferus in Sympatry with Dendrobates pumilio, and Comments on Taxonomic Use of Skin Alkaloids. *American Museum Novitates*, 3144:24.

Myers, C. W., Paolillo, A., and Daly, J. W. (1991). Discovery of a Defensively Malodorous and Nocturnal Frog in the Family Dendrobatidae: Phylogenetic Significance of a New Genus and Species from the Venezuelan Andes. *American Museum Novitates*, 3002:36.

Naumann, C., Hartmann, T., and Ober, D. (2002). Evolutionary recruitment of a flavin-dependent monooxygenase for the detoxification of host plant-acquired pyrrolizidine alkaloids in the alkaloid-defended arctiid moth Tyria jacobaeae. *Proceedings of the National Academy of Sciences*, 99(9):6085–6090.

O'Connell, L. A., LS50: Integrated Science Laboratory Course, O'Connell, J. D., Paulo, J. A., Trauger, S. A., Gygi, S. P., and Murray, A. W. (2020). Rapid toxin sequestration modifies poison frog physiology. Preprint, Physiology.

Opitz, S. E. W. and Müller, C. (2009). Plant chemistry and insect sequestration. *Chemoecology*, 19(3):117–154.

Rivas, L., Luque-Ortega, J. R., and Andreu, D. (2009). Amphibian antimicrobial peptides and Protozoa: Lessons from parasites. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1788(8):1570–1581.

Rodríguez, A., Poth, D., Schulz, S., and Vences, M. (2011). Discovery of skin alkaloids in a miniaturized eleutherodactylid frog from Cuba. *Biology Letters*, 7(3):414–418.

Salgado Costa, C., Bahl, F., Natale, G., Mac Loughlin, T., Marino, D., Venturino, A., Rodriguez-Mozaz, S., and Santos, L. (2023). First evidence of environmental bioaccumulation of pharmaceuticals on adult native anurans (Rhinella arenarum) from Argentina. *Environmental Pollution*, 334:122231.

Sanchez, E., Rodríguez, A., Grau, J. H., Lötters, S., Künzel, S., Saporito, R. A., Ringler, E., Schulz, S., Wollenberg Valero, K. C., and Vences, M. (2019). Transcriptomic Signatures of Experimental Alkaloid Consumption in a Poison Frog. *Genes*, 10(10):733.

Santos, J. C., Coloma, L. A., and Cannatella, D. C. (2003). Multiple, recurring origins of aposematism and diet specialization in poison frogs. *Proceedings of the National Academy of Sciences*, 100(22):12792–12797.

Santos, J. C., Tarvin, R. D., and O'Connell, L. A. (2016). A Review of Chemical Defense in Poison Frogs (Dendrobatidae): Ecology, Pharmacokinetics, and Autoresistance. In Schulte, B. A., Goodwin, T. E., and Ferkin, M. H., editors, *Chemical Signals in Vertebrates 13*, pages 305–337. Springer International Publishing, Cham.

Saporito, A. R., F. Spande, T., Martin Garraffo, H., and A. Donnelly, M. (2009). Arthropod Alkaloids in Poison Frogs: A Review of the 'Dietary Hypothesis'. *HETEROCYCLES*, 79(1):277.

Saporito, R. A., Donnelly, M. A., Garraffo, H. M., Spande, T. F., and Daly, J. W. (2006). Geographic and Seasonal Variation in Alkaloid-Based Chemical Defenses of Dendrobates pumilio from Bocas del Toro, Panama. *Journal of Chemical Ecology*, 32(4):795–814.

Saporito, R. A., Donnelly, M. A., Jain, P., Martin Garraffo, H., Spande, T. F., and Daly, J. W. (2007a). Spatial and temporal patterns of alkaloid variation in the poison frog Oophaga pumilio in Costa Rica and Panama over 30 years. *Toxicon*, 50(6):757–778.

Saporito, R. A., Donnelly, M. A., Madden, A. A., Garraffo, H. M., and Spande, T. F. (2010a). Sex-Related Differences in Alkaloid Chemical Defenses of the Dendrobatid Frog *Oophaga pumilio* from Cayo Nancy, Bocas del Toro, Panama $^{\perp}$. *Journal of Natural Products*, 73(3):317–321.

Saporito, R. A., Donnelly, M. A., Spande, T. F., and Garraffo, H. M. (2012). A review of chemical ecology in poison frogs. *Chemoecology*, 22(3):159–168.

Saporito, R. A., Garraffo, H. M., Donnelly, M. A., Edwards, A. L., Longino, J. T., and Daly, J. W. (2004). Formicine ants: An arthropod source for the pumiliotoxin alkaloids of dendrobatid poison frogs. *Proceedings of the National Academy of Sciences*, 101(21):8045–8050.

Saporito, R. A., Isola, M., Maccachero, V. C., Condon, K., and Donnelly, M. A. (2010b). Ontogenetic scaling of poison glands in a dendrobatid poison frog: Scaling of poison glands in a dendrobatid frog. *Journal of Zoology*, 282(4):238–245.

Saporito, R. A., Norton, R. A., Andriamaharavo, N. R., Garraffo, H. M., and Spande, T. F. (2011). Alkaloids in the Mite Scheloribates laevigatus: Further Alkaloids Common to Oribatid Mites and Poison Frogs. *Journal of Chemical Ecology*, 37(2):213–218.

Saporito, R. A., Zuercher, R., Roberts, M., Gerow, K. G., and Donnelly, M. A. (2007b). Experimental Evidence for Aposematism in the Dendrobatid Poison Frog Oophaga pumilio. *Copeia*, 2007(4):1006–1011.

Silverstone, P. A. (1975). A revision of the poison-arrow frogs of the genus Dendrobates Wagler.

Silverstone, P. A. (1976). A Revision of the Poison-Arrow Frogs of the Genus Phyllobates Bibron in Sagra (Family Ddndrobatidae). *Bulletin of the Natural History Museum of Los Angeles County*, 27:50.

Smith, B. P., Tyler, M. J., Kaneko, T., Garraffo, H. M., Spande, T. F., and Daly, J. W. (2002). Evidence for Biosynthesis of Pseudophrynamine Alkaloids by an Australian Myobatrachid Frog (*Pseudophryne*) and for Sequestration of Dietary Pumiliotoxins. *Journal of Natural Products*, 65(4):439–447.

Stynoski, J. L., Torres-Mendoza, Y., Sasa-Marin, M., and Saporito, R. A. (2014). Evidence of maternal provisioning of alkaloid-based chemical defenses in the strawberry poison frog *Oophaga pumilio*. *Ecology*, 95(3):587–593.

Takada, W., Sakata, T., Shimano, S., Enami, Y., Mori, N., Nishida, R., and Kuwahara, Y. (2005). Scheloribatid Mites as the Source of Pumiliotoxins in Dendrobatid Frogs†. *Journal of Chemical Ecology*, 31(10):2403–2415.

Tarvin, R. D., Santos, J. C., O'Connell, L. A., Zakon, H. H., and Cannatella, D. C. (2016). Convergent Substitutions in a Sodium Channel Suggest Multiple Origins of Toxin Resistance in Poison Frogs. *Molecular Biology and Evolution*, 33(4):1068–1080.

Vandendriessche, T., Abdel-Mottaleb, Y., Maertens, C., Cuypers, E., Sudau, A., Nubbemeyer, U., Mebs, D., and Tytgat, J. (2008). Modulation of voltage-gated Na+ and K+ channels by pumiliotoxin 251D: A "joint venture" alkaloid from arthropods and amphibians. *Toxicon*, 51(3):334–344.

Vences, M. (2003). Convergent evolution of aposematic coloration in Neotropical poison frogs: A molecular phylogenetic perspective. *Organisms Diversity & Evolution*, 3(3):215–226.

Wang, S.-Y. and Wang, G. K. (1999). Batrachotoxin-Resistant Na+ Channels Derived from Point Mutations in Transmembrane Segment D4-S6. *Biophysical Journal*, 76(6):3141–3149.

Webster, N. S. and Taylor, M. W. (2012). Marine sponges and their microbial symbionts: Love and other relationships. *Environmental Microbiology*, 14(2):335–346.

Weldon, P. J., Cardoza, Y. J., Vander Meer, R. K., Hoffmann, W. C., Daly, J. W., and Spande, T. F. (2013). Contact toxicities of anuran skin alkaloids against the fire ant (Solenopsis invicta). *Naturwissenschaften*, 100(2):185–192.

Weldon, P. J., Kramer, M., Gordon, S., Spande, T. F., and Daly, J. W. (2006). A common pumiliotoxin from poison frogs exhibits enantioselective toxicity against mosquitoes. *Proceedings of the National Academy of Sciences*, 103(47):17818–17821.

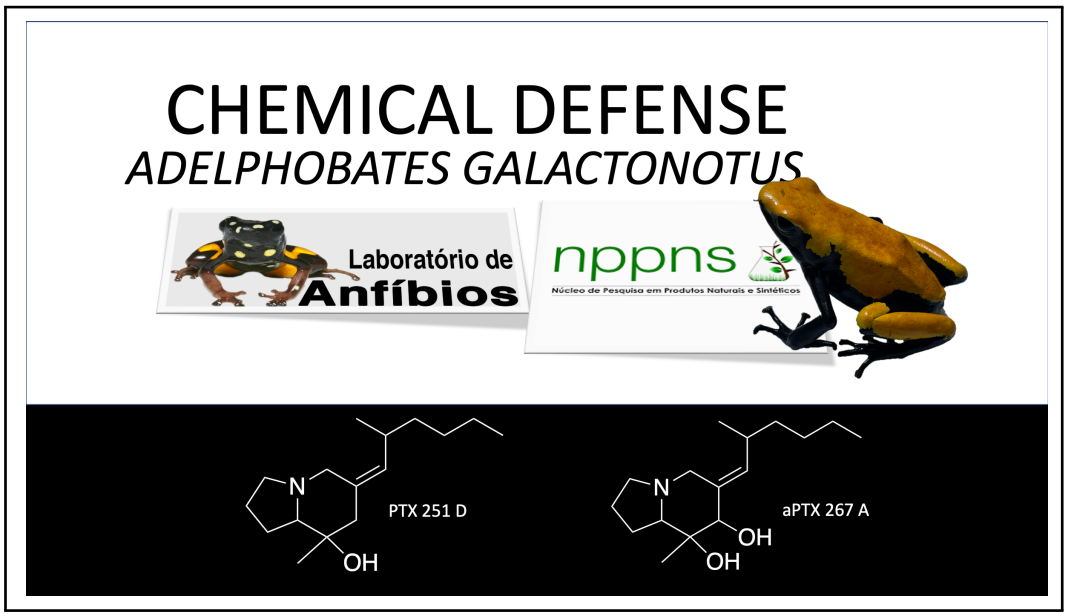
Williams, B. L., Hanifin, C. T., Brodie, E. D., and Brodie, E. D. (2012). Predators usurp prey defenses? Toxicokinetics of tetrodotoxin in common garter snakes after consumption of rough-skinned newts. *Chemoecology*, 22(3):179–185.

Williams, E. A., Welty, S. E., Geske, R. S., Hubble, C. L., Craig, D. M., Quick, G., Meliones, J. N., and Cheifetz, I. M. (2001). Liquid lung ventilation reduces neutrophil sequestration in a neonatal swine model of cardiopulmonary bypass:. *Critical Care Medicine*, 29(4):789–795.

Zvereva, E. L. and Kozlov, M. V. (2015). The costs and effectiveness of chemical defenses in herbivorous insects: A meta-analysis. *Ecological Monographs*, pages 15–0911.1.

Chapter 1

Navigating the Complex Terrain of Alkaloid Biochemistry in Poison Frogs: Methodologies, Challenges, and Insights



1.1 Introduction

The exploration of alkaloid biochemistry within the Dendrobatidae family and broader poison frog species presents a spans multiple interdisciplinary domains within ecology (Saporito et al., 2012; Santos et al., 2016), chemistry (Michael, 2016), and advanced biochemistry (Caty et al., 2019). Driven by their vast array of defensive alkaloids and toxins, research aims to unravel the complexities of alkaloid sequestration (Mebs et al., 2010; Hantak et al., 2013), origin (Takada et al., 2005; Saporito et al., 2004, 2006, 2007; Clark et al., 2005), the chemical composition of skin and internal organs (Rodríguez et al., 2011; Garraffo et al., 2012), metabolic pathways (Alvarez-Buylla et al., 2022), and the protein expression changes stemming from genetic mutations (Sanchez et al., 2019; Posso-Terranova and Andrés, 2020; Roelants et al., 2010). Recent years have seen a marked increase in the scope and creativity of poison frog alkaloid research, attracting notable academic attention as demonstrated by the contributions of Brooks et al. (2023), Jeckel et al. (2022), and Okada et al. (2021). This upswing is characterized by advancements in novel research techniques as described by Krieger et al. (2022) and earlier foundational efforts by Jeckel et al. (2020), moving beyond the traditional scope of gas chromatography-mass spectrometry (GC-MS) analysis (Daly et al., 2008). Innovating research methods is crucial to deepen our comprehension in alkaloid analysis, striving for time and cost efficiency. This chapter presents a thorough overview of methodologies pursued in this master's thesis, focusing specifically on the process of the hydroxylation of PTX 251D to aPTX 267A, contributing to our understanding of alkaloid sequestration and metabolism. While some methods have not been integrated into our final research protocols, their assessment was vital in shaping the direction of our study. As we progress through this chapter, we will detail the care of *Adelphobates galactonotus* and terrarium maintenance, progressing to the applied methodologies in analytical techniques. Our exploration will extend knowledge of gas chromatography and liquid chromatography mass spectrometry (GC-MS, LC-MS), as well as matrix-assisted laser desorption/ionization (MALDI), thereby broadening the analytical perspective, through which these bioactive compounds can be studied. This chapter is not just an exposition of applied methodologies but a narrative of methodological evolution through this thesis. The journey through these methodologies has been marked by challenges, both foreseen and unforeseen, but each has been a stepping stone towards methodological refinement, enhanced accuracy, and, ultimately, a deeper understanding of the possibilities of analyzing poison frog alkaloids. This chapter aims to present various investigative approaches, sharing both negative and positive findings that may not have been further pursued, and providing reflections that may light the path for future explorations in this field.

1.2 Applied Methodologies and Experimental Aproaches

1.2.1 Terrarium Maintance and Animal Husbandary

General Husbandary of Insects

Refined Cultivation Techniques for *Drosophila Melanogaster* The fruit flies, *Drosophila melanogaster*, obtained from Carlos Ribeiro Vilela, Laboratório de Drosofilídeos, IBUSP, were bred in 300 mL glass

jars, containing a portion of culture media, and with sponge lids (Fig. 1.1), to facilitate air circulation. The base mixture for the media was carefully formulated to yield 20 jars. The base mixture consisted of 240g of instant potato puree powder (Purê de Batatas Instantâneo, Aro), 15g of powdered cinnamon (Paita Alimentos) which acts as a fungicide, three cups of instant dry yeast (Fleischmann), and 4.5 espresso mugs of sugar. To prepare each portion, a blend of two large spoons of the base mixture was combined with 30 mL of water and 0.5 mL of rice vinegar (Castelo). The vinegar confers a lower pH to the mixture, functioning as a disinfectant. Upon stirring, the media should form a smooth, light brown paste with a characteristic smell. Any significant change in the scent or color can indicate over-acidification or inconsistency in the paste's homogeneity. After placing the culture media inside the jars, these were filled with thin wood shavings (Fig. 1.1), effectively increasing the surface area for the *Drosophila* breeding environment. Initially we used another method for increasing the surface area: a folded filter paper, vertically inserted into the jar. This version was not efficient, thus we started using wood shavings, which showed a greater contact surface area and durability, maintaining its integrity even five weeks after its initial use, within one or two weeks under high humidity conditions, in the terrarium, with minimal moisture absorption. In contrast, the filter paper construction degraded within a few days, and further offered a smaller surface area, limiting the movement space for the flies.

Figure 1.1: The image on the left shows a glass jar setup, while the image on the right shows a laboratory setup for cultivating *Drosophila melanogaster*. Glass jars filled with culture media and thin wood shavings line the shelves and workbench, each sealed with sponge lids to ensure proper air circulation. This setup is part of a controlled breeding process, with the jars neatly arranged to facilitate easy maintenance and observation. The inclusion of wood shavings represents an innovative approach to increasing the surface area for breeding, showcasing a practical aspect of experimental husbandry in a research environment.

Approaches to Parasite Control and Fungal Prevention in *Drosophila* Breeding To initiate the breeding process, around 20 to 30 adult fruit flies were transferred from an 'old' glass to a new one for reproduction. However, throughout our study, we encountered challenges regarding larvae parasites, leading to jars filled with unhatched cocoons and jeopardizing the survival of the *Drosophila* colony. We detected that during the fly transfer process, we were inadvertently also transferring parasites to the new jar. To mitigate this, we modified our transfer technique. The new methodology involved placing the container with adult flies inside a plastic tub. Only the flies that voluntarily left the container were collected and transferred to a new jar, leaving the parasites in the old one. From one source container, we created four to five new ones. This method helped to prevent the loss of an entire fly generation, in case a jar contained weak flies.

Additionally, we faced problems with fungal growth inside the flies glasses. To prevent fungal growth, the lids were soaked in industrial Javel water (a mixture of sodium hypochlorite and sodium chloride) for at least 24 hours. Afterward, the lids were thoroughly washed, dried, and reused. The glasses were also disinfected with ethanol after each wash and then left to dry. Moreover, direct maintenance of the fly hatchery was conducted to ensure the specimens met adequate quality standards. Fungal growth was also observed when the liquid-to-solid ratio in the breeding mixture was incorrect, often due to inadequate measurements during the procedure.

Effective Contingency Strategies for *Drosophila* Population Management A contingency plan was developed to prevent fly scarcity in case of a *Drosophila* population loss. This strategy allowed us to manage fly production during unforeseen events and ensured a steady supply for the frogs, even in situations of sudden fly population loss. As illustrated in Fig. 1.2 we followed a specific schedule: a new batch of flies (indicated in blue) was placed inside the terrarium and replaced with a fresh batch (in red) after one week. The former blue batch was then placed in a stockpile of glasses, providing additional, vitamin-mixed food for the frogs twice a week. In case of fly population loss, we extended the terrarium stay of the current batch for an additional week and used the stockpile to establish a new colony. Typically, only flies that were never exposed to the terrarium environment were used for breeding to prevent the emergence of winged flies. These native winged flies can lay eggs in the glasses and contaminate the populations, that why they are excluded from reproduction after being in the terrarium. With a weekly growing stockpile, we maintained a constant reserve of fruit flies. Usually, a fly production cycle takes about 12 days to complete, which allowed us to manage up to two weeks without a functioning breeding process, sufficient time to resolve problems or obtain flies from external sources.

Optimal Practices for Collembolas in Amphibian Husbandry Collembolas were maintained in 20 cm x 10 cm x 10 cm plastic containers, which were furnished with water and a thick layer of charcoal. As a food source, we sprinkled beer yeast twice a week over the containers. Collembolas were used in terrarium maintenance for *A. galactonotus* husbandry. According to T. Grant (personal comment), these additions did not interfere with the experiments, as no alkaloids were detected in these animals.

Habitat and Care for Isopods in Terrarium Ecosystems Isopods were housed in 30 cm x 40 cm x 10 cm plastic containers, which were ventilated with netted lids. The substrate within these containers was composed of various natural materials from outdoor settings, including wood sticks, leaves, and organic waste such as salad leaves and banana peels. This mix created an environment that closely mimicked their natural habitat. To maintain optimal conditions for the isopods, we ensured the substrate was kept more moist than dry, providing a suitable environment for their growth and wellbeing. Isopods were also used for maintenance of terrariums, as described previously for the Collembolas.

General Husbandary of *Adelphobates galactonotus*

Innovative Terrarium Design for Optimal *Adelphobates galactonotus* Habitat The initial setup of the Adelphobates terrarium included a base layer of small stones (1 to 2 cm), topped with a thin layer of *Sphag-*

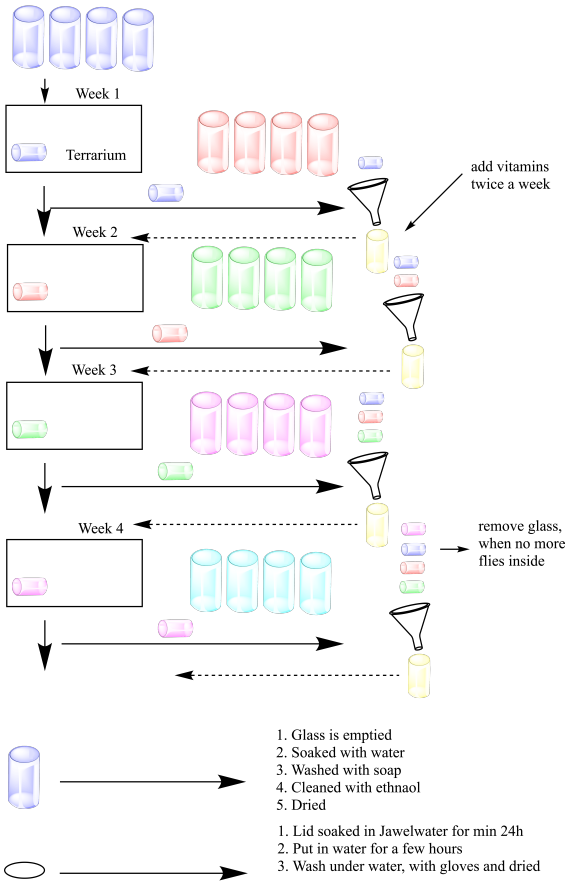


Figure 1.2: The chart visualizes a systematic breeding cycle for *Drosophila melanogaster*, where each color represents a successive generation of fruit flies. On the right side, jars from terrariums accumulate, being repurposed as a reserve. The bottom portion details the cleaning and preparation stages for the jars, ensuring their readiness for future breeding cycles.

num moss, a water bowl, branches with epiphytes, plastic hides, and a dish for daily fly feeding. However, maintaining consistent humidity was challenging due to the room's partial exposure to external weather conditions. During the summer season, the terrariums dried out quickly despite daily water spraying, creating an environment too arid for the frogs. Contrarily, excessive humidity led to overly wet *Sphagnum*, creating swamp-like conditions and potentially causing respiratory infections in frogs, as indicated by their slow movements and trembling. To overcome these problems, we consulted Rafael C. Benetti, a technician from the Institute of Butantan, who provided essential guidance on resolving these habitat challenges. The improved terrarium design (Fig. 1.3) consisted of five additional layers for optimal habitat conditions. The bottom layer, consisted of 3-4 cm of hydroponic beads, designed to trap condensed water while keeping the upper layers dry. On top of the beads, we placed a 1-2 cm layer of pine bark to create as a barrier for the third layer, consisting of 3-4 cm of *Sphagnum* moss, which maintained high humidity. Further the fourth layer consisted of dried leaves to prevent direct contact between the frogs and the wet substrates. These layers made the terrariums lighter and efficient to manage water levels. The top layer included branches, plastic shelters, and a small water pool. This new structure led to more active frogs and reduced maintenance, as it required only 2-3 water sprayings per week. The wood and leaves, sourced from the campus forest, underwent a quarantine period before use to prevent insect infestation, particularly ants. Collembolas and isopods were periodically introduced for terrarium maintenance, serving as decomposers and an alternative

food source for the frogs when fly populations were low. . Additional environmental control measures included large water reservoirs and open aquariums to maintain humidity in the room, and heaters to ensure a minimum temperature of 24°C in winter. During the hot season, room temperature could reach up to 30°C, and the water reservoirs helped to mitigate air dryness. Funding from the Pro-Reitoria de Pesquisa e Inovação has been secured to further renovate the live frog facilities, which will solve many challenges faced in the recent years. The construction is complete, but the transfer of the frogs is pending.

Figure 1.3: Terrarium layer structure from base to top: The foundation consists of 3-4 cm hydroponic beads for moisture control, overlaid by 1-2 cm of pine bark acting as a barrier. Above this is a 3-4 cm layer of *Sphagnum* moss for humidity retention, topped with dried leaves to separate the frogs from wet substrates. The final layer features branches, plastic shelters, and a small water pool, completing the terrarium ecosystem.

Comprehensive Care Strategies for *Adelphobates galactonotus* Colonies Our research group, as per Jeckel (Jeckel et al., 2020), has established a colony of over 40 terrariums each housing one to three *A. galactonotus* individuals. To streamline care and create a more natural environment, five to four specimens, of a similar age, were grouped per terrarium to optimize space, simplify care, and recreate a natural environment. Additionally, an aquarium was maintained for tadpoles, consisting of plastic containers designed with mesh floors to prevent cannibalism during their early developmental stages. Feeding frequency was adjusted to ensure consistency and mimic the natural feeding patterns of the frogs. Initially, frogs were fed daily with a limited quantity of vitamin-mixed fruit flies, from Monday to Friday. This proved suboptimal due to inconsistent *Drosophila* availability. Thus feeding methodology was optimized, as previously described in section 1.2.1. The new methodology closely resembled the feeding habits of *A. galactonotus* in nature and allowed fly reproduction within the terrarium, as natural cause. Additionally, one to two thin slices of bananas were added to the terrariums to attract winged fruit flies, providing an extra food source and encouraging the flies to remain within the terrarium, reducing its loss through the mesh-covered top. Furthermore, an additional portion with vitamin-dusted flies was added semiweekly to maintain food supply and vitamin uptake.

1.2.2 Analyzing Digestion through Dye Tracking

As part of an exploratory experiment, we planned to investigate the metabolic rate of frogs, by observing the transit time of food through their digestive tract. Preliminary tests involved coloring fruit flies with food dye and feeding them to the frogs, anticipating colored fecal matter, which would indicate passage time. However, due to time constraints, challenges with fly populations, as mentioned above, this study was temporary put aside. The intended procedure was as follows: fruit flies would be dyed blue, prior to feeding them to the hyperlapse footage would be hourly captured using a GoPro Hero 9, to accurately determine a precise digestion timeframe. For the tests, we used Brilliant Blue FCF (CAS - 3844-45-9) and Coomassie Brilliant Blue (CAS - 6104-59-2), alongside Blue Curaçao syrup containing 0.01% FD&C Blue #1 (E133), and a pure food coloring FD&C Blue #1 (E133). We observed that the flies turned blue within minutes, after being fed with 3, to 4 drops of food color in sugar water, and well visibly blue within an hour after consuming it. The syrup proved inefficient due to its viscosity and low dye concentration, leading to fungal growth and fly mortality when exposed for a week. Post-feeding, blue flies, the frogs consistently produced blue or green-tinted feces, validating the methodology. As a pretest, some dyed flies were mashed, filtered through a coffee filter and rinsed with pure water, an experiment designed to know, that how well the color is visible in a fly /ethanol suspension. The liquid, initially yellow from undyed, mashed flies, changed to a greenish-blue tint upon addition of the dye. Similar to the dyed frog feces suspended in 70% ethanol produced a green mixture that deepened to blue during prolonged extraction. These preliminary results (Fig. 1.4) contribute to the foundational knowledge necessary for advancing our understanding of amphibian metabolic processes and highlights challenges faced in research with living animals.

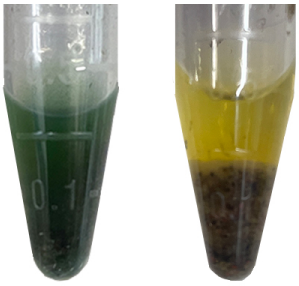


Figure 1.4: The left vial contains the greenish-blue suspension resulting from dyed fly feces mixed with 70% ethanol, showcasing the dye's integration into the digestive system. The right vial displays the baseline mixture of undyed, mashed flies, prior to the addition of any colorant.

1.2.3 Investigating the Hydroxylation Capacity of Fecal Microbiota on PTX 251D

We explored the potential biotransformation capabilities of the frog's gut microbiota in an experimental study by introducing PTX 251D into the medium of control frog fecal samples. We collected eight samples of excrement from frogs in our laboratory that were not part of the experiment and therefore had never consumed any type of toxin. The samples were collected as sterilely as possible after being freshly deposited in the terrarium and split into two sterile vials. One was filled up with a, non gastrointestinental bacteria

specific buffer. The other one was transported within 24h to the lab in Ribeirão Preto and immediately immersed in a sodium phophate buffer (50mL contained 231.7mg NaH_2PO_4 and 435.8mg Na_2HPO_4).We used two approaches to increase the chance of having surviving bacteria in this first trail. We prepared a solution of 98μg PTX 251D in 20μL 50% ethanol and split the solution into the two vials. After 24 hours, both mediums tested positive for aPTX, indicating that the bacteria present in *Adelphobates galactonotus* likely possess peroxidase-like activity, which enables the conversion of PTX 251D to aPTX 267A. However, the taxonomic identities and biochemical roles of symbiotic microbes living within the guts of amphibians remain poorly studied to date (Pryor, 2008; Tong et al., 2020). This finding suggests hydroxylation occurring not only in the host metabolism but also involves some microbial activity taking part in the detoxification and processing of alkaloids in these frogs (An et al., 2017; Hammer and Bowers, 2015; Manson and Thomson, 2009) . Future research may delve into the enzymatic processes and possible symbiotic interactions that enable the hydroxylation of PTX 251D within the digestive system, conducting these investigations within the framework of a well-structured experimental setup.

1.2.4 Evaluating and Refining Analytical Methodologies for Alkaloids Detection in *A. galactonotus*

Gas Chromatography-Mass Spectromety (GC-MS)

To determine the distribution and quantify PTX 251D and aPTX 267A in each frog, and their individual organs, our initial approach was to use Gas Chromatography coupled with Mass Spectrometry (GC-MS) at Dr. Noberto Peporine Lopes's laboratory in the Núcleo de Pesquisa em Produtos Naturais e Sintéticos, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo (Ribeirão Preto). The GC-MS and sample extraction method for poison frog alkaloids, was first developed by Daly et al. (1994), enhanced by Saporito et al. (2010) and established in subsequent work by Jeckel et al. (2015) and modified by McGugan et al. (2016). Although this method allows comparison of retention times across various studies, as reported in Daly et al. (2005), however, its limitation lies in detecting highly hydroxylated and non-volatile molecules. This methodology presents challenges in reproducibility, particularly in quantification and sample preparation, which are subsequently discussed. During the extraction process, there is an inevitable loss of alkaloids, in nanograms, at each vial transfer or pipetting step, demanding a more direct pathway from frog organs to the analytical tool. The final drying of the extract under nitrogen flow adds another layer of inconsistency, as most laboratories have a different set up and do not measure or publish the flow rate of nitrogen (Daly et al., 1994; Saporito et al., 2010; Jeckel et al., 2015, 2022; Caty et al., 2019; Moskowitz et al., 2018; McGugan et al., 2016; Garraffo et al., 2012). The lack of standardization during the nitrogen drying process, combined with the potential loss of alkaloids, presents a challenge regarding alkaloid quantification. Additionally, sublimation rates and the volatility of the alkaloids are not typically accounted for in these procedures. Attaining precision in measurements at the microscale, dealing with quantities like microliters and nanograms, presents considerable difficulties due to the inherent variability and sensitivity of such small quantities. The limited availability and high cost of commercially synthesized alkaloid standards present significant obstacles to precise quantification. The consequent absence of pure standards

turns quantification in a formidable challenge, or impossible. The common method relies on nicotine or decahydroquinoline as an internal standard and back-calculates its concentrations in relation to the studied alkaloid. However, this approach, offers more of an estimation rather than an exact quantity (Gonzalez and Carazzone, 2023). Accurate quantification can only be achieved with an internal calibration curve, yet, this crucial methodological detail is not consistently reported in the literature, as seen in works by Saporito et al. (2006, 2010, 2011) and Jeckel et al. (2015, 2022), McGugan et al. (2016), Garraffo et al. (2012). Recently, the review "Eco-Metabolomics Applied to the Chemical Ecology of Poison Frogs (Dendrobatoidea)" by Gonzalez and Carazzone (2023) first emphasized and discussed this critical point. Given these concerns we chose to establish a novel methodology, different from the traditional ones. We simply conducted GC-MS tests by using these traditional methods to verify sample quality and explored alternative internal standards, given the unavailability in the laboratory and high toxicity of the commonly used nicotine. Since we were working with an isolated form of a specific alkaloid, PTX (+)-251D, we could inject it directly into a mass spectrometer and determine its retention times, independently of an existing dataset, which was confirmed by the obtained spectra detailed in (Minder et al., 2023). Unfortunately, impurities identified in the PTX (+)-251D supply precluded its use for creating a calibration curve, and precise quantification, casting doubt on whether these contaminants were the result of degradation or byproducts of synthesis. With attempt to optimize methodologies and minimize sample loss, we explored several extraction and sample preparation methods beyond the standard acid-base extraction (Saporito et al., 2010). We used methanol extracts from carcasses of *A. galactonotus*, from the main study, as source for PTX(+)-251D and aPTX(+)-267A. These samples were not included in our main experiments, but they provided a rich matrix containing fatty acids and proteins, valuable for method development. We had 3 mL of methanol based extract per frog carcass to our disposal, totaling 36 mL across all samples, each rich in both alkaloids. In the process of extraction and purification of carcass solutions, we employed a variety of non-aqueous solvents to constitute the organic phase. These included Dichloromethane (DCM), tert-Butyl Methyl Ether (MTBE), and Butyl Acetate (Bu-O-AC). These solvents were utilized in conjunction with a saturated solution of Sodium Chloride (NaCl) acting as the aqueous phase, facilitating a more efficient biphasic separation and enhanced clarification of the extracts. Additionally, we trialed hexane to clarify our samples by 3 x μL Hexane extraction and 200 μL 2% NaCl solution per 1mL sample. This technique successfully facilitated the targeted transfer of PTX (+)-251D into the first two hexane fraction and aPTX in the third; however, it concurrently extracted fatty acids and other lipophilic substances, which led to complex chromatograms characterized by overlapping peaks, thereby complicating the spectral analysis. To remedy the issue of co-extraction, we meticulously optimized the extraction matrix and the respective volumes of each component. Through extensive experimentation, it was determined that a mixture comprising 1 mL of methanol extract, 200 μL of Milli-Q water, 400 μL of saturated NaCl solution, and 300 μL Dichloromethane (DCM) provided the most efficient phase separation. This particular ratio resulted in three clearly defined DCM fractions amenable to subsequent analytical evaluation, outperforming other tested ratios which exhibited slower or incomplete phase separation. Simultaneously, we implemented Sephadex-LH20 for filtering out salts and fatty acids from our extracts prior injection into GC-MS. Sephadex, a versatile sugar polymer, serves for filtering out fatty acids, NaCl ions, and other salts present in the sample. The method involved a simple filtration process using a glass pipette

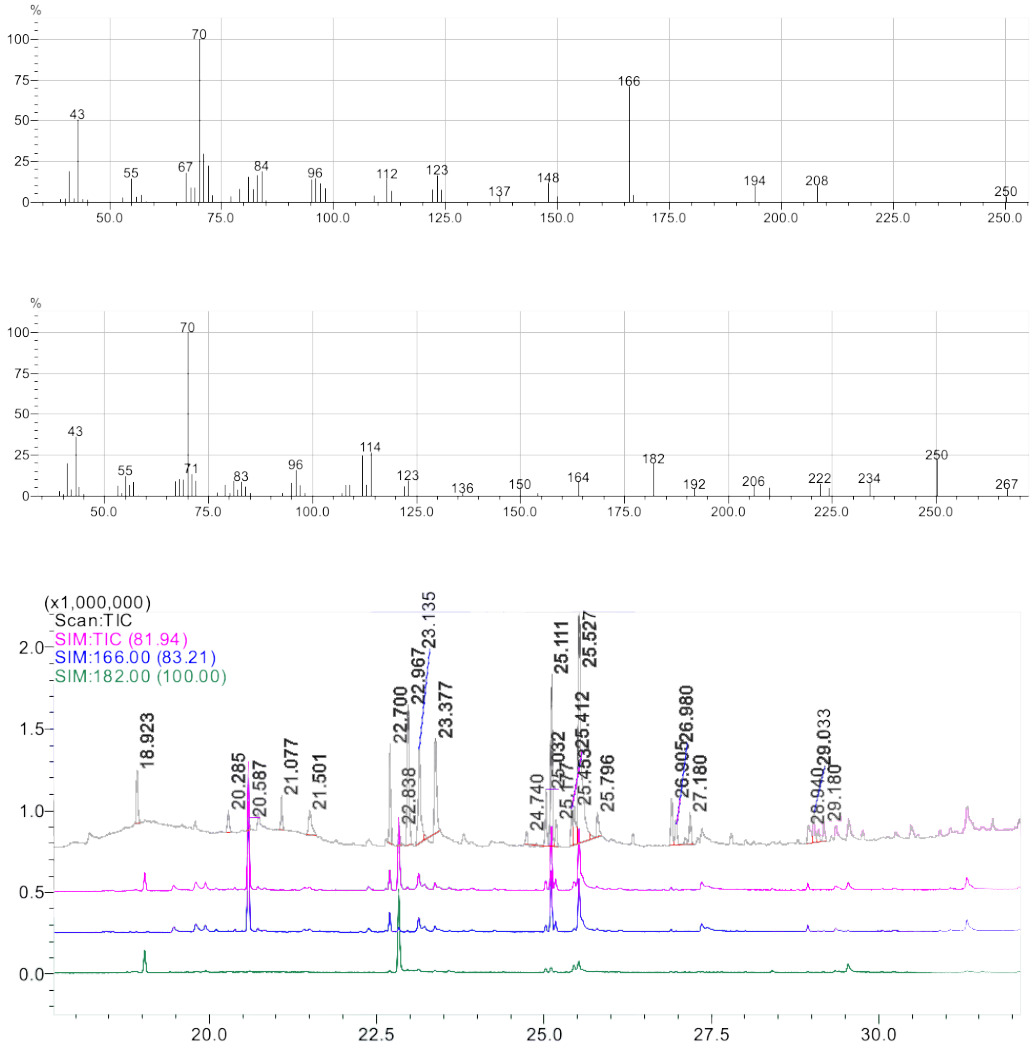


Figure 1.5: The mass spectra distinctly highlight the unique profiles of PTX 251D and aPTX 267A, marked by significant ions at specific mass-to-charge ratios: *m/z* 70 and *m/z* 166 for PTX 251D, and *m/z* 70 and *m/z* 182 for aPTX 267A, delineating their precise mass spectrometric characteristics. Enhanced by Sephadex treatment to minimize fatty acid interference, the lowest spectra improved readability a lot.

packed with a fingertip of cotton and topped with the Sephadex-solvent mixture, providing rapid and efficient filtration under normal pressure and gravity. The obtained results (Fig. 1.5) were compared with those from acid-base extraction, following the protocol of Jeckel et al. (2015). For GC-MS analysis, we utilized a Shimadzu GC-2010 coupled with a Shimadzu GCMS-QP2010 and a ZB-5ms Zebron column. The temperature program ranged from 60 to 280°C, increasing by 10°C/min, using helium as the carrier gas (Jeckel et al., 2015). In an effort to find alternatives to nicotine as an internal standard, we tested four different compounds: 2-Piperidineethanol, Diethylaminopyridine, 2-Methylpyridine, and Trimethylpyridine. The selection of Trimethylpyridine as the preferred standard was based on its accessibility within our facilities and its comparatively lower health risk than nicotine. Notably, Trimethylpyridine demonstrated a retention time of 7 minutes and produced a substantial signal intensity of 14×10^6 at an analyte concentration of 0.1 μg/μL and 20 μg per sample. The alternative compounds were deemed unsuitable as they either failed to

generate a strong signal at the desired concentration or exhibited non-optimal retention times. Later on, we decided to switch our analysis entirely to liquid chromatography, as GC-MS was inadequate for detecting hypothesized hydrophilic or multiple hydroxylated metabolites. It is noteworthy to mention the identified retention times of the target compounds: 20.580 to 20.600 minutes for PTX 251D and 22.830 to 22.850 minutes for aPTX 267A. The chromatography mass spectra showcase the distinctive profiles of PTX 251D and aPTX 267A (Fig. 1.5). Notably, the spectra for PTX 251D are characterized by prominent ions at mass-to-charge ratios *m/z* 70 ($C_4H_8N^+$) and *m/z* 166 ($C_{10}H_{16}NO^+$), whereas for aPTX 267A the key ions are at *m/z* 70 ($C_4H_8N^+$) and *m/z* 182 ($C_{10}H_{16}NO_2^+$), demonstrating the precise mass spectrometric identification of these alkaloids. These measurements were taken after treating the samples with SEPHADEX, which notably enhanced peak clarity, by removing fatty acids. This refinement of the sample preparation process was essential for achieving precise results, herein presented.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

In addition to the use of liquid chromatography spectrometers, our objective was to represent the data in a visual format. Matrix-assisted laser desorption/ionization (MALDI) imaging, a standard method in research, was employed. This technique utilizes a laser for ionization, which might result in molecular ionization distinct from that observed in GC and LC methodologies. To investigate the potential interaction of PTX 251D with the standard matrix (ionization facilitator) in the MALDI-MS/MS, we conducted experiments using four different matrices.The optimization of matrix was performed to PTX 251D standard using four different matrix, titanium dioxide (TiO_2), graphene, α-cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxybenzoic acid (DHB). The analysis was performed on a MALDI-TOF/TOF ultrafleXtreme (Bruker®Daltonics, UltrafleXtreme, Bremen, Germany) system equipped with a smartbeam-II laser system controlled by a FleXcontrol v.3.3 (Bruker®Daltonics, Bremen, Germany). The settings of the instrument were as follows: positive ion reflector mode; ion source 1, 25.00 kV; ion source 2, 22.35 kV; lens, 7.75 kV; reflector, 26.60 kV; reflector 2, 13.30 kV; pulsed ion extraction, 100 ns; laser frequency, 100 Hz; and matrix suppression mass cutoff, m/z 500. The spectra were recorded across a mass range of m/z 100-600 and accumulate 500 shots per spectrum. The sample of standard of PTX 251D was diluted in methanol to obtain a solution of 1 mg/mL. The matrix was prepared in ACN/H20 1:1 contained 0.1% of TFA. For analysis, 1 μL of matrix was mixed with 1 μL of sample and 1 μL was spotted in MALDI plate and drying into ambient conditions. As a result, HCCA works best for ionization (Fig. 1.6), while DHB will be the best choice to use, because of the large amount of matrix contaminating signals observed in HCCA in this mass range. The idea for using MALDI was to localize PTX 251D and aPTX 267A in the animal through cross-section and MALDI-imaging analyses. Nevertheless, given the superior resolution achieved with UPLC-MS, as detailed in chapter 2 and 3, we elected not to pursue MALDI imaging further. This decision was based on the consideration that MALDI imaging would not contribute additional insights beyond what had already been elucidated through our findings.

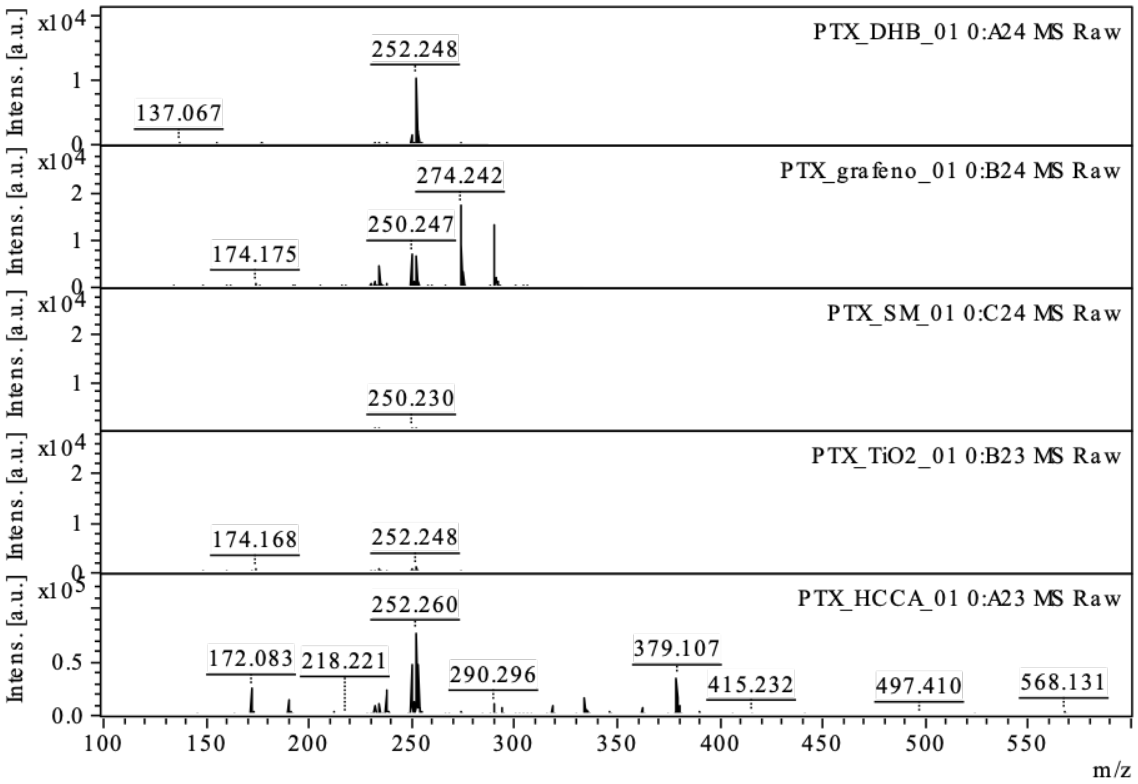


Figure 1.6: These MALDI spectra, show the massfractions of PTX 251D in combination with the different matrices. Top to bottom: 2,5-dihydroxybenzoic acid (DHB), graphene, SM,titanium dioxide (TiO_2), α-cyano-4-hydroxycinnamic acid (HCCA)

Electrospray Ionization Tandem Mass Spectrometry Coupled Quadrupole Time-of-Flight Mass Spectrometry (ESI-MS-MS /qTOF)

As a potential alternative to GC-MS, we investigated the use of direct injection into a micrOTOF-Q II mass spectrometer for measuring PTX 251D. This system combines a quadrupole with a time-of-flight (TOF) analyzer, allowing us to explore ionization via ESI, which facilitates soft ionization and the study of fragmentation patterns at various electron volt energies. Unlike conventional GC-MS that operates at a fixed impact energy of 70 eV, we experimented with a range of 5 to 50 eV. This approach helps identify optimal voltages for fragmentation and provides useful for subsequent multiple reaction monitoring (MRM) experiments. The detailed methodology and results of this investigation were already published in (Minder et al., 2023) and further elaborated in 2.

Ultra Pressure Liquid Mass Spectrometry Coupled Triplo Quandropole (UPLC-TQMS), Neutral Loss

The analysis was performed using an Acquity UPLC-MS System (Waters Corp., Massachusetts, EUA) and the chromatographic separation was achieved on a Kinetex XB-C18 column 50 mm x 2.1 mm, 1.7 μm (Phenomenex, California, EUA) with a pre-column of the same material, maintained at 30°C. The mobile phase was composed by solvent A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The elution program was as follows: 10% B (initial), 10-100% B (12 min), 100% B (15 min), 100-10% B (16 min), 10% B (20 min). The flow rate was 0.3 $mL \times min^{-1}$), the injection volume was 2 μL, and the

autosampler temperature was set to 10°C. The MS was operated in the positive ion and neutral loss mode using an electroypray voltage of .2.5 kV temperature of 150°C and desolvation temperature of 400°C. Nitrogen was used as desolvation gas (1,000 $L \times h^{-1}$)) and as a cone gas (50 $L \times h^{-1}$)). Data interpretation was facilitated by MassLynx V 4.1 Software (Waters Corp.). The calibration of the mass spectrometer was specifically set to identify mass losses of 18 Da and 56 Da, which correspond to water and butane, respectively. This was based on the findings presented in chapter 2, targeting molecules within the mass range of 50 to 600. For the molecular mass of 252, a retention time of 5.61 minutes was observed, while for a mass of 268, the retention time was recorded at 4.2 minutes, both under 25 eV. It's notable that at 30 eV, these specificities were maintained, whereas no detection occurred at 40 eV. The objective in this case was to identify metabolites or more highly hydroxylated alkaloids by employing the same degradation mechanism that was noted in aPTX 267A and PTX251D.

Solid Phase Extraction (SPE)

To prepare the samples for direct injection into the UPLC, we used solid phase extraction (SPE). SPE is a method used for rapid and selective sample preparation, enabling purification before chromatographic analysis. It involves isolating analytes from a liquid sample by extracting them onto a solid stationary phase, essentially filtering through column material used in the UPLC. This ensures that the column pores remain unclogged during analysis. We utilized Supelclean LC-18 SPE 100 mg, 1 mL tubes from Supelco, mounted on a Supelco Visiprep 24 DL to expedite the process under vacuum. The column was primed with 1 mL of 100% methanol and 2 mL miliQ water, followed by 1 mL of sample solution (200 μL sample + 1800 μL water), which was then washed twice with 500 μL water. To elute the alkaloids, we added 200 μL of methanol to the column, and then collected it into a glass vial via a micro glass tube for subsequent UPLC analysis.

1.3 Synthesis and Reflections on Research Endeavors

As we wrap up this chapter, we take a step back to look at the broader picture that has emerged from the interplay of caring for *A. galactonotus* and improving our analytical skills. It's been a blend of learning curves, unexpected hiccups, and those 'aha' moments that are the elements of science. The strategies we employed to navigate the breeding of *A. galactonotus* and *D. melanogaster,* though not perfect, were the best solutions within the constraints of time, materials, and unchangeable factors, like seasons and the consequent temperature fluctuation or facility conditions. The practicalities of nurturing *D. melanogaster* for feeding *A. galactonotus* offered a firsthand understanding of the delicate balance between controlled conditions and the unpredictable nature of biological systems. The loss of fly populations, though a setback, provided a compelling lesson in the resilience required for scientific investigation. The first year of this thesis, demanded significant time, effort, and learning to keep the frogs alive and breeding successfully. Managing living animals under time pressure presented unique challenges, particularly when rapid solutions were necessary to prevent dire consequences. My years of experience as a Senior Lab Technician in the pharmaceutical industry proved invaluable in problem-solving, drawing on physical and chemical

principles. It is always easy to work under ideal conditions but the creative solving of problems is fun and challenging the same time. Methodologically, the chapter dissected the adaptation of various analytical methods, underscoring our venture into the mass spectrometry based on GC, LC and MALDI. Through these explorations, we've not only enriched our methodological toolkit but also contributed valuable data to the scientific community's growing databases. Our endeavors in refining sample preparation techniques, particularly for LC injection, present a leap toward more efficient and precise analysis. Such methodological advancements are crucial in a research landscape where recent years have seen a surge of interest in these analytical techniques, as evidenced by Jeckel et al. (2022, 2020), Gonzalez and Carazzone (2023), Minder et al. (2023), Krieger et al. (2022), Pearson and Tarvin (2022), Alvarez-Buylla et al. (2022), O'Connell et al. (2021) and others. While acknowledging the resource-intensive nature of state-of-the-art extraction techniques, we have endeavored to optimize these methods within our means, aiming to contribute significantly to the field. In this research, we aimed to enrich the LC databases with information beyond what is typically confined to GC-MS databases (Daly et al., 2008), acknowledging their methodological and sensitivity limitations as highlighted by Gonzalez and Carazzone (2023). Our work contributes previously unavailable data on PTX 251D and aPTX 267A, complementing the findings in Minder et al. (2023). Reflecting upon the complex process of extracting and quantifying alkaloids, the lessons learned extend beyond the laboratory's walls. In the process of sample preparation, we aimed at minimizing risks such as possible oxidation or UV-induced modifications in the alkaloids post-quenching, with methanol. We acknowledge the gaps in data regarding the stability and preservation of these sensitive compounds. We operated under the assumption that these factors pose a potential threat, and therefore we take comprehensive precautions. This included rapid processing to counteract the evaporation rate and the temperature-dependent density changes of methanol, which could otherwise lead to undesirable concentration shifts in the samples. Our approach was to safeguard the integrity of the alkaloids as effectively as possible within the scope of current knowledge and available techniques. Reproducibility demands meticulous recording of environmental conditions, such as laboratory temperature and sample exposure duration, which are often overlooked in existing literature. We documented mass of methanol used, although volume metrics were then considered sufficient due to the focus on alkaloid ratios independent of solvent volume. Ultimately, rapid sample handling is crucial, given the largely unknown chemical and physical properties of many toxins under study. The acquisition of pure alkaloid would be pivotal for accurate quantification, yet this endeavor is often hindered by the prohibitive market costs or sheer unavailability within the industry. Recognizing the importance of these substances, one prospective strategy could be to implement a system for the recovery and subsequent reuse of the alkaloids utilized in experiments. While such a process of recycling may present as complex and time-intensive, especially when working on a smaller scale, it holds potential for maximizing the utility of these valuable compounds. As elucidated in subsequent chapters, this research endeavor focused on optimizing various steps that have been critiqued or identified as needing improvement. By employing a diverse array of techniques and analyses in an exploratory manner, we aimed to advance this field of study. This was made possible thanks to the available expertise, facilities, and funding, which collectively facilitated significant progress in this research area. As we close this chapter, we recognize that each experiment, each observation, and each conclusion drawn has been instrumental in broadening our understanding of

amphibian metabolic processes. The collective efforts and findings articulated here not only pave the way for future studies but also underscore the evolving nature of scientific methods. With every methodological refinement and each piece of data meticulously recorded, we edge closer to the precise delineation of alkaloid profiles in these fascinating creatures, contributing a vital chapter to the ever-unfolding story of poison frog research. The paths we've trod have laid the groundwork for those who will follow, looking to unravel even more secrets of poison frog alkaloids. It's been a chapter of growth, challenges, and, most importantly, learning—the kind that you can only get with your sleeves rolled up and your hands dirty.

Bibliography

Alvarez-Buylla, A., Payne, C. Y., Vidoudez, C., Trauger, S. A., and O'Connell, L. A. (2022). Molecular physiology of pumiliotoxin sequestration in a poison frog. *PLOS ONE*, 17(3):e0264540.

An, C.-Y., Sun, Z.-Z., Shen, L., and Ji, H.-F. (2017). Biotransformation of food spice curcumin by gut bacterium *Bacillus megaterium* DCMB-002 and its pharmacological implications. *Food & Nutrition Research*, 61(1):1412814.

Brooks, O. L., James, J. J., and Saporito, R. A. (2023). Maternal chemical defenses predict offspring defenses in a dendrobatid poison frog. *Oecologia*.

Caty, S. N., Alvarez-Buylla, A., Byrd, G. D., Vidoudez, C., Roland, A. B., Tapia, E. E., Budnik, B., Trauger, S. A., Coloma, L. A., and O'Connell, L. A. (2019). Molecular physiology of chemical defenses in a poison frog. *The Journal of Experimental Biology*, 222(12):jeb204149.

Clark, V. C., Raxworthy, C. J., Rakotomalala, V., Sierwald, P., and Fisher, B. L. (2005). Convergent evolution of chemical defense in poison frogs and arthropod prey between Madagascar and the Neotropics. *Proceedings of the National Academy of Sciences*, 102(33):11617–11622.

Daly, J. W., Garraffo, H. M., Spande, T. F., Giddings, L.-A., Saporito, R. A., Vieites, D. R., and Vences, M. (2008). Individual and Geographic Variation of Skin Alkaloids in Three Species of Madagascan Poison Frogs (Mantella). *Journal of Chemical Ecology*, 34(2):252–279.

Daly, J. W., Gusovsky, F., Myers, C. W., Yotsu-Yamashita, M., and Yasumoto, T. (1994). First occurrence of tetrodotoxin in a dendrobatid frog (Colostethus inguinalis), with further reports for the bufonid genus Atelopus. *Toxicon*, 32(3):279–285.

Daly, J. W., Spande, T. F., and Garraffo, H. M. (2005). Alkaloids from Amphibian Skin: A Tabulation of Over Eight-Hundred Compounds. *Journal of Natural Products*, 68(10):1556–1575.

Garraffo, H. M., Andriamaharavo, N. R., Vaira, M., Quiroga, M. F., Heit, C., and Spande, T. F. (2012). Alkaloids from single skins of the Argentinian toad Melanophryniscus rubriventris (ANURA, BUFONIDAE): An unexpected variability in alkaloid profiles and a profusion of new structures. *SpringerPlus*, 1(1):51.

Gonzalez, M. and Carazzone, C. (2023). Eco-Metabolomics Applied to the Chemical Ecology of Poison Frogs (Dendrobatoidea). *Journal of Chemical Ecology*.

Hammer, T. J. and Bowers, M. D. (2015). Gut microbes may facilitate insect herbivory of chemically defended plants. *Oecologia*, 179(1):1–14.

Hantak, M. M., Grant, T., Reinsch, S., Mcginnity, D., Loring, M., Toyooka, N., and Saporito, R. A. (2013). Dietary Alkaloid Sequestration in a Poison Frog: An Experimental Test of Alkaloid Uptake in Melanophryniscus stelzneri (Bufonidae). *Journal of Chemical Ecology*, 39(11-12):1400–1406.

Jeckel, A. M., Bolton, S. K., Waters, K. R., Antoniazzi, M. M., Jared, C., Matsumura, K., Nishikawa, K., Morimoto, Y., Grant, T., and Saporito, R. A. (2022). Dose-dependent alkaloid sequestration and *N* -methylation of decahydroquinoline in poison frogs. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 337(5):537–546.

Jeckel, A. M., Grant, T., and Saporito, R. A. (2015). Sequestered and Synthesized Chemical Defenses in the Poison Frog Melanophryniscus moreirae. *Journal of Chemical Ecology*, 41(5):505–512.

Jeckel, A. M., Matsumura, K., Nishikawa, K., Morimoto, Y., Saporito, R. A., Grant, T., and Ifa, D. R. (2020). Use of whole-body cryosectioning and desorption electrospray ionization mass spectrometry imaging to visualize alkaloid distribution in poison frogs. *Journal of Mass Spectrometry*, 55(6):e4520.

Krieger, A. C., Povilaitis, S. C., Gowda, P., O'Connell, L. A., and Eberlin, L. S. (2022). Noninvasive Detection of Chemical Defenses in Poison Frogs Using the MasSpec Pen. *ACS Measurement Science Au*, 2(5):475–484.

Manson, J. S. and Thomson, J. D. (2009). Post-ingestive effects of nectar alkaloids depend on dominance status of bumblebees. *Ecological Entomology*, 34(4):421–426.

McGugan, J. R., Byrd, G. D., Roland, A. B., Caty, S. N., Kabir, N., Tapia, E. E., Trauger, S. A., Coloma, L. A., and O'Connell, L. A. (2016). Ant and Mite Diversity Drives Toxin Variation in the Little Devil Poison Frog. *Journal of Chemical Ecology*, 42(6):537–551.

Mebs, D., Arakawa, O., and Yotsu-Yamashita, M. (2010). Tissue distribution of tetrodotoxin in the red-spotted newt Notophthalmus viridescens. *Toxicon*, 55(7):1353–1357.

Michael, J. P. (2016). Simple Indolizidine and Quinolizidine Alkaloids. In *The Alkaloids: Chemistry and Biology*, volume 75, pages 1–498. Elsevier.

Minder, B., Mendonça, J., Grant, T., and Lopes, N. (2023). Gas-Phase Fragmentation Reactions of Protonated Pumiliotoxin (+)-251D and Allopumiliotoxin (+)-267A in ESI-MS/MS. *Journal of the Brazilian Chemical Society*.

Moskowitz, N. A., Roland, A. B., Fischer, E. K., Ranaivorazo, N., Vidoudez, C., Aguilar, M. T., Caldera, S. M., Chea, J., Cristus, M. G., Crowdis, J. P., DeMessie, B., desJardins-Park, C. R., Effenberger, A. H., Flores, F., Giles, M., He, E. Y., Izmaylov, N. S., Lee, C. C., Pagel, N. A., Phu, K. K., Rosen, L. U., Seda, D. A., Shen, Y., Vargas, S., Murray, A. W., Abebe, E., Trauger, S. A., Donoso, D. A., Vences, M., and O'Connell, L. A. (2018). Seasonal changes in diet and chemical defense in the Climbing Mantella frog (Mantella laevigata). *PLOS ONE*, 13(12):e0207940.

O'Connell, L. A., LS50: Integrated Science Laboratory Course, O'Connell, J. D., Paulo, J. A., Trauger, S. A., Gygi, S. P., and Murray, A. W. (2021). Rapid toxin sequestration modifies poison frog physiology. *Journal of Experimental Biology*, 224(3):jeb230342.

Okada, T., Ozaki, T., Yamamoto, T., Kasahara, H., Kawasaki, M., and Toyooka, N. (2021). Divergent Syntheses of Pumiliotoxin-Type Poison-Frog Alkaloids. *ChemistrySelect*, 6(8):1939–1945.

Pearson, K. C. and Tarvin, R. D. (2022). A review of chemical defense in harlequin toads (Bufonidae: Atelopus). *Toxicon: X*, 13:100092.

Posso-Terranova, A. and Andrés, J. (2020). Skin transcriptional profiles in Oophaga poison frogs. *Genetics and Molecular Biology*, 43(4):e20190401.

Pryor, G. S. (2008). Anaerobic Bacteria Isolated from the Gastrointestinal Tracts of Bullfrog Tadpoles (Rana catesbeiana). *Herpetological Conservation and Biology*.

Rodríguez, A., Poth, D., Schulz, S., and Vences, M. (2011). Discovery of skin alkaloids in a miniaturized eleutherodactylid frog from Cuba. *Biology Letters*, 7(3):414–418.

Roelants, K., Fry, B. G., Norman, J. A., Clynen, E., Schoofs, L., and Bossuyt, F. (2010). Identical Skin Toxins by Convergent Molecular Adaptation in Frogs. *Current Biology*, 20(2):125–130.

Sanchez, E., Rodríguez, A., Grau, J. H., Lötters, S., Künzel, S., Saporito, R. A., Ringler, E., Schulz, S., Wollenberg Valero, K. C., and Vences, M. (2019). Transcriptomic Signatures of Experimental Alkaloid Consumption in a Poison Frog. *Genes*, 10(10):733.

Santos, J. C., Tarvin, R. D., and O'Connell, L. A. (2016). A Review of Chemical Defense in Poison Frogs (Dendrobatidae): Ecology, Pharmacokinetics, and Autoresistance. In Schulte, B. A., Goodwin, T. E., and Ferkin, M. H., editors, *Chemical Signals in Vertebrates 13*, pages 305–337. Springer International Publishing, Cham.

Saporito, R. A., Donnelly, M. A., Garraffo, H. M., Spande, T. F., and Daly, J. W. (2006). Geographic and Seasonal Variation in Alkaloid-Based Chemical Defenses of Dendrobates pumilio from Bocas del Toro, Panama. *Journal of Chemical Ecology*, 32(4):795–814.

Saporito, R. A., Donnelly, M. A., Madden, A. A., Garraffo, H. M., and Spande, T. F. (2010). Sex-Related Differences in Alkaloid Chemical Defenses of the Dendrobatid Frog *Oophaga pumilio* from Cayo Nancy, Bocas del Toro, Panama $^{\perp}$. *Journal of Natural Products*, 73(3):317–321.

Saporito, R. A., Donnelly, M. A., Spande, T. F., and Garraffo, H. M. (2012). A review of chemical ecology in poison frogs. *Chemoecology*, 22(3):159–168.

Saporito, R. A., Garraffo, H. M., Donnelly, M. A., Edwards, A. L., Longino, J. T., and Daly, J. W. (2004). Formicine ants: An arthropod source for the pumiliotoxin alkaloids of dendrobatid poison frogs. *Proceedings of the National Academy of Sciences*, 101(21):8045–8050.

Saporito, R. A., Norton, R. A., Andriamaharavo, N. R., Garraffo, H. M., and Spande, T. F. (2011). Alkaloids in the Mite Scheloribates laevigatus: Further Alkaloids Common to Oribatid Mites and Poison Frogs. *Journal of Chemical Ecology*, 37(2):213–218.

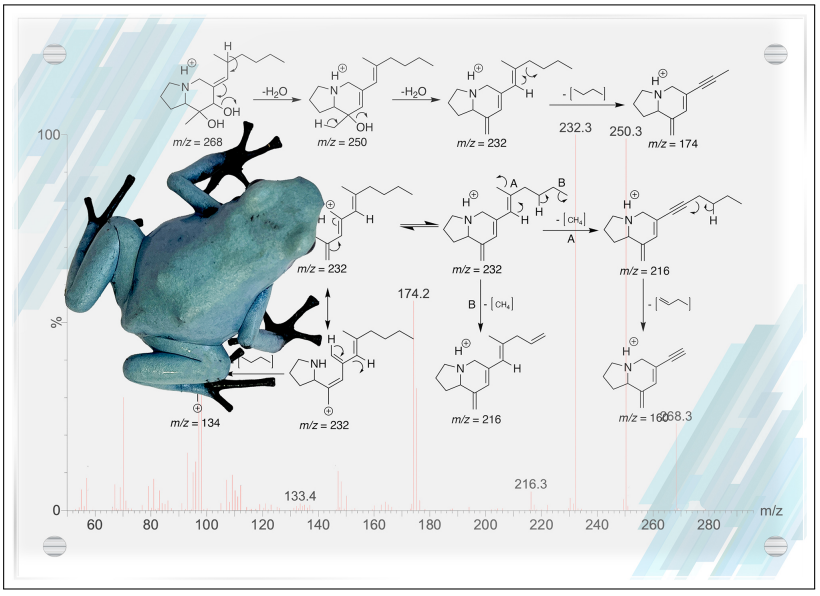
Saporito, R. A., Zuercher, R., Roberts, M., Gerow, K. G., and Donnelly, M. A. (2007). Experimental Evidence for Aposematism in the Dendrobatid Poison Frog Oophaga pumilio. *Copeia*, 2007(4):1006–1011.

Takada, W., Sakata, T., Shimano, S., Enami, Y., Mori, N., Nishida, R., and Kuwahara, Y. (2005). Scheloribatid Mites as the Source of Pumiliotoxins in Dendrobatid Frogs†. *Journal of Chemical Ecology*, 31(10):2403–2415.

Tong, Q., Cui, L.-Y., Du, X.-P., Hu, Z.-F., Bie, J., Xiao, J.-H., Wang, H.-B., and Zhang, J.-T. (2020). Comparison of Gut Microbiota Diversity and Predicted Functions Between Healthy and Diseased Captive Rana dybowskii. *Frontiers in Microbiology*, 11:2096.

Chapter 2

Gas-Phase Fragmentation Reactions of Protonated Pumiliotoxin (+)-251D and Allopumiliotoxin (+)-267A in ESI-MS/MS



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Short Report

Gas-Phase Fragmentation Reactions of Protonated Pumiliotoxin (+)-251D and Allopumiliotoxin (+)-267A in ESI-MS/MS

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In recent years, there has been great interest in understanding the chemistry of natural products from different organisms and their role in ecological processes. Some species of frogs sequester and metabolize dietary alkaloids obtained primarily from mites and ants as chemical defense. Most of these studies have employed gas chromatography techniques coupled with mass spectrometry, which restricts the possibility of observing more polar metabolites. In the case of pumiliotoxin (+)-251D and allopumiliotoxin (+)-267A, the fragmentation mechanisms in electrospray ionization systems with collision-induced fragmentation are undescribed. The present study aims to elucidate the fragmentation pathways of these two toxins. For this purpose, we used direct infusion of toxins in a time-of-flight hyphenated quadrupole electrospray ionization tandem mass spectrometry (ESI-MS/MS) system. Different collision energies were applied, and the data rationalized from the concepts of reaction mechanisms. The joint analysis allowed to present a robust map of fragmentation opening perspectives for their application in studies of the occurrence of new polar analogues.

Keywords: allopumiliotoxin (+)-267A, alkaloid, fragmentation mechanism, mass spectrometry, pumiliotoxin (+)-251D

Introduction

Amphibians have evolved a large arsenal of chemical signals, including volatile and nonvolatile pheromones for sexual communication[1,2] and toxins to deter predation,[3-5] parasitization,[6,7] and pathogenic infections.[8-10] Although most amphibian defensive chemicals are endogenous, controlled experiments involving the oral administration of lipophilic alkaloids have revealed that so-called "poison frogs" (a polyphyletic collection of diurnal, brightly colored, microphagous frogs distributed in the families Bufonidae,[3] Dendrobatidae,[11,12] Eleutherodactylidae,[13] Mantellidae[14] and Myobatrachidae)[3] sequester their defenses[15-17] from dietary arthropods, primarily ants and mites.[10,16,18-20]

Alkaloids accumulate in the skin and are detected in the liver in higher concentrations as well as muscles and kidney in lower amounts.[21,22] The accumulation occurs within a couple of hours.[15,22-25] Further, biomodifications of sequestered alkaloids are known, including hydroxylation of the pumiliotoxin (PTX) (+)-251D to the allopumiliotoxin (aPTX) (+)-267A in the dendrobatids *Oophaga pumilio*, *Adelphobates galactonotus*, *Dendrobates auratus* and *Dendrobates tinctorius*,[10,15,25] *N*-methylation in decahydroquinoline (DHQ) in *Adelphobates galactonotus*,[24] and enzymatic reduction and/or hydroxylation of dietary PTX 307A into PTXs of molecular weights 309, 323, and 325 in species of the myobatrachid *Pseudophryne*.[26]

To date classical gas chromatography combined with electron ionization mass spectrometry (GC-MS) has been the preferred tool for chemical analysis and structural elucidation.[22,24,27-32] Although GC-MS is still one of the best strategies for thermally stable and volatile compounds,[12,33] recent technological advances have allowed a more complete analysis of chemical signals at various molecular levels by different sources configurations in mass spectrometry.[34] For example, liquid chromatography coupled to mass spectrometry with an electrospray (ESI) source is a powerful tool in the analysis and identification of a greater number of substances since charge transfer is a lighter process and allows analyzing from medium polarity compounds to polar substances.[35] Recently, the investigation of biomodification of PTX (+)-251D into the more potent aPTX (+)-267A used for the first time

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high performance liquid cromatography technique coupled mass spectrometry with electrospray ionization (HPLC-ESI-MS).[25] Although, this more sensitive technique lacks commercially available internal standards which can present a challenge for structural confirmation and quantitative analysis.[36] The authors describe ions related to metabolites with two additional hydroxylation structures that would not be possible to observe in GC-MS, and they also report problems in detecting one of the substances that could result from its rapid metabolization or ionization problems. Reactions at the source can lead to unexpected ions such as radical cations,[37,38] coordination with metals other than sodium,[39] in addition to all ionic suppression phenomena,[40,41] indicating the need for additional techniques to characterize new PTX, aPTX, and homopumiliotoxin (hPTX) derivatives. With this in mind, the objective of this study was to carry out a systematic analysis of the fragmentation reactions in ESI in order to characterize the structure of all fragment ions in MS/MS experiments of PTX (+)-251D and aPTX (+)-267A for application in metabolization studies.

Experimental

Chemicals and reagents

High performance liquid chromatography (HPLC) grade methanol and P.A. grade acetic acid were purchased from JTBaker (Phillipsburg, NJ, USA). The pumiliotoxin (PTX (+)-251D) used for feeding the frogs as for the ultra performance liquid chromatography (UPLC) analysis was obtained from Ralph Saporito.[42]

Animals: *Adelphobates galactonotus* toxin administration

The carcass of *Adelphobates galactonotus* frogs was used as source of the aPTX (+)-267A. The carcass was extracted with methanol following the article published by Saporito *et al.*[43] and Jeckel *et al.*[31] The sampling authorization was obtained with the number SISBIO: 54640-1b and the research was registered and approved by the Comissão de Ética no Uso de Animais under No. 402/2023 - CEUDo Institute of Biosciences of the University of São Paulo. The samples were stored at the Laboratório de Anfíbos da Universidade de São Paulo.

Mass spectrometry

High-resolution electrospray ionization (ESI) mass spectrometry analyses were performed on a micrOTOF-QII mass spectrometer (Bruker Daltonics, USA) fitted with time-of-flight analyzer (TOF). The spectrometer was operated in the positive ionization mode and the capillary voltage was fixed at 3.5 kV. For accurate mass analysis, the TOF analyzer was calibrate with sodium trifluoroacetic acid TFA–Na^+ as the internal standard. For MS/MS spectra, protonated molecule was selected and fragmented at different laboratory frame collision energies, Elab, (ranging from 5 to 50 eV). N_2 was employed as collision gas. Energy-resolved plots were obtained from ion intensities variation for each Elab applied. Finally, samples were inserted by direct infusion in a syringe pump.

Results and Discussion

Interpretation of the product ion spectrum showed stability of the protonated molecule, and only the water elimination fragment could be observed up to collision energies greater than 30 eV (Figure 1). Some natural products present this difficulty, being a characteristic directly related to the physical chemistry properties of the analyte and requiring a careful strategy in data analysis.[44,45] Figure 1 clearly demonstrates the need for data acquisition, the structural determination of analogues, using energies higher than 45 eV. Thus, as with some natural products containing fused rings, all other observed fragment ions are formed from $[M - 18]^+$.[44,45]

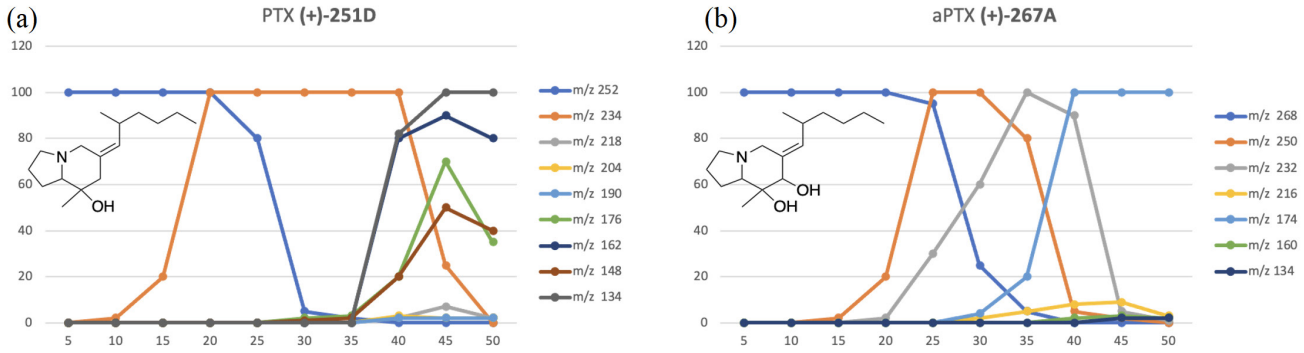

Figure 1. Energy-resolved plots from the ESI-MS/MS spectra of pumiliotoxin (+)-251D (a) and allopumiliotoxin (+)-267A (b). All the mass spectra and energy plots are available at Supplementary Information section.

As commented above, the main ion for the beginning of the fragmentation is the elimination of water. This elimination can occur by two mechanisms, charge retention fragmentations (CRF) or charge migration fragmentations (CMF).[46] In the comparison between the amine and the alcohol, protonation is more likely to occur at the N atom (which favors the CRF mechanism), as previously described for similar models of six- and five-membered cycles containing N.[47] Furthermore, CMF reactions pass through a stage of carbocation formation, which would be unfavorable compared to a possible CRF reaction, like a remote hydrogen rearrangement.[46] An additional gain of stability can occur by the formation of a fragment where the double bonds stay conjugated, confirming the mechanism.[46]

From the $[M - 18]^+$ (Figure 2), a series of other fragments is formed by the elimination of alkanes and alkenes (Figure 2). All these CRF reactions allowed the ions at *m/z* 204, 190, and 176 resulting from the elimination of alkanes, which require a higher transition energy, but they are viable in the gas phase, as previously observed in other cyclic alkaloids.[48]

Finally, in the second collection, the ions were observed at low intensity. These ions can be obtained after an open ring process lead by a conjugate carbocation formation. The new ion can be in equilibrium returning to the exactly $[M - 18]^+$ or the electrons form the N-atom can attack in the opposite side leavening a new cycle formation (Figure 1), which can eliminate the side chain by a CMF mechanism resulting in the minor ion at *m/z* = 148.[46] Another two ions can occur after ring opening via an initial Grob-Watter gas phase reaction[44,45] that yielded the ion at *m/z* = 218, which will form the ion at *m/z* = 162 after the CRF reaction.[46]

The next step was the analysis of the gas phase fragmentation reaction of the aPTX in electrospray source ionization with quadrupole time of flight (ESI-QTof). As expected, the introduction of a second hydroxyl group at alpha position of the oxo-ring double bound and near by the previous hydroxyl group does not increase the number of observed ions or the intensity of fragmentation. This fact can be explained by two restrictions effects for water elimination.[46] Then, to form an equilibrium between CMF and CRF mechanisms it requires two steps. An initial 1,4 water removal (Figure 3) followed by a similar E2 mechanism leading to a stable conjugate system.[46] The comparison of

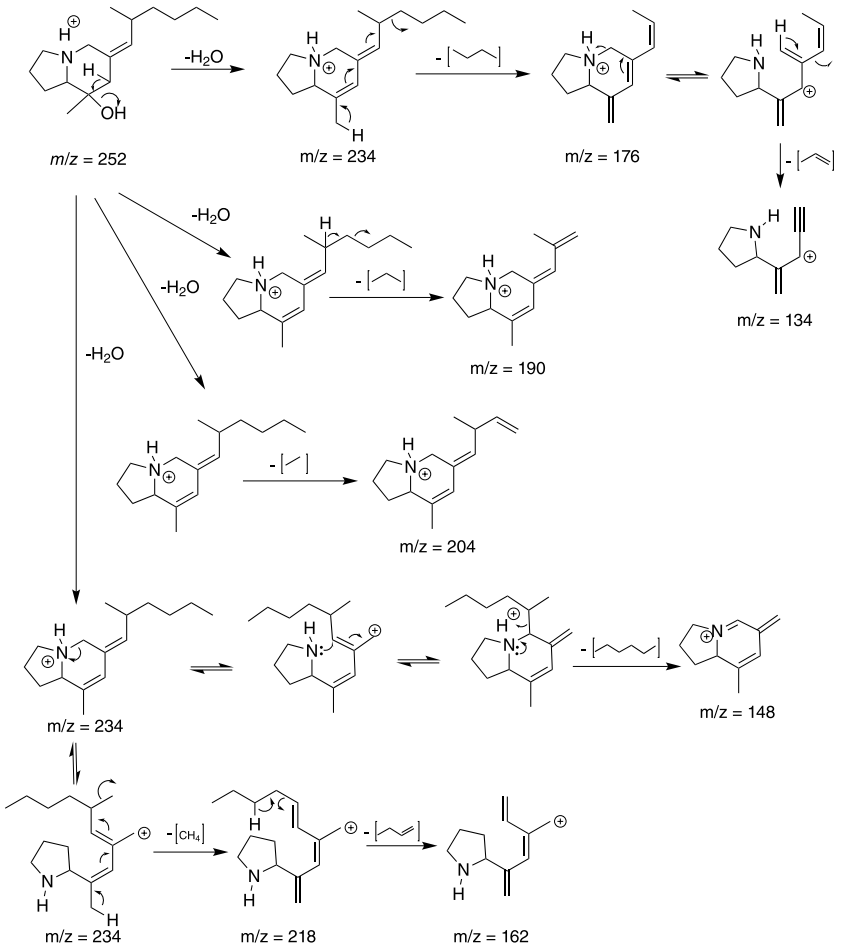

Figure 2. Fragmentation mechanism proposed for PTX (+)-251D.

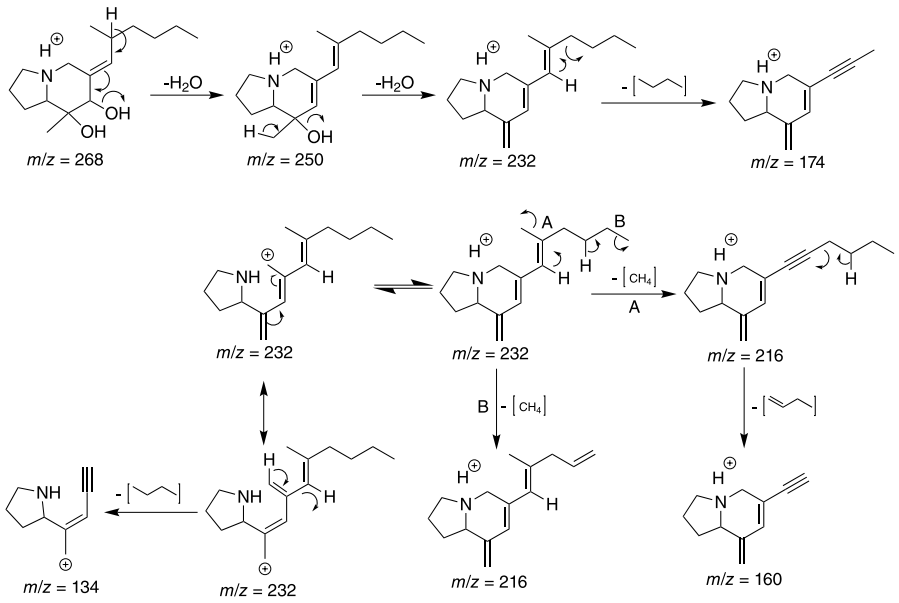

Figure 3. Fragmentation mechanism proposed for aPTX (+)-267A.

the MS/MS of both toxins suggest that the ions after loss of water do not show a collection of neutral eliminations of alkanes and alkenes, at least with some intensity. Again, the strong stability is responsible for the inhibition, and only the ion at *m/*z = 174 has some significance. The other signal at *m/*z 216, 134, and 160 cannot be observed at more than 10% in all applied energies (see Figure 1 and Supplementary Information section). Finally, our results open the perspective for biologists to investigate more possible alkaloid metabolites through liquid chromatography coupled tandem mass spectrometer (LC-MS/MS) instead of GC-MS, which cannot detect the more polar compounds.

Conclusions

Our results show that the structural determination of new analogues is possible, since the first elimination of water opens three different consecutive fragmentation pathways, and that substitutions in different positions, as with aPTX, can alter this balance. Further, the different pathways are eliminated from different parts of the skeleton, allowing us to better understand the location of any new functionalization.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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References

1. Brunetti, A. E.; Lyra, M. L.; Melo, W. G. P.; Andrade, L. E. A.; Palacios-Rodríguez, P.; Prado, B. M.; Haddad, C. F. B.; Pupo, M. T.; Lopes, N. P.; *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116*, 2124. [Crossref]
2. Willaert, B.; Bossuyt, F.; Janssenswillen, S.; Adriaens, D.; Baggerman, G.; Matthijs, S.; Pauwels, E.; Proost, P.; Raepsaet, A.; Schoofs, L.; Stegen, G.; Treer, D.; Van Hoorebeke, L.; Vandebergh, W.; Van Bocxlaer, I; *J. Exp. Biol.* **2013**, *22*, 4139. [Crossref]
3. Daly, J. W.; Highet, R. J.; Myers, C. W.; *Toxicon* **1984**, *22*, 905. [Crossref]
4. Naumann, C.; Hartmann, T.; Ober, D.; *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 6085. [Crossref]
5. Hantak, M. M.; Grant, T.; Reinsch, S.; Mcginnity, D.; Loring, M.; Toyooka, N.; Saporito, R. A.; *J. Chem. Ecol.* **2013**, *39*, 1400. [Crossref]
6. Weldon, P. J.; Kramer, M.; Gordon, S.; Spande, T. F.; Daly, J. W.; *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 1091. [Crossref]
7. Weldon, P. J.; Cardoza, Y. J.; Vander Meer, R. K.; Hoffmann, W. C.; Daly, J. W.; Spande, T. F.; *Naturwissenschaften* **2013**, *100*, 1432. [Crossref]

8. Hovey, K. J.; Seiter, E. M.; Johnson, E. E.; Saporito, R. A.; *J. Chem. Ecol.* **2018**, *44*, 312. [Crossref]
9. Saporito, R. A.; Donnelly, M. A.; Hoffman, R. L.; Garraffo, H. M.; Daly, J. W.; *J. Chem. Ecol*. **2003**, *29*, 2781. [Crossref]
10. Saporito, R. A.; Donnelly, M. A.; Spande, T. F.; Garraffo, H. M.; *Chemoecology* **2012**, *22*, 159. [Crossref]
11. Grant, T.; Rada, M.; Anganoy-Criollo, M.; Batista, A.; Dias, P. H.; Jeckel, A. M.; Machado, D. J.; Rueda-Almonacid, J. V.; *S. Am. J. Herpetol.* **2017**, *12*, 1. [Crossref]
12. Gonzalez, M.; Palacios-Rodriguez, P.; Hernandez-Restrepo, J.; González-Santoro, M.; Amézquita, A.; Brunetti, A. E.; Carazzone, C.; *Front. Zool*. **2021**, *18*, 39. [Crossref]
13. Rodríguez, A; de la Nuez, D.; Alonso, R.; *J. Herpetol*. **2010**, *44*, 457. [Crossref]
14. Garraffo, H. M.; Caceres, J.; Daly, J. W.; Spande, T. F.; Andriamaharavo, N. R.; Andriantsiferana, M.; *J. Nat. Prod.* **1993**, *56*, 1016. [Crossref]
15. Daly, J. W.; Garraffo, H. M.; Spande, T. F.; Clark, V. C.; Ma, J.; Ziffer, H.; Cover Jr., J. F.; *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11092. [Crossref]
16. Daly, J. W.; Secunda, S. I.; Garraffo, H. M.; Spande, T. F.; Wisnieski, A.; Cover Jr., J. F.; *Toxicon* **1994**, *32*, 657. [Crossref]
17. Saporito, R. A.; Spande, T. F.; Garraffo, H. M.; Donnelly, M. A.; *J. Heterocycl. Chem*. **2009**, *79*, 277. [Crossref]
18. Daly, J. W.; Padgett, W. L.; Saunders, R. L.; Cover Jr., J. F.; *Toxicon* **1997**, *35*, 705. [Crossref]
19. Daly, J. W.; *J. Nat. Prod.* **1998**, *61*, 162. [Crossref]
20. Mebs, D.; *Toxicon* **2001**, *39*, 87. [Crossref]
21. O'Connell, L. A.; LS50: Integrated Science Laboratory Course; O'Connell, J. D.; Paulo, J. A.; Trauger, S. A.; Gygi, S. P.; Murray, A. W.; *J. Exp. Biol*. **2021**, *224*, jeb230342. [Crossref]
22. Jeckel, A. M.; Bolton, S. K.; Waters, K. R.; Antoniazzi, M. M.; Jared, C.; Matsumura, K.; Nishikawa, K.; Morimoto, Y.; Grant, T.; Saporito, R. A.; *J. Exp. Zool., Part A* **2022**, *337*, 537. [Crossref]
23. Caty, S. N.; Alvarez-Buylla, A.; Byrd, G. D.; Vidoudez, C.; Roland, A. B.; Tapia, E. E.; Budnik, B; Trauger, S. A.; Coloma, L. A.; O'Connell, L. A.; *J. Exp. Biol.* **2019**, *222*, jeb204149. [Crossref]
24. Jeckel, A. M.: *Sequestration Efficiency and Alkaloid Composition in Poison Frogs of the Family Dendrobatidae*; PhD Thesis, University of São Paulo, São Paulo, Brazil, 2020. [Crossref]
25. Alvarez-Buylla, A.; Payne, C. Y.; Vidoudez, C.; Trauger, S. A.; O'Connell, L. A; *PLoS One* **2022**, *17*, e0264540. [Crossref]
26. Smith, B. P.; Tyler, M. J.; Kaneko, T.; Garraffo, H. M.; Spande, T. F.; Daly, J. W; *J. Nat. Prod.* **2002**, *65*, 439. [Crossref]
27. Saporito, R. A.; Garraffo, H. M.; Donnelly, M. A.; Edwards, A. L.; Longino, J. T.; Daly, J. W.; *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 8045. [Crossref]
28. Saporito, R. A.; Donnelly, M. A.; Jain, P.; Garraffo, H. M.; Spande, T. F.; Daly, J. W.; *Toxicon* **2007**, *50*, 757. [Crossref]
29. Saporito, R. A.; Norton, R. A.; Andriamaharavo, N. R.; Garraffo, H. M.; Spande, T. F.; *J. Chem. Ecol.* **2011**, *37*, 213. [Crossref]
30. Takada, W.; Sakata, T.; Shimano, S.; Enami, Y.; Mori, N.; Nishida, R.; Kuwahara, Y.; *J. Chem. Ecol.* **2005**, *31*, 2403. [Crossref]
31. Jeckel, A. M.; Kocheff, S.; Saporito, R. A.; Grant, T.; *Chemoecology* **2019**, *29*, 225. [Crossref]
32. Brooks, O. L.; James, J. J.; Saporito, R. A.; *Oecologia* **2023**, *201*, 385. [Crossref]
33. Demarque, D. P.; Dusi, R. G.; de Sousa, F. D.; Grossi, S. M.; Silvério, M. R.; Lopes, N. P.; Espindola, L. S.; *Sci. Rep.* **2020**, *10*, 1051. [Crossref]
34. Brunetti, A. E.; Carnevale Neto, F.; Vera, M. C.; Taboada, C.; Pavarini, D. P.; Bauermeister, A.; Lopes, N. P.; *Chem. Soc. Rev.* **2018**, *47*, 1547. [Crossref]
35. Crotti, A. E. M.; Vessecchi, R.; Lopes, J. L. C.; Lopes, N. P.; *Quim. Nova* **2006**, *29*, 287. [Crossref]
36. McGugan, J. R.; Byrd, G. D.; Roland, A. B.; Caty, S. N.; Kabir, N.; Tapia, E. E.; Trauger, S. A.; Coloma, L. A.; O'Connell, L. A.; *J. Chem. Ecol.* **2016**, *42*, 537. [Crossref]
37. Guaratini, T.; Vessecchi, R. L.; Lavarda, F. C.; Campos, P. M. M.; Naal, Z.; Gates, P. J.; Lopes, N. P.; *Analyst* **2004**, *129*, 1223. [Crossref]
38. Guaratini, T.; Vessecchi, R.; Pinto, E.; Colepicolo, P.; Lopes, N. P.; *J. Mass Spectrom.* **2005**, *40*, 963. [Crossref]
39. Butler, M.; Arroyo Mañez, P.; Cabrera, G. M.; *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 545. [Crossref]
40. Draper, W. M.; Xu, D.; Perera, S. K.; *Anal. Chem.* **2009**, *81*, 4153. [Crossref]
41. García-Fonseca, S.; Rubio, S.; *Talanta* **2016**, *148*, 370. [Crossref]
42. Daly, J. W.; Spande, T. F.; Garraffo, H. M.; *J. Nat. Prod.* **2005**, *68*, 1556. [Crossref]
43. Saporito, R. A.; Donnelly, M. A.; Jain, P.; Garraffo, H. M.; Spande, T. F.; Daly, J. W.; *J. Chem. Ecol.* **2006**, *32*, 795. [Crossref]
44. Lopes, N. P.; Stark, C. B.; Hong, H.; Gates, P. J.; Staunton, J.; *Rapid. Commun. Mass Spectrom.* **2002**, *16*, 414. [Crossref]
45. Lopes, N. P.; Stark, C. B.; Gates, P. J.; Staunton, J.; *Analyst* **2002**, *127*, 503. [Crossref]
46. Demarque, D. P.; Crotti, A. E.; Vessecchi, R.; Lopes, J. L.; Lopes, N. P.; *Nat. Prod. Rep.* **2016**, *33*, 432. [Crossref]
47. Furtado, N. A.; Vessecchi, R.; Tomaz, J. C.; Galembeck, S. E.; Bastos, J. K.; Lopes, N. P.; Crotti, A. E.; *J. Mass Spectrom.* **2007**, *42*, 1279. [Crossref]
48. Aguiar, G. P.; Wakabayashi, K. A.; Luz, G. F.; Oliveira, V. B.; Mathias, L.; Vieira, I. J.; Braz-Filho, R.; Crotti, A. E.; *Rapid Commun. Mass Spectrom.* **2010**, *24*, 295. [Crossref]

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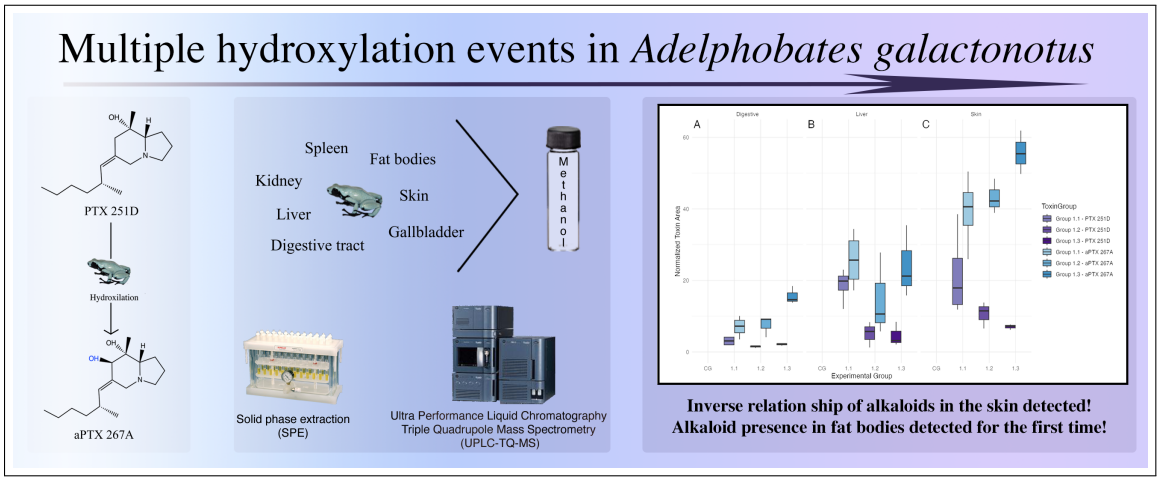
Chapter Title 3

Hydroylation of Pumiliotoxin (+)-251D to Allopumiliotoxin (+)-267A in *Adelphobates galactonotus*



Hydroxylation of Pumiliotoxin (+)-251D to Allopumiliotoxin (+)-267A in *Adelphobates galactonotus*

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Abstract

Chemical defense stands as a pivotal strategy for predation avoidance in anurans, characterized by their capacity to sequester dietary alkaloids, a trait that has evolved multiple times independently. Despite over 1200 compounds being isolated in anuran toxins through extensive research, the mechanisms underlying alkaloid sequestration remain largely unknown. This study delves into the biochemical pathways employed by *Adelphobates galactonotus*(Steindachner 1864), a species notable for its ability to modify ingested alkaloids. We focused on the hydroxylation of consumed pumiliotoxin PTX (+)-251D into allopumiliotoxin aPTX (+)-267A. Using advanced analytical techniques like Ultra Performance Liquid Chromatography - Triple Quadrupole Mass Spectrometry (UPLC-TQ-MS), we examined the distribution and transformation of these alkaloids across various organs, notably the liver, skin, and digestive tract. Introducing a novel protocol for sample preparation using Solid-Phase-Extraction (SPE), our study elucidates the translocation patterns of these alkaloids within the body, along with the ratios changes. Our findings highlight the liver's role in initial alkaloid metabolism and the skin's function as a primary depot for alkaloid storage. Additionally, we report the presence of these alkaloids in the fat bodies, suggesting a passive storage mechanism This study advances our understanding of anuran chemical defense and metabolic adaptations, offering insights for species conservation and pharmacological research.

1. Introduction

Poison frogs encompass a diversity of anuran lineages among five families Cardall et al. (2004), and are distinguished by their remarkable arsenal of defensive chemicals and varied ecological behaviors Grant et al. (2017). Species of the South American Bufonidae (*Melanophryniscus*) Daly et al. (1984), Cuban Eleutherodactylidae (*Eleutherodactylus limbatus* group), Madagascan Mantellidae (*Mantella*) Garraffo et al. (1993), Australian Myobatrachidae (*Pseudophryne*) Daly et al. (1984), and Neotropical Dendrobatidae are all known for their potent lipohilic alkaloids. These poison frog species are the focus of intense study due to complexity of their alkaloid profiles Mebs et al. (2008); Daly et al. (1992), which are strongly influenced by their habitats and reflect significant evolutionary adaptations Myers et al. (1995); Saporito et al. (2006). More than 1200 lipophilic alkaloids have been identified where they play a role in chemical defence against predators and microbial infection Saporito et al. (2012); Hovey (2018); Toledo and Jared

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(1995). Frog alkaloid profiles vary considerably across species, populations, and individuals Saporito et al. (2010a, 2012); Mebs et al. (2008); Daly et al. (1992). The observed chemical diversity is influenced by geographic location Saporito et al. (2006); Daly et al. (2007), seasonality Saporito et al. (2007a), ontogeny Saporito et al. (2010b), and age and body size Jeckel et al. (2015).

However, the mechanisms by which these frogs incorporate alkaloids into their dermal skin glands are still not fully understood Santos et al. (2016); Caty et al. (2019). Dietary sources, and in particular formicine ants and oribatid mites Saporito et al. (2004, 2007b); Daly et al. (1994b); Saporito et al. (2011); Takada et al. (2005), have been identified as primary contributors to the alkaloid profiles in poison frogs. Experimental evidence has consistently shown these arthropods as significant sources, especially of pumiliotoxin (PTX) alkaloids Saporito et al. (2007b, 2009, 2012); Daly (1998). which were first identified and characterized in *Oophaga pumilio* by Daly and Myers (1967) , but are now known to be a widespread class of alkaloids that includes ca. 50 different pumiliotoxins and allopumiliotoxins (aPTX), along with numerous deoxy and desmethyl analogs Michael (2016); Daly et al. (2005). The most notable examples of PTX derivatives are based on quinolizidine and indolizidine structures, such as homopumiliotoxins (hPTX) and aPTX, which are among the most prevalent in these frogs Saporito et al. (2012); Michael (2016).

A subset of poison frogs is able to chemically modify certain dietary lipophilic alkaloids Daly et al. (2003); Jeckel et al. (2022); Daly et al. (1997a); Saporito et al. (2012); Smith et al. (2002), a trait not universally observed, as most species appear to accumulate alkaloids without alteration Saporito et al. (2012). Certain species of *Pseudophryne* (Myobatrachidae) perform modifications such as reductions and hydroxylations Daly et al. (1994a, 1997b, 2003); Saporito et al. (2012) and appear to enzymatically alter PTX 307A into derivatives with molecular masses of 309, 323, and 325 Smith et al. (2002); Sague et al. (2023).

The enantioselective transformation Daly et al. (2003) of the naturally occurring enantiomer PTX (+)-251D into the hydroxylated aPTX (+)-267A, Fig. 1, represents a notable biochemical process with implications for both defense and pharmacology Daly et al. (2003); Weldon et al. (2006, 2013); Küpfer and Branch (1985); Zhu et al. (2022). Recently methylation of decahydroquinoline (DHQ) was described in *Adelphobates galactonotus* Jeckel et al. (2022); Daly et al. (2009). Such modifications suggest a broader spectrum of enzymatic activity within these amphibians than previously understood Smith et al. (2002).

While such modifications can expedite the excretion of xenobiotics, enhancing detoxification by making the compounds more polar and thus more readily excreted Gibson and Skett (2001), this particular transformation of PTX 251D results in a hydroxylated compound five times more toxic when subcutaneously injected into mice Daly et al. (2003). Feeding experiments have found that certain dendrobatid frogs, such as *A. galactonotus* and *A. cataneoticus*, can metabolize up to 70% of dietary provided PTX 251D into the more toxic aPTX 267A Daly et al. (2003), suggesting the presence of an enzyme responsible for the 7'-hydroxylation process. However, this enzymatic activity is not yet fully understood

and limited to some sequestering species Alvarez-Buylla et al. (2022b,a, 2020); for example, only PTX 251D has been found in species like *Phyllobates bicolor* and *Epipedobates tricolor* Daly et al. (2003); Alvarez-Buylla et al. (2022b). In our study, the objective was the investigation of the organs involved in the process of hydroxylation and storage of PTX 251D and aPTX 267A in *A. galactonotus*, native to the southern Amazon River basin in Brazil. This species emerges as an exemplary model organism for the study of the intricate connections between ecology, evolution, and biochemistry Hoogmoed and Avila-Pires (2012); Jeckel et al. (2019).

The hydroxylation, is a process not only observed in *A. galactonotus* Daly et al. (2003) but also in other Dendrobatinae species such as *O. pumilio* Daly et al. (2003); Mebs et al. (2008); Caty et al. (2019) and *Dendrobates tinctorius* Alvarez-Buylla et al. (2022b); O'Connell et al. (2020); Saporito et al. (2012). In this study, we conducted two experiments targeting the skin, liver, digestive tract, fat bodies, kidney, and gallbladder of *A. galactonotus*. Our aim was to determine where hydroxylation occurs within these organs and to quantify the extent of hydroxylation over time, expressed in the hydroxylation ratio aPTX 267A to PTX 251D. To this end, adult specimens of *A. galactonotus* were subjected to a regimen of varying doses of PTX 251D, allowing us to observe and compare metabolic responses across their diverse tissues. Herein, we present for the first time evidence of hydroxylation occurring in the skin, the presence of alkaloids in the fat bodies, and the excretion of these compounds through urine, adding to *A. galactonotus* skin based chemical defense mechanism.

2. Material and Methods

2.1. Poison Frogs: *Adelphobates galactonotus*

The specimens of *A. galactonotus* used in this study were F1 captive-bred at the University of São Paulo Institute of Biosciences adults collected at Floresta Nacional de Caxiuanã, Pará, Brazil, 1°48′16.87″ S, 51°26′45.31″ W Jeckel et al. (2019), SISBIO: 54640-1b. The research was registered and approved by the Comissão de Eťica no Uso de Animais under No. 402/2023 - CEUDo Institute of Biosciences of the University of São Paulo. In the first experiment, the average weight of the frogs was 2.76 ± 0.579 g, while in the second experiment, it was 3.48 ± 0.858 g. Assignment to groups was based on weight, to counter act for bias in organ size, and therefore different metabolization rates. Each frog weighing at least 2.0 g. Sex could not be determined prior to euthanasia. The frogs were maintained in terraria in groups of 3 - 5 individuals at 70 - 80% relative humidity, 28°C, and natural light cycle of close to 13h. Their diet consisted of continuous feeding with wingless *Drosophila melanogaster*, Collembola, and isopods, supplemented twice weekly with flies dusted with NEKTON-Rep vitamin powder (Nekton Produkte). During the experiments, each frog was housed individually in a 15 x 15 x 13 cm plastic container with a moist paper towel substrate, a water dish, and a shelter and was provided *Drosophila ad libitum* every other day. The research was registered and approved by the

Comissão de Ética no Uso de Animais, under No. 402/2023 - CEUA at the Institute of Biosciences of the University of São Paulo.

2.2. Chemicals and reagents

High-performance liquid chromatography (HPLC) grade methanol, acetonitrile, and pro analysis (P.A.) grade acetic acid were procured from J.T.Baker (Phillipsburg, NJ, USA). Ultra-pure-water was obtained using a MilliQ water purification system (Millipore, Billerica, MA, USA). Pumiliotoxin (+)-251D, utilized for both frog feeding and ultra-performance liquid chromatography (UPLC) analysis, was sourced from Richard W. Fitch and Ralph Saporito Daly et al. (2003). The PTX 251D was received as a white powder. A Thermogravimetric Analyzer (TGA-51) from Shimadzu, located in the laboratory of Jivaldo do Rosário Matos at the Departamento de Química Fundamental, USP, was employed to prepare the stock solution for the experiments. During the experimental work we observed the hygroscopic nature of PTX 251D, by water accumulation in the vials when uncovered. Mass analysis revealed four minor impurities with a Indolizidine structure in the supplied PTX 251D with masses of $m/z = 238$, 250, 268, and 308, among others, with unidentified base structures that have no significant amount, less than 5% of alkaloid signal in mass spectrometry analysis. It remains uncertain whether these impurities resulted from degradation or were byproducts of synthesis, which rendered the obtained PTX 251D unsuitable as a standard for precise quantification.

To ascertain the hydroxylation of PTX 251D into its derivative aPTX 267A in the samples, we utilized gas-phase fragmentation reactions of protonated PTX 251D and aPTX 267A in high-resolution electrospray ionization (ESI) mass spectrometry, performed on a micrOTOF-QII mass spectrometer equipped with a time-of-flight (TOF) analyzer (see Minder et al. (2023) for details).

2.3. Experimental

2.3.1. Alkaloid administration and sample collection

Consistent with the protocol etablished by Jeckel et al. Jeckel et al. (2022), we administered a daily dose of 10 µg of PTX 251D to each frog of the experimental group. The alkaloid was orally delivered in a 5 µL volume of a 50% ethanol solution, following the method outlined by Jeckel et al. Jeckel et al. (2022). The control group (CG) was administered 5 µl of 50% ethanol without the PTX 251D. The administration involved gently opening the frog's oral cavity laterally with a 1000 µL pipette tip, allowing for the precise deposit of the alkaloid solution with a 5 µL micropipette, thus minimizing spillage and ensuring the frog did not close its mouth prematurely. A pilot study on a single individual indicated no adverse behavioral effects post-administration, except for initial tongue protrusion and approximately 8 minutes of rapid breathing. Post-dose, frogs were immediately offered fruit flies to mitigate any potential effects of the alcohol and to encourage natural predatory behavior. In the experiment, each frog's behavior was observed post-treatment to ensure the absence of any abnormalities. We incidentally collected urine samples from two individuals

while they were hovering over the to falcon tubes, before inserting them, prior to euthanasia. Euthanasia was conducted by cooling in a falcon tube, followed by flash-freezing in liquid nitrogen, as described by Lillywhite et al. Lillywhite et al. (2017), Shine et al. Shine et al. (2015), and Jeckel et al. Jeckel et al. (2022). Individual organs (see below) and carcasses were excised and wet-weighed individual organs (see below) using a Tecnal analytical balance ($\pm 0.0001g$) and stored at -20°C in 1 mL of 100% methanol, noted by weight to account for volume variability due to temperature changes and evaporation. We first secured the 4 mL glass vials containing the samples with Teflon-lined caps and then meticulously sealed them using Parafilm to prevent evaporation. Owing to their larger size, the skin and carcass were preserved in 2 mL and 3 mL of methanol, respectively.

2.3.2. Experiment 1 – Constant amount and variable time

Experiment 1 was designed as a preliminarily exploration to inform experiment 2, by determining the duration of the hydroxylation process, and enable us to chose an appropriate timeframe and identify the organs involved. From the initial n = 13 individuals selected and allocated into three experimental groups (1.1, 1.2, 1.3) with four independent replicates, and one negative control group, two individuals were subsequently excluded from the final analysis (see 2.4.3). A single control group was deemed sufficient given that the hydroxylation of PTX 251D to aPTX 267A had already been established by Daly et al.Daly et al. (2003). Group 1.1 comprised two males and two females; group 1.2 included only females; group 1.3 comprised three females and one male; and the control group was represented by a single male. The groups varied by the interval between the final dose of alkaloid and euthanization. All groups were administered a second dose 45 h after the initial dose. Group 1.1 was euthanized 3 hours after the last dose, while the subsequent groups were euthanized at 27 hours and 51 hours, respectively, with each individual receiving a total of two times approximately 10 µg of alkaloid, Fig. 2. The control group was administered 5 µl of 50% ethanol without the alkaloid, following in the same time intervals as the protocol of the third experimental group, 1.3. Following euthanasia, we excised the digestive tract (stomach and intestine), liver, skin for chemical analysis.

2.3.3. Experiment 2 – Variable amount, variable time

The purpose of this experiment was to test the correlation between escalating consumed PTX 251D amounts and the efficacy of hydroxylation, with a particular focus on understanding if the hydroxylation reached a plateau, exhibited linearity, exponentiality, or manifested any distinct biodistribution phenomena. To avoid risking the health of the frogs, the higher doses were administered over several days. We assigned n = 21 individuals to this experiment, which were distributed across four experimental groups, each with five independent replicates, and one negative control, Fig. 3. The sex distribution was following: group 2.1 comprised one male and four females; group 2.2 had four females and one male; groups 2.4 and 2.4 each had three females and two males; the control was a single female. Each experimental group was administered a daily dose of 10 µg of alkaloid. The control group was administered 5 µl of 50% ethanol

without the alkaloid, following the same time interval protocol of the fourth experimental group, 2.4.
Group 1.1 individuals received a single 10 µg dose and were euthanized 4 days post-administration. Group 2 received two 10 µg doses administered 24 hours apart, with euthanasia occurring 4 days after the final dose. Group 3 was given three 10 µg doses, each separated by 24 hours, with individuals euthanized 4 days subsequent to the last dose. Lastly, Group 4 received 4 daily doses of 10 µg each, culminating in euthanasia 4 days after the final dose. Euthanization occurred 4 days after the final dose to ensure any unsequestered alkaloids were cleared prior to chemical analysis and maximize the potential hydroxylation of PTX 251D (Fig. 3). The control group was administered 5 µl of 50% ethanol for 4 days. Following euthanasia, we excised the digestive tract, liver, skin, spleen, fat bodies (abdominal), and kidneys for chemical analysis. In the case of the gallbladder, its weight was below the measurement error margin of our balance, allowing only for qualitative analysis.

2.4. Ultra pressure liquid mass spectrometry analysis

2.4.1. Sample preparation with solid phase extraction (SPE)

For direct injection into the Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS) system, we utilized Supelclean LC-18 SPE 100 mg, 1mL tubes from Supelco, attached to a Supelco Visiprep 24 DL to expedite the filtration process via vacuum. The column was conditioned with 1 mL of methanol and 2 mL of milipore water, followed by the addition of 1 mL of sample solution (comprising 200 µL of methanolic organ extract and 1800 µL of H_2O). This was followed by two washes with 500 µL of H_2O and an elution with 200 µL of methanol, which was then collected directly into a glass vial using a micro glass tube for UPLC analysis. Due to the higher dilution of the skin samples, we doubled the volume of sample solution, to compensate. Prior to sealing the vial, we added 10 µL of a 4.2 µg/mL caffeine solution as an internal standard to facilitate area comparison in subsequent analyses.

2.4.2. Mass spectrometry

The analysis was performed using an Acquity Ultra Performance Liquid Chromatography Triple Quadrupole Mass Spectrometry (UPLC-TQ-MS) System (Waters Corp., Massachusetts, EUA) and the chromatographic separation was achieved on a Kinetex XB-C18 column 50 mm x 2.1 mm, 1.7 µm (Phenomenex, California, EUA) with a pre-column of the same material, maintained at 30°C. The mobile phase was composed of solvent A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The elution program was as follows: 10% B (initial), 10-100% B (12 min), 100% B (15 min), 100-10% B (16 min), 10% B (20 min). The flow rate was 0.3 mL min-1, the injection volume was 2 µL, and the autosampler temperature was set to 10°C. The MS was operated in the positive ion and multiple reaction monitoring (MRM) mode using an electrospray voltage of 2.5 kV, source temperature of 150°C and desolvation temperature of 400°C. Nitrogen was used as a desolvation gas (1,000 L h-1) and as a cone gas (50 L h-1). Data interpretation was facilitated by MassLynx V 4.1 Software (Waters Corp.). To preclude potential cross-contamination of alkaloids

or other molecules between samples, we injected, the same organs together in sequence, for example all the liver samples followed by a blank and the sequence of all the digestive tract samples. Control samples were processed first, followed by respective organ samples, with a blank run between each organ type to guarantee no carryover. The mass spectrometer was calibrated to detect specific transitions: PTX 251D at *m/z* = 252 to 134, aPTX267 at *m/z* = 268 to 174, "PTX233" at *m/z* = 234 to 176, and "PTX 249" at *m/z* = 250 to 174. An alkaloid was considered present and confidently detected only if the signal-to-noise ratio (S/N) of the mass spectra surpassed a threshold of three times the baseline height.

2.4.3. Statistical analysis

The data obtained from the spectrometer (area) was normalized in relation to both the internal standard (caffeine) and organ size by dividing the alkaloid area by the caffeine area then the weight of the organ. We refer to this value as the normalized area or area. The normalized area of aPTX 267A was divided by the normalized area of PTX 251D to obtain the aPTX 267A:PTX 251D hydroxylation ratio. Individuals with z-scores greater than 3.00 for the hydroxylation ratio were excluded to remove outliers more than three standard deviations from the mean, preventing undue influence on our analysis. We tested for normality and homogeneity of variances using the Shapiro-Wilk and Levene tests, respectively, and proceeded to conduct one-way analyses of variance (ANOVA) with Tukey's honestly significantly different (HSD) test for parametric analyses and the Kruskal-Wallis test with Dunn's post-hoc test for non-parametric analyses. We used a linear single regression model to test for trends of the areas of the alkaloids of the skin within groups. Due to the limited sample size, and to mitigate the influence of extreme values, the median was employed as the measure of central tendency in hydroxylation ratios and areas for comparison within organs. Statistical analyses and graphical representations were executed using the ggplot2 package Wickham (2016) in R Studio, R-4.3.1 Team (2022). Statistical language was used in accordance with the guidelines presented by Muff et al. (2022) Muff et al. (2022).

3. Results

3.1. Experiment 1:

We detected both PTX251D and aPTX267A all organs of all experimental groups (Fig. 4). For the skin, we found evidence for an inverse relationship between PTX251D and aPTX267A, with the amount of aPTX 267A increasing (F(1, 8) = 7.357, β = 7.958, p = 0.027, R^2_{adj} = 0.414) and the amount of PTX251D decreasing (F(1, 8) = 6.055, β = -7.413, p = 0.04, R^2_{adj} = 0.36) among subsequent groups. For the digestive tract we found evidence for a positive trend in aPTX (F(1, 8) = 11.15, β = 4.134, p = 0.01, R^2_{adj} = 0.53) and no significant trend in PTX 251D (F(1, 8) = 1.817, β = -0.513, p = 0.215, R^2_{adj} = 0.083). The liver evidenced a negative trend in PTX 251D (F(1, 8) =

14.52, β = -7.336, $p = 0.005$, $R^2_{\text{adj}} = 0.6$), and a non significant one of aPTX 267A (F(1, 8) = 0.09702, β = -1.242, $p = 0.763$, $R^2_{\text{adj}} = -0.112$).

We detected very strong evidence for a group effect on the hydroxylation ratio (ANOVA; F(2, 27) = 32.6, $p = 6.31 \times 10^{-8}$) Median ratios increased across groups: 1.86 (group 1.1), 3.68 (group 1.2), 7.61 (group 1.3), indicating greater hydroxylation with increasing amounts of alkaloid and time. This result represented a ratio increase of a factor of 1.98 after the initial 24 h and 2.07 in the subsequent 24 h (Fig. 5). There is moderate evidence for variation among the groups for the digestive tract (F(2, 7) = 7.24, $p = 0.02$; med = 2.23, 5.6, 7.99) and strong evidence for the liver (F(2, 7) = 12.62, $p = 0.005$; med = 1.54, 3.34, 6.70) and the skin (F(2, 7) = 19.06, $p = 0.002$; med = 2.06, 3.68, 7.70), specifically groups 1.1 and 1.3 differed for the digestive tract (Tukey HSD, $p = 0.02$) and liver (Tukey HSD , $p = 0.004$) and groups 1.1 and 1.3 (Tukey HSD, $p = 0.001$) and 1.2 and 1.3 (Tukey HSD, $p = 0.023$) for the skin. For all organs, the median hydroxylation ratios were greatest for group 1.3 (digestive tract: med = 7.99; liver: med = 6.70; skin: med = 7.70; (Fig. 5).

3.2. Experiment 2:

In the course of the second experiment, which initially comprised n = 21 individuals, one individual from each group was excluded (see 2.4.3). These exclusions were necessitated by two instances of data loss and two cases in which the individuals presented anomalous alkaloid distribution profiles. We detected both aPTX 267A and PTX 251D and in all organs of all experimental groups (Fig. 6 and 7), except the spleen. Most individuals (n = 15, of 17) accumulated aPTX 267A in the kidney and the fat bodies (Fig. 7 and 8). We found in 10 of 15 individuals qualitative evidence (see 2.3.2) in the gallbladder for both alkaloids. In the skin we found strong evidence of variation among groups in the amounts of aPTX 267A (ANOVA, F(3, 12) = 8.277, $p = 0.003$) but not PTX 251D (Kruskal-Wallis, $\chi^2(3) = 6.441, p = 0.092$). A trend analysis revealed an increase in the skin of PTX251D (F(1, 14) = 8.723, β = 8.111, $p = 0.01$, $R^2_{\text{adj}} = 0.34$) and aPTX (F (1, 14) = 18.35, $\beta = 24.91$, $p < 0.001$, $R^2_{\text{adj}} = 0.5363$) among subsequent groups (Fig. 6. For the other organs no significant trend could be observed (Fig. 6 and 7). Hydroxylation ratios did not differm among the four groups for skin, digestive tract, and liver (Kurskal-Wallis test: $\chi^2(3) = 3.9201, p = 0.2702$). The group median hydroxylation ratios were 5.28 (group 2.1), 4.39 (group 2.2), 6.35 (group 2.3), and 5.75 (group 2.4). Likewise, differences in the hydroxylation ratios among groups were not significant for the digestive tract (med = 4.96, 4.38, 10.64, 8.43), liver (med = 3.72, 5.15, 6.35, 5.57), skin (med = 6.55, 4.45, 5.99, 4.97), or fat bodies (med = 6.0, 4.8, 6.6, 3.4). An increase of median hydroxylation ratios in the kidney was found (F (1, 2) = 19.7, $\beta = 67.03$, $p = 0.048$, $R^2_{\text{adj}} = 0.8617$; med =11.3, 12.5, 25.2, 29.3; Fig. 7), although the differences among groups were not significant (Kruskal-Wallis, $\chi^2(3) = 3.5792, p = 0.31$),

4. Discussion

This study identified the main organs involved in the hydroxylation of PTX 251D, including their roles as accumulation sites, metabolic pass-through sites, or hydroxylation sites. The liver, skin, digestive tract, fat bodies, kidney, and gall bladder in *A. galactonotus* all possessed PTX 251D and its metabolite aPTX 267A; however the spleen did not contain PTX 251D or aPTX 267A. On the basis of our findings, the skin appears to serve primary alkaloid accumulation site, the liver as a hydroxylation and accumulation site, and the possible accumulation in fat bodies. Our findings indicate that *A. galactonotus* does not fully hydroxylate PTX 251D 4 days post-consumption, indicating that, although hydroxylation of PTX 251D begins immediately after consumption, it progresses more slowly than previously thought Alvarez-Buylla et al. (2022b). Although PTX 251D and aPTX 267A detection relied solely on their exact masses, which precludes detection of complexes or metabolites formed during sequestration and hydroxylation and could lead to underestimation alkaloid quantities. Below we employ comparisons of the ratios of aPTX 267A to PTX 251D (hydroxylation ratios) and quantities, we reconstruct potential metabolic responses and pathways during ingestion, excretion, and sequestration. Xenobiotic metabolization transforms organic molecules into more hydrophilic forms through hydroxylation, sometimes producing more toxic substances like aPTX 267A for easier excretion, enhancing detoxification and increasing compound polarity for urinary elimination Remmer (1970); Casarett (2008); Daly et al. (2003); Ose et al. (2017). It is important to note that PTX 251D and aPTX 267A detection relied solely on their exact masses, excluding potential complexes or metabolites formed during sequestration and hydroxylation, which could lead to underestimation of detected amounts due to unrecognized alkaloid-protein complexes.

4.1. Liver

In experiment 1, we tested the effect of time on hydroxylation, and we found progressive increase in hydroxylation ratio every 24hours (Fig. 5B). In experiment 2, all test groups of experiment 2 remained positive for both alkaloids 4 days post-ingestion, (Fig. 6B).

Our findings, support the liver's key function in detoxifying and metabolizing xenobiotics Casarett (2008); Alvarez-Buylla et al. (2020, 2022b); Caty et al. (2019).Xenobiotic metabolization oxidize lipid-soluble molecules into more hydrophilic, more polar forms through hydroxylation, over a variety of microsomes, cytochromes, and enzymes, sometimes producing more toxic substances such as probably PTX 267A Daly et al. (2003); Remmer (1970); Grant (1991). These water-soluble substances can be directly eliminated or they can be conjugated to highly polar acids Remmer (1970); Almazroo et al. (2017), aiding urinary excretion and enhancing detoxification Casarett (2008); Ose et al. (2017).

Our experiments indicated that sustained consumption of substantial PTX 251D quantities, may lead to their accumulation in the body, the liver, leaving the possibly at very high doses causing organ-specific toxicity Remmer

(1970); Omura (1999). A literature search showed that cytochrome P450 plays a pivotal role, shielding the organism from external toxins through hydroxylation reactions, including aromatic and aliphatic hydroxylation and N-oxidation Jandacek and Tso (2001); Omura (1999) and possibly the recently discovered methylation of DHQ in *A. galactonotus* Jeckel et al. (2022).). This scenario arises when liver enzymes, specifically cytochrome P450 in the endoplasmic reticulum, are unable to efficiently metabolize hydrophobic, chemically inert compounds into excretable, water-soluble metabolites at a sufficient rate Remmer (1970).

In scenarios where enzyme specificity is lacking, as it could be the case here, competition for binding sites emerges within liver cells, at high xenobiotic concentrations Remmer (1970), which can have a dose pendent effect on experimental out comes. Therefore, we recommend avoiding ethanol in future cytochrome reaction studies due to its possible competitive oxidation with PTX 251D, potentially impacting early hydroxylation efficiency Kurose et al. (1996); Zakhari (2006); Chacín et al. (1991); Cederlund et al. (1991). An oil-based transporter would better enable cytochrome activity to be assessed Zhang et al. (2016)), expected to yield a higher hydroxylation ratio, especially in the early hours of the experiment.

In the last century, Remmer (1970) Remmer (1970) suggested that the liver acts as a temporary storage for xenobiotics and alkaloids. We cannot confirm the same hypothesis as them, , but our data showed accumulation of both alkaloids in the liver. The same article showed that a higher liver bodyweight ratio led to a higher cytochrome P450 availability in a rat human comparison. In our study, the liver of *A. galactonotus* constituted an average of 3.9% of body weight across 34 frogs, implying a 4 to 6 times higher cytochrome P450 availability than in humans, facilitating more efficient processing of high xenobiotic exposure Remmer (1970). Contrasting with human liver cytochrome CYP2D6, where PTX 251D clearance occurs within 90 minutes to an unidentified hydroxylation product Alvarez-Buylla et al. (2022b). Our results reveal a notably slower hydroxylation process in the vivo animals, not completed after 4 days, supporting Remmer (1970) statment, that the hydroxylation is a rate-limiting and relatively slow step. We would need to isolate microsomes, to test under the same experimental conditions as Alvarez-Buylla (2022) Alvarez-Buylla et al. (2022b) to have a fair comparison with their found hydroxylation time.

Further, variability in P450 levels across individuals underscores the complexity of xenobiotic metabolism, influencing the effectiveness of detoxification processes Remmer (1970); Omura (1999). Genetic diversity of P450s affect its availability and may account for the differential capabilities among frog species to hydroxylate PTX251D Thomas (2007); Fink-Gremmels (2008); Ingelman-Sundberg et al. (2007), as some species exhibit this ability while others do not Daly et al. (2003); Alvarez-Buylla et al. (2022b). Future research should address the cytochrome variability in *A. galactonotus* and, more broadly, poison frogs. Additional, longer-duration experiments are necessary to understand hydroxylation dynamics thoroughly. Furthermore, it's essential to test the limits of liver hydroxylation and its capacity to process alkaloids, especially under high PTX 251D exposure, to ensure experimental accuracy.

4.2. Digestive tract

The digestive tract of *A. galactonotus* exhibits lower PTX / aPTX amounts than the liver and skin but is crucial in alkaloid uptake. Our findings corroborate the findings of Alvarez-Buylla et al. Alvarez-Buylla et al. (2022b) in *Dendrobates tinctorius*, that metabolic activities related to PTX 251D occur in the digestive tract. Alkaloid detection in the digestive tract 4 days after ingestion suggests either a rerouting of aPTX 267A to the intestine following liver hydroxylation or local hydroxylation, potentially involving cytochromes or microbial flora.

O'Connell et al. (2021) reported an increase in fatty acid binding proteins in the intestine, which are responsible for transporting lipophilic substances, following alkaloid exposure in poison frogs. Given its lipophilicity, pumiliotoxin absorption likely occurs via passive via passive diffusion through cell membrane Gibson and Skett (2001) or, alternatively via ABC transporter proteins Santos et al. (2016) into the blood or plasma. Investigating the transport of lipophilic molecules, either through lymphatic chylomicrons Randolph and Miller (2014) or in the bloodstream with liver-synthesized carrier proteins Alvarez-Buylla et al. (2023), is a critical yet understudied aspect of poison frog physiology O'Connell et al. (2021). Despite the intestines' lower cytochrome abundance Remmer (1970); Kirischian and Wilson (2012), the increase in Cytochrome P450, specifically CYP3A29, post PTX 251D ingestion Alvarez-Buylla et al. (2022b), needs further exploration of enzymatic pathways and symbiotic interactions that prove PTX 251D hydroxylation in the digestive tracts of poison frogs, as literature shows. Our study did not separate stomach and intestine for analysis, which limits further conclusions in this area. An other potentially important aspect of xenobiotic metabolism, is the biliary excretion. In this process, xenobiotics are transported to the gut and subsequently expulsed from the body Bardal et al. (2011); Jandacek and Tso (2001). Moreover, investigating bile acid-related pathways is compelling, particularly with findings of bile acid derivatives in mantellid poison frogs' skin Clark et al. (2012). This suggests the presence of a bile acid-based transport system for certain alkaloids in poison frogs, a hypothesis that merits further investigation in the context of *A. galactonotus* and other related species.

4.3. Skin

Experiment 1 revealed an inverse relationship between PTX 251D and aPTX 267A amounts in the skin: as aPTX 267A increased, PTX 251D decreased (Fig. 4C). Experiment 2 further supported these findings, demonstrating a significant rise in aPTX 267A and a modest increase in PTX 251D, consistent with increased administered doses of PTX 251D (Fig. 6C). Aligning with the literature Dumbacher et al. (2009), our study confirms that the skin of *A. galactonotus* serves as a key site for accumulation of PTX 251D and aPTX 267A (Fig. 4C and 6C).

Both experiments clearly demonstrate an increase in the hydroxylation ratio over time and at higher greater amounts of PTX 251 are consumed, and pointing towards a specific behavior of PTX 251D. We propose three potential explanations for our observed pattern. First, PTX 251D may exhibit a greater propensity for excretion due to its

lower polarity as compared to the hydrogen bonds of allopumiliotoxin, therefore favoring the accumulation of the more hydrophilic, hydroxylated alkaloid aPTX 267A. Second, aPTX could be transported to the skin faster than PTX. And third, additional hydroxylation of PTX 251D could occur in the skin. Additional research is required to test these different hypotheses. The precise chemical mechanism responsible for PTX 251D hydroxylation is unknown, but a number of potential mechanisms exist, particularly the impact of reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$), which are crucial to consider Proctor and Reynolds (1984). Such ROS are regulated in aerobic organisms by systems like cytochrome oxidase and cytochrome P-450 monooxygenase Black (1987); Proctor and Reynolds (1984). Amphibians' distinctive skin respiration Tattersall (2007), likely enhances the interaction between stored alkaloids and both endogenous and exogenous ROS Black (1987). Moreover, the presence of cytochromes in the skin, which, as shown in mammalian studies, may have lower but as well significant metabolizing activities Oesch et al. (2007); Huang et al. (2016); Svensson (2009) points to enzymatic processes occurring within amphibian skin as well. Given the precedents in animal physiology and human pharmacology, the occurrence of hydroxylation within the skin should not be entirely unexpected, though it may occur on a much smaller scale than other organs.

This raises the prospect of a more complex alkaloid metabolism in the skin, necessitating prolonged studies with consistent alkaloid exposure to determine if complete hydroxylation of PTX 251D occurs. A secondary hydroxylation mechanism also opens up intriguing questions about the modification of other alkaloids in the skin. A deeper understanding of the skin's metabolic activities could shed light on the origins of various alkaloids, especially where dietary sources remain unidentified. This could significantly alter our current understanding of alkaloid profiles and alkaloid origins in poison frogs, with many still unidentified Saporito et al. (2012). Future research should expand to cover both liver metabolism but also skin-based processes, potentially unveiling new aspects of alkaloid dynamics. However, delineating the precise hydroxylation mechanisms in *A. galactonotus* requires further empirical exploration. The current absence of definitive evidence highlights the important need for caution in speculation, emphasizing the necessity for additional research to confirm these proposed pathways.

4.4. Fat bodies

Within the abdominal cavities of frogs, fat bodies serve as vital sources of energy for reproduction and hibernation Jackson and Ultsch (2010) but also, as our findings indicate, can accumulate alkaloids. In *Adelphobates*, sequestration of aPTX 267A and PTX 251D within fat bodies is delayed, taking 3 to 4 days to reach detectable amounts, as illustrated in Fig. 8 and 7B. Specimens subjected to higher dosages in experiment 2 exhibited increased alkaloid amounts. This suggests a potential metabolic saturation in other organs, that prompts temporary storage in fat bodies, evidenced

by a lower hydroxylation ratio reflecting greater PTX 251D retention. However, these quantities in fat bodies are considerably less than those in primary organs (Fig. 8).

To date, no reports have documented the presence of alkaloids within fat bodies of poison frogs since they were left out, with research primarily emphasizing organ-specific studies Jeckel et al. (2022); Saporito et al. (2006); Bolton et al. (2017) or analyses of whole animal extracts Mebs et al. (2007). But, pharmaceutically active compounds (PhACs) have been found in the fat bodies of *Rhinella arenarum* Salgado Costa et al. (2023).

Alkaloids stored in fat bodies suggest 'environmental acquisition', where individuals in this concept transiently increase their internal toxin amounts temporary, caused by normal metabolic activity. Analogously, grasshoppers consuming toxic plants accumulat these substances within their digestive tract, gaining protection against predators Sword (1999), as long as the digestion takes place. Greyer et al. Geyer et al. (1993) hypothesized that the substance toxicity might inversely correlate with the amount of body fat in animals, proposing fat as a reservoir limiting toxic exposure to vital organs. This concept becomes particularly intriguing when considering the primary sequestration of alkaloids in the skin of poison frogs, suggesting that skin storage is an active process, while fat body absorption is passive. Our data, however, neither fully support nor refute this hypothesis for *Adelphobates*, the presence of traces is more consistent with expectations than it is an unforeseen anomaly.

Research has shown that xenobiotics like Tetrahydrocannabinol (THC), a cannabinoid, are passively sequestered in fat and can be released back into circulation, a process known as 'reintoxication' Gunasekaran et al. (2009); Nahas (2001). A similar mechanism may apply to the much more polar alkaloids in our study, ultimately impacting absorption efficiency.

In *A. galactonotus*, it is conceivable that older and larger individuals may exhibit more robust defense mechanisms due to their greater fat reserves, which may serve as a temporary alkaloid repository. Studying wild-caught *A. galactonotus* may reveal how fat-body alkaloid accumulation confers ecological benefits, potentially enhancing predator-prey interactions and overall fitness, or not at all. The relationship between body fat and alkaloid distribution, like the THC diffusion in other species, needs further investigation to clarify its significance in poison frogs.

4.5. Kidney and urine

We detected PTX 251D and aPTX 267A in the kidneys 4 days post-ingestion (Fig. 7B), with amounts comparable to those in fat bodies (Fig. 8). Notably, the detection of lower amounts of these alkaloids at earlier time points, specifically two days post-ingestion, indicates an excretion process in the kidneys. The kidneys showed much higher hydroxylation ratios than other organs (Fig. 7A), highlighting their role in excreting the more soluble aPTX 267A. Our findings suggest two primary routes for alkaloid clearance in *A. galactonotus*: renal excretion, which preferentially handles low amounts of the more hydrophilic aPTX 267A, and skin sequestration, which seems to be more effective, and quicker

in dealing with the major amounts. Thus, leading to the evolution driven chemical defense ability over the skin organ in these frogs. The data reveal further a proportionate increase in aPTX 267A amounts with higher doses of PTX 251D administered, indicating dose-dependent renal processing. However, the relatively low amounts of alkaloids in the kidneys imply that only a small fraction is excreted via urine.

Renal excretion, a key detoxification mechanism in vertebrates, may operate similarly in *A. galactonotus*, where reflexive urination under stress could contribute to a broader defense strategy Forzán and Horney (2020), alongside skin sequestration, with renal excretion offering a weaker yet less energy-intensive method of defense. We cannot definitively say whether this is truly an evolutionarily developed chemical defense, or simply a benefit arising from natural urination.

Amphibian urine collection is a chance depended task Forzán and Horney (2020), leading to the sampling of our study, where we were able to collect two urine by chance. This is not a sufficient sample size for any scientific conclusion, else then confirming presence in urine of PTX 251D and aPTX 267A in *A. galactonotus*. Further, we may not have detected all forms of these alkaloids in the kidney, such as acidic derivatives or partially metabolized variants. Future research, with improved urine collection techniques, should aim to investigate these aspects more thoroughly, considering the limited samples collected in our study. Dedicated sampling of urine or bladder content, could yield more definitive, statistically relevant insights into the renal excretion patterns of *A. galactonotus*.

5. Conclusion

We found the widespread presence of alkaloids in the main organs of *A. galactonotus*, such as the liver, skin, and digestive tract, with a notable preference for accumulating and metabolizing the hydroxylated form, aPTX 267A, over PTX 251D. We report for the first time the presence of these alkaloids in the fat bodies of poison frogs, suggesting a passive mechanism for temporary alkaloid storage. Our findings corroborate the skin's essential role as a primary site for long-term alkaloid storage, while also positing a possible secondary hydroxylation process in the skin, thereby adding a new dimension to our understanding of alkaloid profiles in poison frogs.

Additionally, this study pioneers the use of solid phase extraction (SPE) in sample purification for poison frog research. This innovative approach presents a viable alternative to traditional methods, significantly reducing the risk of alkaloid loss during sample preparation by reducing the number of preparations steps. Coupled with the utilization of UPLC-TQ-MS, known for its heightened sensitivity to multiple hydroxylated molecules and metabolites, our methodology enhances the detection and analysis of these complex substances, albeit without identifying the expected metabolites in this particular study. Unfortunately, we were unable to identify higher hydroxylated derivatives of Pumiliotoxin.

In conclusion, while our study sheds light on several aspects of alkaloid metabolism in *A. galactonotus*, it also unveils new queries, particularly regarding the hydroxylation pathways and their evolutionary significance in poison frogs.

Field observations indicate that several species of dendrobatid poison frogs, including *Dendrobates tinctorious*, *D. azureus*, and *Oophaga histrionica*, lack PTX 251D but contain only aPTX 267A, which Daly et al. Daly et al. (2003) hypothesized might originate directly from their diet. However, given that laboratory experiments have shown that *Dendrobates tinctorious* is able to convert PTX 251D to aPTX 267A Alvarez-Buylla et al. (2022b), the absence of PTX 251D in wild-caught frogs could also be due to through hydroxylation within the skin.

Future research should concentrate on dissecting the enzymatic pathways across various organs and detailing the role of specific cytochromes as well in the in the skin and the kidney. Given the diverse expression of these enzymes in organs central to absorption, metabolism, and excretion, a comparative analysis of these systems in different poison frog species, particularly focusing on hydroxylation rates, could offer profound insights into the efficiency and mechanisms of PTX metabolism among varying species. This research provided insights into the amphibian's metabolic adaptations for handling xenobiotics, revealing potential applications in human medicine. Our findings also highlighted the ecological significance and adaptive strategies of *A. galactonotus*, contributing to both species conservation and pharmacological research.

6. Conflicts of interest

The authors declare that there are no conflicts of interest.

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CRediT authorship contribution statement

Basil Minder: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Project administration. **Andres E. Brunetti:** Writing - Review & Editing. **Jacqueline N. Mendonça:** Methodology, Formal analysis. **Rodrigo Moreira da Silva:** Methodology. **Ralph A. Saporito:** Conceptualization,Resources, Writing - Review & Editing. **Noberto P. Lopes:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Funding acquisition. **Taran Grant:** Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding aquisition.

References

Almazroo, O. A., Miah, M. K., and Venkataramanan, R. (2017). Drug Metabolism in the Liver. *Clinics in Liver Disease*, 21(1):1–20.

Alvarez-Buylla, A., Fischer, M.-T., Moya Garzon, M. D., Rangel, A. E., Tapia, E. E., Tanzo, J. T., Soh, H. T., Coloma, L. A., Long, J. Z., and O'Connell, L. A. (2023). Binding and sequestration of poison frog alkaloids by a plasma globulin. *eLife*, 12:e85096.

Alvarez-Buylla, A., Moya-Garzon, M. D., Rangel, A. E., Tapia, E. E., Tanzo, J., Soh, H. T., Coloma, L. A., Long, J. Z., and O'Connell, L. A. (2022a). Binding and sequestration of poison frog alkaloids by a plasma globulin. Preprint, Physiology.

Alvarez-Buylla, A., Payne, C. Y., Vidoudez, C., Trauger, S. A., and O'Connell, L. A. (2020). Pumiliotoxin metabolism and molecular physiology in a poison frog. Preprint, Physiology.

Alvarez-Buylla, A., Payne, C. Y., Vidoudez, C., Trauger, S. A., and O'Connell, L. A. (2022b). Molecular physiology of pumiliotoxin sequestration in a poison frog. *PLOS ONE*, 17(3):e0264540.

Bardal, S. K., Waechter, J. E., and Martin, D. S. (2011). Chapter 2 - Pharmacokinetics. In Bardal, S. K., Waechter, J. E., and Martin, D. S., editors, *Applied Pharmacology*, pages 17–34. W.B. Saunders, Philadelphia.

Black, H. S. (1987). Potential Involvement Of Free Radical Reactions In Ultraviolet Light-Mediated Cutaneous Damage*. *Photochemistry and Photobiology*, 46(2):213–221.

Bolton, S. K., Dickerson, K., and Saporito, R. A. (2017). Variable Alkaloid Defenses in the Dendrobatid Poison Frog Oophaga pumilio are Perceived as Differences in Palatability to Arthropods. *Journal of Chemical Ecology*, 43(3):273–289.

Cardall, B. L., Brodie, E. D., Brodie, E. D., and Hanifin, C. T. (2004). Secretion and regeneration of tetrodotoxin in the rough-skin newt (Taricha granulosa). *Toxicon*, 44(8):933–938.

Casarett, L. J. (2008). *Casarett and Doull's Toxicology: The Basic Science of Poisons*, volume 71470514. McGraw-Hill New York.

Caty, S. N., Alvarez-Buylla, A., Byrd, G. D., Vidoudez, C., Roland, A. B., Tapia, E. E., Budnik, B., Trauger, S. A., Coloma, L. A., and O'Connell, L. A. (2019). Molecular physiology of chemical defenses in a poison frog. *The Journal of Experimental Biology*, 222(12):jeb204149.

Cederlund, E., Peralba, J. M., Pares, X., and Jörnvall, H. (1991). Amphibian alcohol dehydrogenase, the major frog liver enzyme. Relationships to other forms and assessment of an early gene duplication separating vertebrate class I and class III alcohol dehydrogenases. *Biochemistry*, 30(11):2811–2816.

Chacín, J., Cárdenas, P., Lobo, P., and Hernández, I. (1991). Secretory and Metabolic Effects of Ethanol in the Isolated Amphibian Gastric Mucosa. *Gastroenterology*, 100(5, Part 1):1288–1295.

Clark, V. C., Harinantenaina, L., Zeller, M., Ronto, W., Rocca, J., Dossey, A. T., Rakotondravony, D., Kingston, D. G. I., and Shaw, C. (2012). An Endogenous Bile Acid and Dietary Sucrose from Skin Secretions of Alkaloid-Sequestering Poison Frogs. *Journal of Natural Products*, 75(3):473–478.

Daly, J. W. (1998). Thirty Years of Discovering Arthropod Alkaloids in Amphibian Skin †. *Journal of Natural Products*, 61(1):162–172.

Daly, J. W., Garraffo, H. M., Spande, T. F., Clark, V. C., Ma, J., Ziffer, H., and Cover, J. F. (2003). Evidence for an enantioselective pumiliotoxin 7-hydroxylase in dendrobatid poison frogs of the genus Dendrobates.

Proceedings of the National Academy of Sciences, 100(19):11092–11097.

Daly, J. W., Gusovsky, F., Myers, C. W., Yotsu-Yamashita, M., and Yasumoto, T. (1994a). First occurrence of tetrodotoxin in a dendrobatid frog (Colostethus inguinalis), with further reports for the bufonid genus Atelopus. *Toxicon*, 32(3):279–285.

Daly, J. W., Highet, R. J., and Myers, C. W. (1984). Occurrence of skin alkaloids in non-dendrobatid frogs from Brazil (Bufonidae), Australia (Myobatrachidae) and Madagascar (Mantellinae). *Toxicon*, 22(6):905–919.

Daly, J. W., Martin Garraffo, H., Hall, G. S., and Cover, J. F. (1997a). Absence of skin alkaloids in captive-raised Madagascan mantelline frogs (Mantella) and sequestration of dietary alkaloids. *Toxicon*, 35(7):1131–1135.

Daly, J. W., Martin Garraffo, H., Spande, T. F., Jaramillo, C., and Stanley Rand, A. (1994b). Dietary source for skin alkaloids of poison frogs (Dendrobatidae)? *Journal of Chemical Ecology*, 20(4):943–955.

Daly, J. W. and Myers, C. W. (1967). Toxicity of Panamanian Poison Frogs (Dendrobates): Some Biological and Chemical Aspects. *Science*, 156(3777):970–973.

Daly, J. W., Padgett, W. L., Saunders, R. L., and Cover, J. F. (1997b). Absence of tetrodotoxins in a captive-raised riparian frog, Atelopus varius. *Toxicon*, 35(5):705–709.

Daly, J. W., Secunda, S. I., Garraffo, H., Spande, T. F., Wisnieski, A., Nishihira, C., and Cover, J. F. (1992). Variability in alkaloid profiles in neotropical poison frogs (Dendrobatidae): Genetic versus environmental determinants. *Toxicon*, 30(8):887–898.

Daly, J. W., Spande, T. F., and Garraffo, H. M. (2005). Alkaloids from Amphibian Skin: A Tabulation of Over Eight-Hundred Compounds. *Journal of Natural Products*, 68(10):1556–1575.

Daly, J. W., Ware, N., Saporito, R. A., Spande, T. F., and Garraffo, H. M. (2009). *N* -Methyldecahydroquinolines: An Unexpected Class of Alkaloids from Amazonian Poison Frogs (Dendrobatidae). *Journal of Natural Products*, 72(6):1110–1114.

Daly, J. W., Wilham, J. M., Spande, T. F., Garraffo, H. M., Gil, R. R., Silva, G. L., and Vaira, M. (2007). Alkaloids in Bufonid Toads (Melanophryniscus): Temporal and Geographic Determinants for Two Argentinian Species. *Journal of Chemical Ecology*, 33(4):871–887.

Dumbacher, J. P., Menon, G. K., and Daly, J. W. (2009). Skin as a Toxin Storage Organ in the Endemic New Guinean Genus *Pitohui*. *The Auk*, 126(3):520–530.

Fink-Gremmels, J. (2008). Implications of hepatic cytochrome P450-related biotransformation processes in veterinary sciences. *European Journal of Pharmacology*, 585(2-3):502–509.

Forzán, M. J. and Horney, B. S. (2020). Amphibians. In Heatley, J. J. and Russell, K. E., editors, *Exotic Animal Laboratory Diagnosis*, pages 347–368. Wiley, 1 edition.

Garraffo, H. M., Caceres, J., Daly, J. W., Spande, T. F., Andriamaharavo, N. R., and Andriantsiferana, M. (1993). Alkaloids in Madagascan Frogs (Mantella): Pumiliotoxins, Indolizidines, Quinolizidines, and Pyrrolizidines. *Journal of Natural Products*, 56(7):1016–1038.

Geyer, H. J., Schheunert I., Rapp, K., Gebefugi, I., Steinberg, C., and Kettrup, A. (1993). The Relevance of Fat Content in Toxicity of Lipophilic Chemicals to Terrestrial Animals with Special Reference to Dieldrin and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). *Ecotoxicology and Environmental Safety*, 26(1):45–60.

Gibson, G. G. and Skett, P. (2001). *Introduction to Drug Metabolism*. Nelson Thornes Publishers, Cheltenham, UK, 3rd ed edition.

Grant, D. M. (1991). Detoxification pathways in the liver. *Journal of Inherited Metabolic Disease*, 14(4):421–430.

Grant, T., Rada, M., Anganoy-Criollo, M., Batista, A., Dias, P. H., Jeckel, A. M., Machado, D. J., and Rueda-Almonacid, J. V. (2017). Phylogenetic Systematics of Dart-Poison Frogs and Their Relatives Revisited (Anura: Dendrobatoidea). *South American Journal of Herpetology*, 12(s1):S1–S90.

Gunasekaran, N., Long, L., Dawson, B., Hansen, G., Richardson, D., Li, K., Arnold, J., and McGregor, I. (2009). Reintoxication: The release of fat-stored Δ^9 -tetrahydrocannabinol (THC) into blood is enhanced by food deprivation or ACTH exposure. *British Journal of Pharmacology*, 158(5):1330–1337.

Hoogmoed, M. S. and Avila-Pires, T. C. S. (2012). Inventory of color polymorphism in populations of Dendrobates galactonotus (Anura: Dendrobatidae), a poison frog endemic to Brazil. page 21.

Hovey, K. J. (2018). Sequestered Alkaloid Defenses in the Dendrobatid Poison Frog Oophaga pumilio Provide Variable Protection from Microbial Pathogens. *J Chem Ecol*, page 14.

Huang, L., Li, J., Anboukaria, H., Luo, Z., Zhao, M., and Wu, H. (2016). Comparative transcriptome analyses of seven anurans reveal functions and adaptations of amphibian skin. *Scientific Reports*, 6(1):24069.

Ingelman-Sundberg, M., Sim, S. C., Gomez, A., and Rodriguez-Antona, C. (2007). Influence of cytochrome P450 polymorphisms on drug therapies: Pharmacogenetic, pharmacoepigenetic and clinical aspects. *Pharmacology & Therapeutics*, 116(3):496–526.

Jackson, D. C. and Ultsch, G. R. (2010). Physiology of hibernation under the ice by turtles and frogs. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 313A(6):311–327.

Jandacek, R. J. and Tso, P. (2001). Factors affecting the storage and excretion of toxic lipophilic xenobiotics. *Lipids*, 36(12):1289–1305.

Jeckel, A. M., Bolton, S. K., Waters, K. R., Antoniazzi, M. M., Jared, C., Matsumura, K., Nishikawa, K., Morimoto, Y., Grant, T., and Saporito, R. A. (2022). Dose-dependent alkaloid sequestration and *N* -methylation of decahydroquinoline in poison frogs. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 337(5):537–546.

Jeckel, A. M., Grant, T., and Saporito, R. A. (2015). Sequestered and Synthesized Chemical Defenses in the Poison Frog Melanophryniscus moreirae. *Journal of Chemical Ecology*, 41(5):505–512.

Jeckel, A. M., Kocheff, S., Saporito, R. A., and Grant, T. (2019). Geographically separated orange and blue populations of the Amazonian poison frog Adelphobates galactonotus (Anura, Dendrobatidae) do not differ in alkaloid composition or palatability. *Chemoecology*, 29(5-6):225–234.

Kirischian, N. L. and Wilson, J. Y. (2012). Phylogenetic and functional analyses of the cytochrome P450 family 4. *Molecular Phylogenetics and Evolution*, 62(1):458–471.

Küpfer, A. and Branch, R. A. (1985). Stereoselective mephobarbital hydroxylation cosegregates with mephenytoin hydroxylation. *Clinical Pharmacology & Therapeutics*, 38(4):414–418.

Kurose, I., Higuchi, H., Kato, S., Miura, S., and Ishii, H. (1996). Ethanol-Induced Oxidative Stress in the Liver. *Alcoholism: Clinical and Experimental Research*, 20(s1).

Lillywhite, H. B., Shine, R., Jacobson, E., DeNardo, D. F., Gordon, M. S., Navas, C. A., Wang, T., Seymour, R. S., Storey, K. B., Heatwole, H., Heard, D., Brattstrom, B., and Burghardt, G. M. (2017). Anesthesia and Euthanasia of Amphibians and Reptiles Used in Scientific Research: Should Hypothermia and Freezing Be Prohibited? *BioScience*, 67(1):53–61.

Mebs, D., Maneyro, R., and Pogoda, W. (2007). Further studies on pumiliotoxin 251D and hydroquinone content of the skin secretion of Melanophryniscus species (Anura, Bufonidae) from Uruguay. *Toxicon*, 50(1):166–169.

Mebs, D., Pogoda, W., Batista, A., Ponce, M., Köhler, G., and Kauert, G. (2008). Variability of alkaloid profiles in Oophaga pumilio (Amphibia: Anura: Dendrobatidae) from western Panama and southern Nicaragua. *Salamandra*, 44(4):7.

Michael, J. P. (2016). Simple Indolizidine and Quinolizidine Alkaloids. In *The Alkaloids: Chemistry and Biology*, volume 75, pages 1–498. Elsevier.

Minder, B., Mendonça, J., Grant, T., and Lopes, N. (2023). Gas-Phase Fragmentation Reactions of Protonated Pumiliotoxin (+)-251D and Allopumiliotoxin (+)-267A in ESI-MS/MS. *Journal of the Brazilian Chemical Society*.

Muff, S., Nilsen, E. B., O'Hara, R. B., and Nater, C. R. (2022). Rewriting results sections in the language of evidence. *Trends in Ecology & Evolution*, 37(3):203–210.

Myers, C. W., Daly, J. W., Garraffo, H. M., Wisnieski, A., and Cover, J. F. (1995). Discovery of the Costa Rican Poison Frog Dendrobates granuliferus in Sympatry with Dendrobates pumilio, and Comments on Taxonomic Use of Skin Alkaloids. *American Museum Novitates*, 3144:24.

Nahas, G. G. (2001). The pharmacokinetics of THC in fat and brain: Resulting functional responses to marihuana smoking. *Human Psychopharmacology: Clinical and Experimental*, 16(3):247–255.

O'Connell, L. A., LS50: Integrated Science Laboratory Course, O'Connell, J. D., Paulo, J. A., Trauger, S. A., Gygi, S. P., and Murray, A. W. (2020). Rapid toxin sequestration modifies poison frog physiology. Preprint, Physiology.

O'Connell, L. A., LS50: Integrated Science Laboratory Course, O'Connell, J. D., Paulo, J. A., Trauger, S. A., Gygi, S. P., and Murray, A. W. (2021). Rapid toxin sequestration modifies poison frog physiology. *Journal of Experimental Biology*, 224(3):jeb230342.

Oesch, F., Fabian, E., Oesch-Bartlomowicz, B., Werner, C., and Landsiedel, R. (2007). Drug-Metabolizing Enzymes in the Skin of Man, Rat, and Pig. *Drug Metabolism Reviews*, 39(4):659–698.

Omura, T. (1999). Forty Years of Cytochrome P450. *Biochemical and Biophysical Research Communications*, 266(3):690–698.

Ose, K., Miyamoto, M., Fujisawa, T., and Katagi, T. (2017). Bioconcentration and Metabolism of Pyriproxyfen in Tadpoles of African Clawed Frogs, Xenopus laevis. *Journal of Agricultural and Food Chemistry*, 65(46):9980–9986.

Proctor, P. and Reynolds, E. (1984). Free radicals and disease in man. *Physiological chemistry and physics and medical NMR*, 16:175–95.

Randolph, G. J. and Miller, N. E. (2014). Lymphatic transport of high-density lipoproteins and chylomicrons. *The Journal of Clinical Investigation*, 124(3):929–935.

Remmer, H. (1970). The role of the liver in drug metabolism. *The American Journal of Medicine*, 49(5):617–629.

Sague, M., Dudaitis, V., Plumert, L., Umbers, K. D. L., Saporito, R. A., and Lawrence, J. P. (2023). Alkaloid-based chemical defenses and diet in six species of Australian poison frogs in the genus Pseudophryne (Myobatrachidae). *Evolutionary Ecology*.

Salgado Costa, C., Bahl, F., Natale, G., Mac Loughlin, T., Marino, D., Venturino, A., Rodriguez-Mozaz, S., and Santos, L. (2023). First evidence of environmental bioaccumulation of pharmaceuticals on adult native anurans (Rhinella arenarum) from Argentina. *Environmental Pollution*, 334:122231.

Santos, J. C., Tarvin, R. D., and O'Connell, L. A. (2016). A Review of Chemical Defense in Poison Frogs (Dendrobatidae): Ecology, Pharmacokinetics, and Autoresistance. In Schulte, B. A., Goodwin, T. E., and Ferkin, M. H., editors, *Chemical Signals in Vertebrates 13*, pages 305–337. Springer International Publishing, Cham.

Saporito, A. R., F. Spande, T., Martin Garraffo, H., and A. Donnelly, M. (2009). Arthropod Alkaloids in Poison Frogs: A Review of the 'Dietary Hypothesis'. *HETEROCYCLES*, 79(1):277.

Saporito, R. A., Donnelly, M. A., Garraffo, H. M., Spande, T. F., and Daly, J. W. (2006). Geographic and Seasonal Variation in Alkaloid-Based Chemical Defenses of Dendrobates pumilio from Bocas del Toro, Panama. *Journal of Chemical Ecology*, 32(4):795–814.

Saporito, R. A., Donnelly, M. A., Jain, P., Martin Garraffo, H., Spande, T. F., and Daly, J. W. (2007a). Spatial and temporal patterns of alkaloid variation in the poison frog Oophaga pumilio in Costa Rica and Panama over 30 years. *Toxicon*, 50(6):757–778.

Saporito, R. A., Donnelly, M. A., Madden, A. A., Garraffo, H. M., and Spande, T. F. (2010a). Sex-Related Differences in Alkaloid Chemical Defenses of the Dendrobatid Frog *Oophaga pumilio* from Cayo Nancy, Bocas del Toro, Panama $^{\perp}$. *Journal of Natural Products*, 73(3):317–321.

Saporito, R. A., Donnelly, M. A., Norton, R. A., Garraffo, H. M., Spande, T. F., and Daly, J. W. (2007b). Oribatid mites as a major dietary source for alkaloids in poison frogs. *Proceedings of the National Academy of Sciences*, 104(21):8885–8890.

Saporito, R. A., Donnelly, M. A., Spande, T. F., and Garraffo, H. M. (2012). A review of chemical ecology in poison frogs. *Chemoecology*, 22(3):159–168.

Saporito, R. A., Garraffo, H. M., Donnelly, M. A., Edwards, A. L., Longino, J. T., and Daly, J. W. (2004). Formicine ants: An arthropod source for the pumiliotoxin alkaloids of dendrobatid poison frogs. *Proceedings of the National Academy of Sciences*, 101(21):8045–8050.

Saporito, R. A., Isola, M., Maccachero, V. C., Condon, K., and Donnelly, M. A. (2010b). Ontogenetic scaling of poison glands in a dendrobatid poison frog: Scaling of poison glands in a dendrobatid frog. *Journal of Zoology*, 282(4):238–245.

Saporito, R. A., Norton, R. A., Andriamaharavo, N. R., Garraffo, H. M., and Spande, T. F. (2011). Alkaloids in the Mite Scheloribates laevigatus: Further Alkaloids Common to Oribatid Mites and Poison Frogs. *Journal of Chemical Ecology*, 37(2):213–218.

Shine, R., Amiel, J., Munn, A. J., Stewart, M., Vyssotski, A. L., and Lesku, J. A. (2015). Is "cooling then freezing" a humane way to kill amphibians and reptiles? *Biology Open*, 4(7):760–763.

Smith, B. P., Tyler, M. J., Kaneko, T., Garraffo, H. M., Spande, T. F., and Daly, J. W. (2002). Evidence for Biosynthesis of Pseudophrynamine

Alkaloids by an Australian Myobatrachid Frog (*Pseudophryne*) and for Sequestration of Dietary Pumiliotoxins. *Journal of Natural Products*, 65(4):439–447.

Svensson, C. K. (2009). Biotransformation of Drugs in Human Skin. *Drug Metabolism and Disposition*, 37(2):247–253.

Sword, G. A. (1999). Density-dependent warning coloration. *Nature*, 397(6716):217–217.

Takada, W., Sakata, T., Shimano, S., Enami, Y., Mori, N., Nishida, R., and Kuwahara, Y. (2005). Scheloribatid Mites as the Source of Pumiliotoxins in Dendrobatid Frogs†. *Journal of Chemical Ecology*, 31(10):2403–2415.

Tattersall, G. (2007). Skin Breathing in Amphibians. In *Endothelial Biomedicine*, pages 85–91. Cambridge University Press.

Team, R. (2022). RStudio: Integrated Development Environment for R. RStudio, PBC.

Thomas, J. H. (2007). Rapid Birth–Death Evolution Specific to Xenobiotic Cytochrome P450 Genes in Vertebrates. *PLoS Genetics*, 3(5):e67.

Toledo, R. and Jared, C. (1995). Cutaneous granular glands and amphibian venoms. *Comparative Biochemistry and Physiology Part A: Physiology*, 111(1):1–29.

Weldon, P. J., Cardoza, Y. J., Vander Meer, R. K., Hoffmann, W. C., Daly, J. W., and Spande, T. F. (2013). Contact toxicities of anuran skin alkaloids against the fire ant (Solenopsis invicta). *Naturwissenschaften*, 100(2):185–192.

Weldon, P. J., Kramer, M., Gordon, S., Spande, T. F., and Daly, J. W. (2006). A common pumiliotoxin from poison frogs exhibits enantioselective toxicity against mosquitoes. *Proceedings of the National Academy of Sciences*, 103(47):17818–17821.

Wickham, H. (2016). *Ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Zakhari, S. (2006). Overview: How Is Alcohol Metabolized by the Body? 29(4).

Zhang, C.-x., Huang, K.-k., le Lu, K., Wang, L., Song, K., Zhang, L., and Li, P. (2016). Effects of different lipid sources on growth performance, body composition and lipid metabolism of bullfrog Lithobates catesbeiana. *Aquaculture*, 457:104–108.

Zhu, R., Liu, Y., Yang, Y., Min, Q., Li, H., and Chen, L. (2022). Cytochrome P450 Monooxygenases Catalyse Steroid Nucleus Hydroxylation with Regio- and Stereo-Selectivity. *Advanced Synthesis & Catalysis*, 364(16):2701–2719.

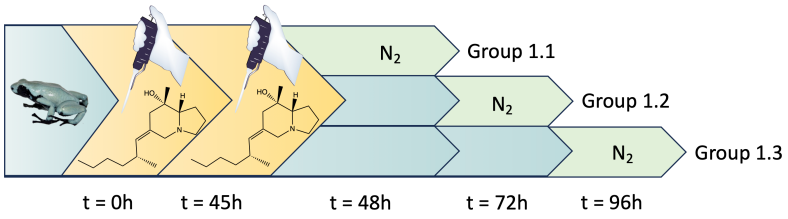

Figure 2: Experiment 1: Three experimental groups (1.1, 1.2, 1.3) and one control (not shown), were subjected to alkaloid dosing (10 µg PTX 251D per dose) and subsequent euthanasia at specified time points (t = 0h initial dose; t = 45h second dose; at t = 48h, t = 72h, and t = 96h euthanasia, by flash freezing in liquid Nitrogen.) Yellow = time of PTX 251D administration, blue = waiting days, green = time of euthanization

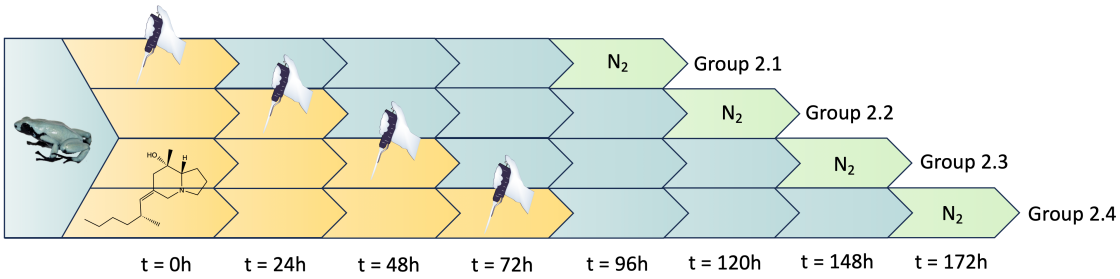

Figure 3: Experiment 2: Four experimental groups and one control (not shown), were subjected to different alkaloid dosings (10 µg PTX 251D per dose) and subsequent euthanasia at 4 days after the last administration. t = 0h initial dose (all groups); t = 24h second dose (group 2 - 4); t = 48h third dose (group 3 and 4), t = 72h (group 4). Yellow = time of PTX 251D administration , blue = waiting days, green = time of euthanization

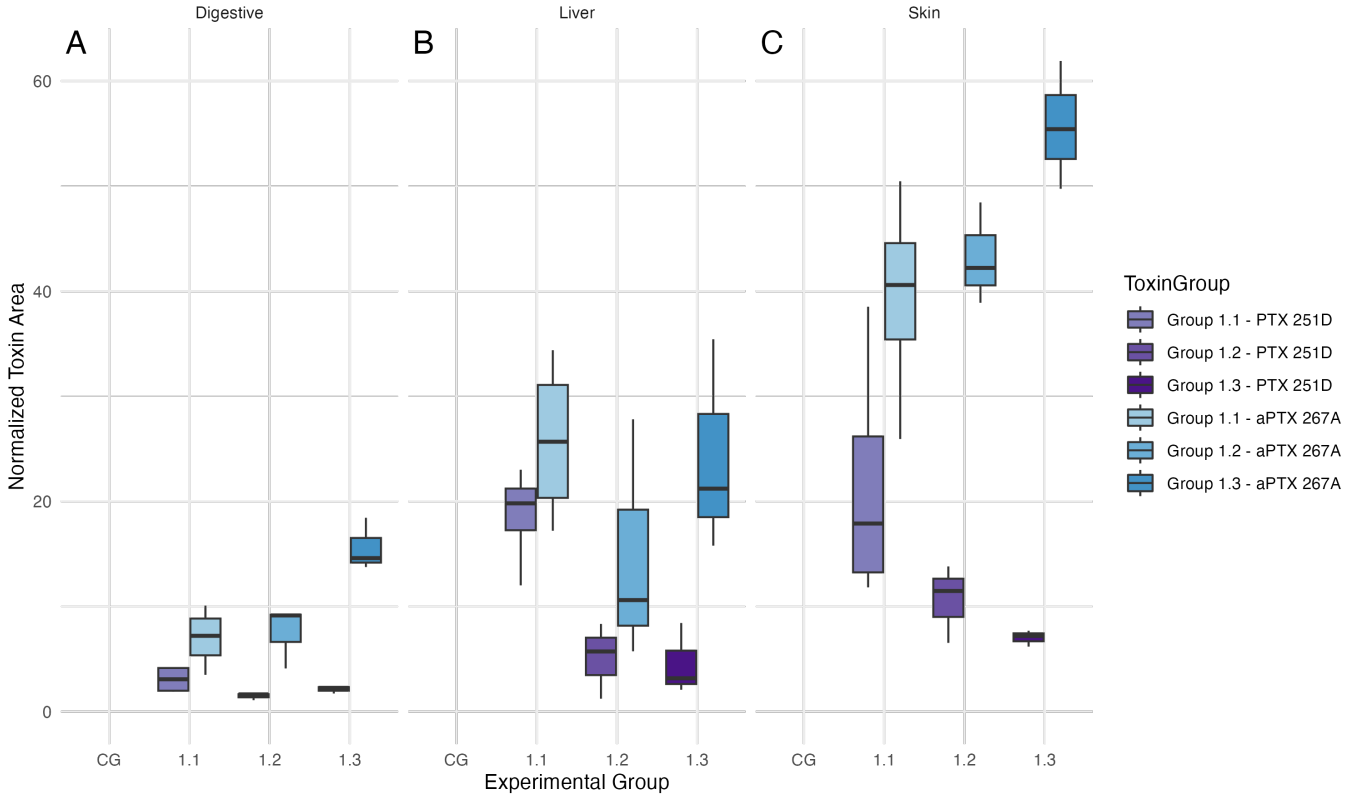

Figure 4: Experiment 1: Normalized alkaloid amount profiles in the digestive tract (A), liver (B), and skin (C) of *A. galactonotus* across different time intervals post-administration of PTX 251D. Control group (CG) was treated with ethanol only, while experimental groups 1.1, 1.2, and 1.3 received 20 µg of alkaloid each, followed by euthanasia at 48, 72, and 96 hours, respectively, to observe the hydroxylation process.

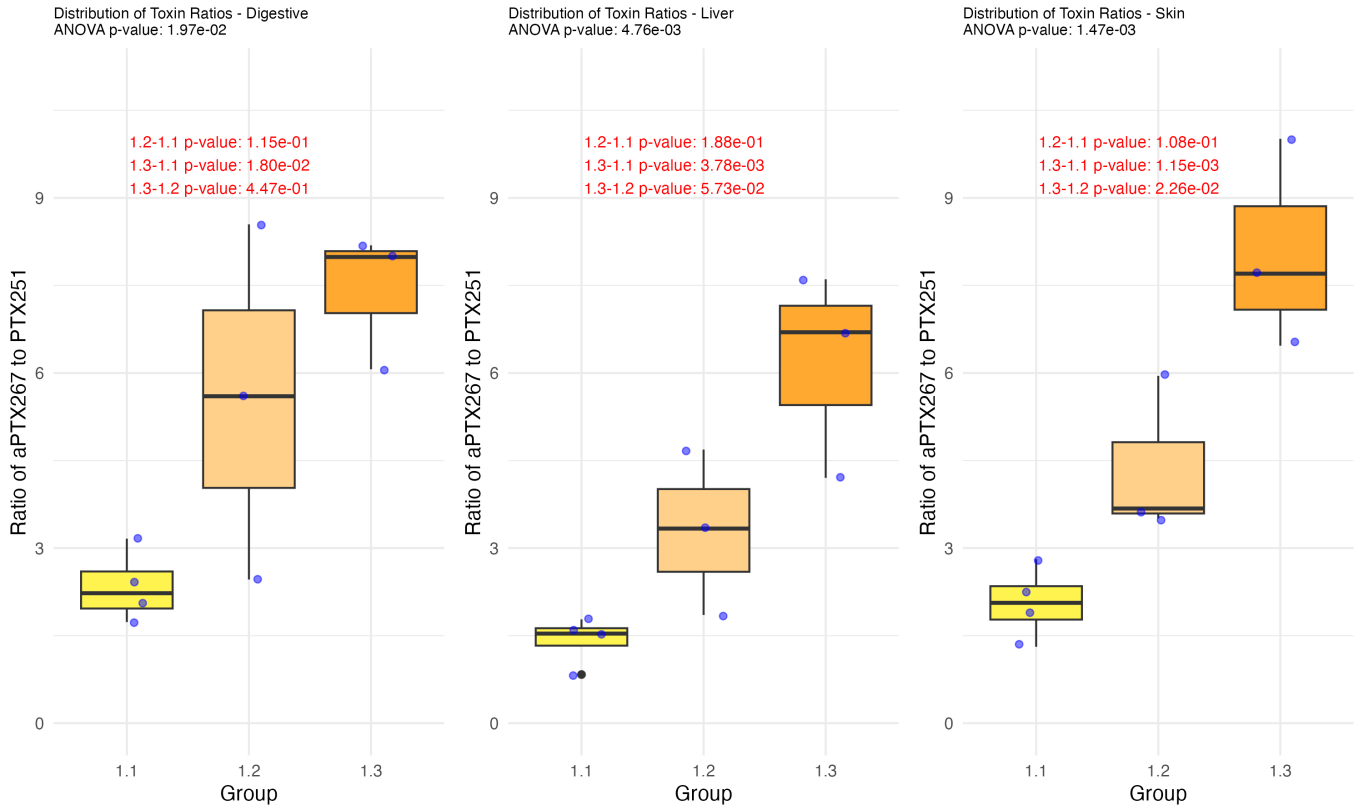

Figure 5: These Boxplots illustrating the distribution of the ratios of hydroxylated pumiliotoxin (aPTX267) to pumiliotoxin (PTX251) across digestive tract, liver and skin in *A. galactonotus*. Experimental groups 1.1 to 1.3 correspond to cohorts administered same doses of PTX 251D, with subsequent analysis performed at different time intervals post-administration. Data points are blue and outliers are highlighted with black points. ANOVA p-values are provided for each organ to indicate the statistical significance of differences between groups, with significant post-hoc Tukey HSD test results annotated in red text.

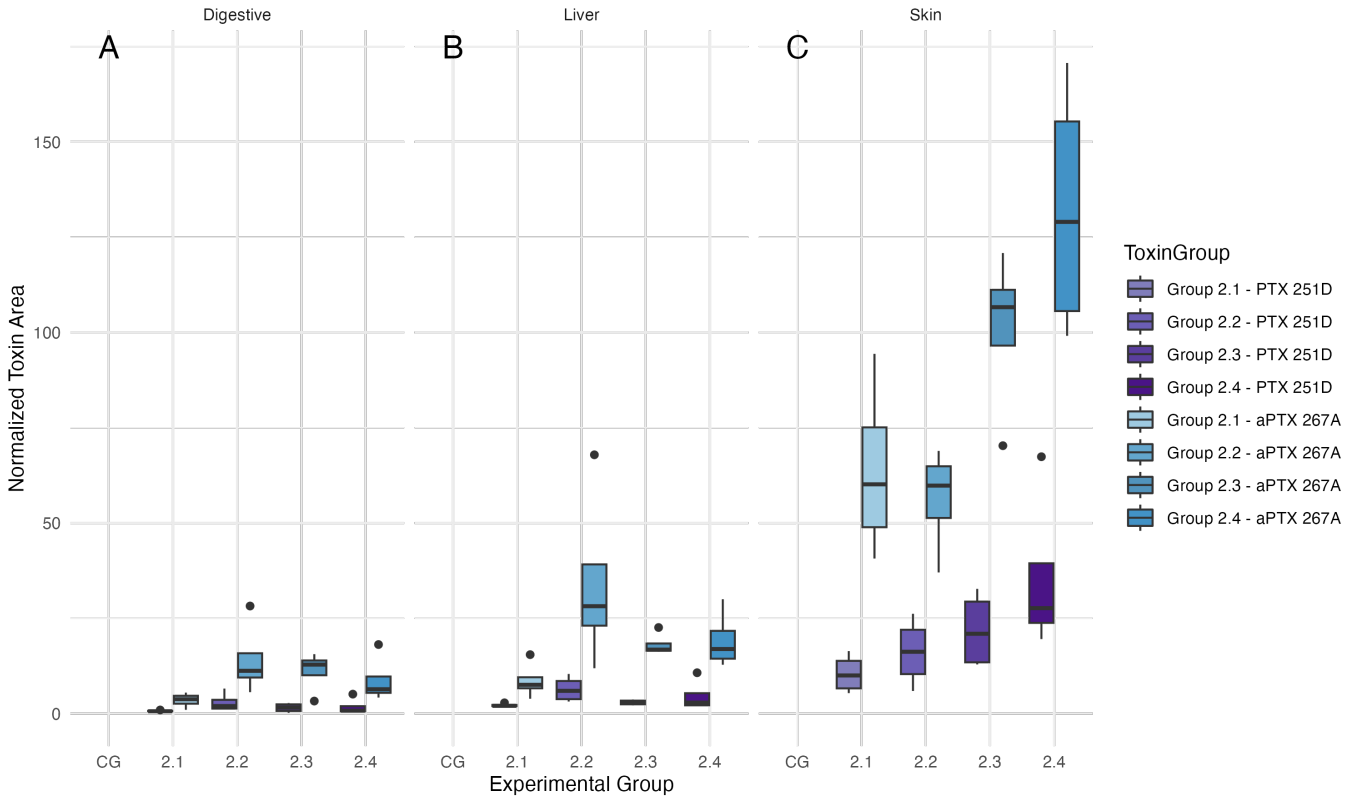

Figure 6: Boxplots depicting the normalized ratios of allopumiliotoxin (aPTX 267A) to pumiliotoxin (PTX 251D) across varying experimental groups in the digestive tract (A), liver (B), and skin (C) of *A. galactonotus* from Experiment 2. Control group (CG) received only the carrier solution. Experimental groups were administered increasing doses of PTX 251D: Group 2.1 received a single dose of 10 µg, Group 2.2 a twice dose of 10 µg, Group 2.3 a three times dose of 10 µg, and Group 2.4 a 4 doses of 10 µg. The time post-administration at euthanasia was four days, resulting in an experiment duration for Group 2.1 of 4 days, 5 days for Group 2.2, 6 days for Group 2.3, and 7 days for Group 2.4. Outliers are marked with black dots, annotated with the corresponding frog ID and organ, highlighting variations within and between groups.

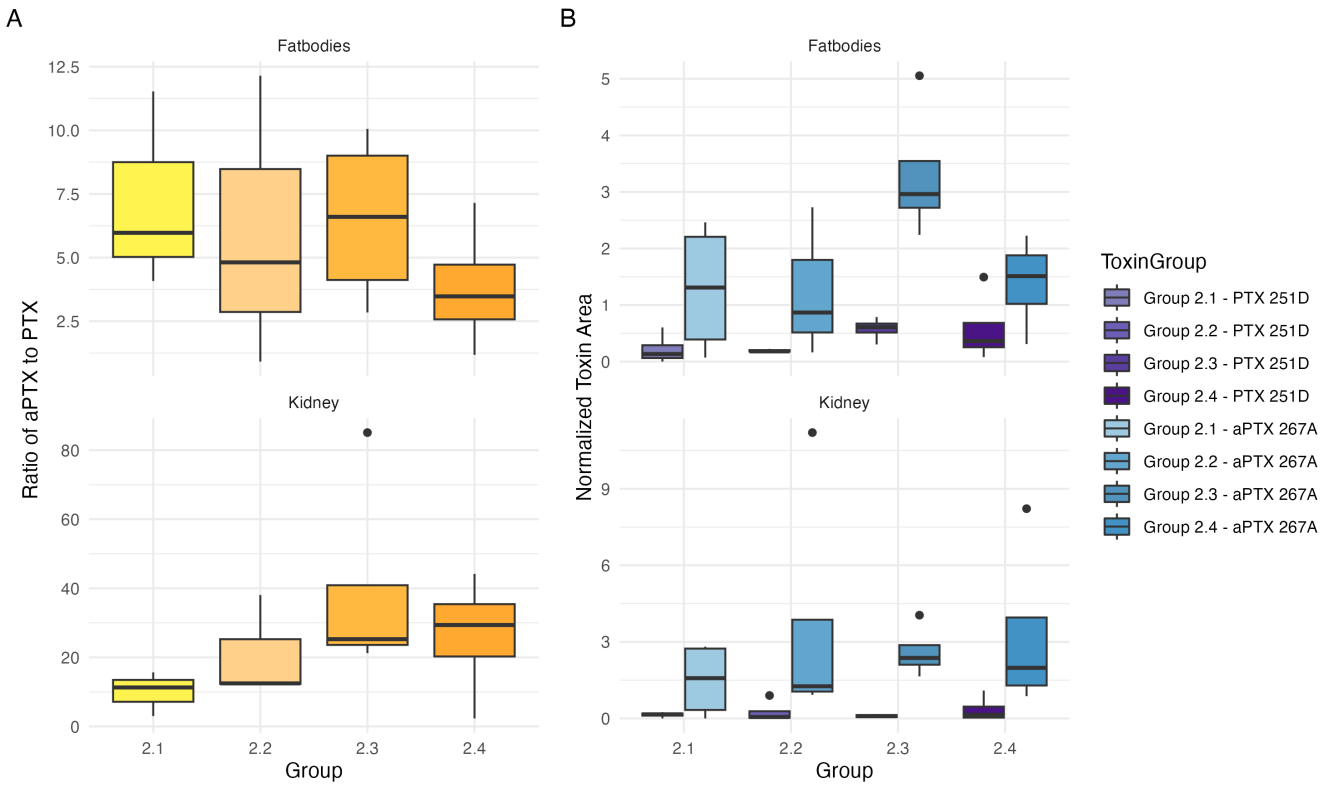

Figure 7: Differential hydroxylation and accumulation of pumiliotoxin (PTX251D) and allopumiliotoxin (aPTX267A) in *A. galactonotus*. Each graph has an adapted y-scale for better visualization. (A) Boxplots depict the ratio of aPTX267A to PTX251D within the kidneys and fat bodies, highlighting group-specific variations in alkaloid metabolism correlated with incremental doses and study durations (Groups 2.0 to 2.4, spanning 4 to 7 days). (B) Presentation of PTX251D and aPTX267A areas, demonstrating the organ-specific alkaloid distribution and amounts over time.

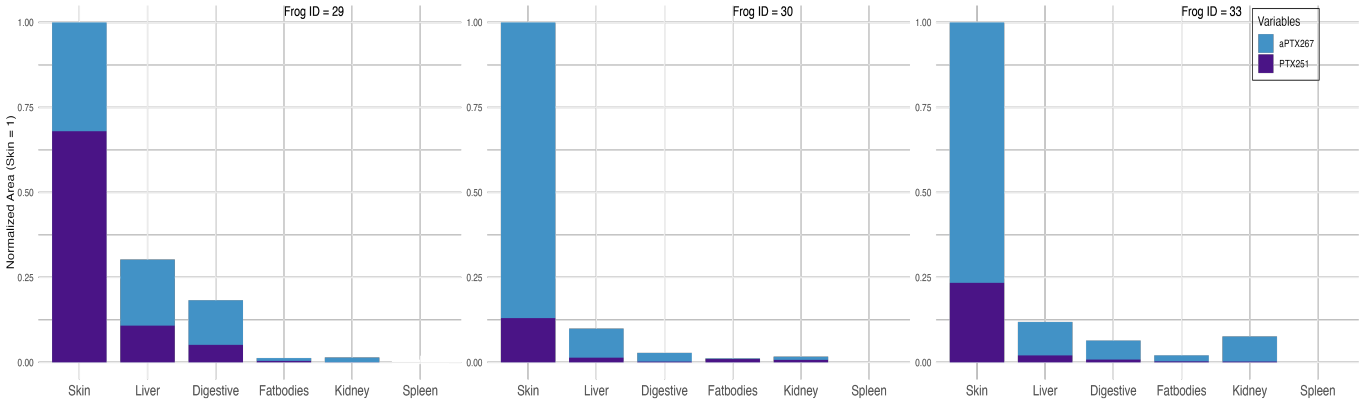

Figure 8: This composite plot showcases normalized alkaloid amounts in various organs for selected individual frogs (Frog IDs: 29, 30, 33). Each subplot corresponds to a specific frog, with the alkaloid amounts in organs normalized relative to the skin value set as 1 for clearer comparison. The purple bars represent normalized areas of PTX 251D, and the blue bars indicate normalized amounts of hydroxylated aPTX 267A. The y-axis indicates the normalized alkaloid area, facilitating direct inter-organ comparison within each frog. This visualization underscores the differential accumulation and metabolism of these alkaloids within the frog.

Conclusion

In conclusion this thesis this marks a significant leap forward in unraveling the complex biochemistry of alkaloid modification within *A. galactonotus*. Through two main experiments, this research has shed light on the processes by which these amphibians not only sequester but also modify dietary alkaloids for defense. We implement advanced sample preparation and analytical techniques, such as solid-phase extraction (SPE) and electrospray ionization tandem mass spectrometry (ESI-MS/MS). This has provided an unprecedented glimpse into the metabolic process of PTX (+)-251D and its hydroxylated counterpart, aPTX (+)-267A. Our findings underscore the liver's critical role in initial metabolism and we found the skin not only functioning as a primary depot for alkaloid storage, but as an additional hydroxylation site. Additionally, the identification of these alkaloids in the fat bodies introduces a novel perspective on passive storage mechanisms within poison frogs. This discovery not only provides more insights in the field of herpetology and biochemistry but also bridges connections to pharmacological applications, where such natural processes can inform drug discovery and development. The scientific journey through the master thesis yielded a rich amount of experience and data which opens several avenues for future research, particularly concerning the enzymatic pathways across different organs. This work lays a foundation for comparative analyses on hydroxylation rates among poison frog species, potentially offering profound insights into the efficiency and mechanisms of pumiliotoxin metabolism. In summary, this master thesis not only illuminates several facets of alkaloid metabolism in *Adelphobates galactonotus* but also raises many new questions about the hydroxylation pathways and their evolutionary relevance in poison frogs. As we look to the future, the enzymatic processes, particularly the role of cytochromes in the skin and kidneys, beckon further exploration. The potential applications of these findings in human medicine and the conservation of these remarkable creatures remain an exciting prospect, testament to the interdisciplinary nature of this work.

Resumo

A tese apresenta um estudo abrangente sobre as vias bioquímicas de modificação de alcaloides em *Adelphobates galactonotus*, espécie notável pela capacidade única de alterar alcaloides ingeridos, especificamente a hidroxilação da pumiliotoxina PTX (+)-251D para allopumiliotoxina aPTX (+)-267A. Utiliza uma abordagem metodológica multifacetada, integrando extração em fase sólida (SPE) para purificação dos alcaloides e espectrometria de massa em tandem com ionização por electrospray (ESI-MS/MS) para análise estrutural, além de exame detalhado do processamento metabólico nos diversos órgãos. Foram conduzidos experimentos fundamentais para investigar os órgãos envolvidos no processo de hidroxilação e para elucidar os padrões de translocação desses alcaloides nos sapos venenosos. As descobertas fornecem insights significativos sobre as vias metabólicas no metabolismo de alcaloides, ressaltando o papel crucial do fígado e da pele na rápida absorção e transformação desses compostos, e dos corpos gordurosos como locais de absorção. Mapeando os padrões de translocação, esta pesquisa avança o entendimento dos mecanismos de defesa química dos anuros, oferecendo perspectivas inéditas sobre as adaptações evolutivas que facilitam a sequestração e modificação dos alcaloides. Os resultados contribuem para o campo da herpetologia e bioquímica e trazem implicações potenciais para a pesquisa farmacológica, destacando a complexidade das estratégias de defesa química dos sapos venenosos e abrindo caminho para futuras investigações sobre a importância ecológica e evolutiva da modificação dos alcaloides.

Abstract

This thesis presents a comprehensive study on the biochemical pathways of alkaloid modification in *Adelphobates galactonotus*, a species renowned for its unique ability to modify ingested alkaloids, specifically the hydroxylation of pumiliotoxin PTX (+)-251D into allopumiliotoxin aPTX (+)-267A. The study employs a multifaceted methodological approach, integrating solid-phase extraction (SPE) for alkaloid purification and including gas-phase fragmentation reactions in advanced electrospray ionization tandem mass spectrometry (ESI-MS/MS) for structural analysis and a detailed examination of metabolic processing within various organs. Two core experiments were conducted to investigate the organs involved in the hydroxylation process and to elucidate the translocation patterns of these alkaloids within poison frogs. The findings reveal significant insights into the metabolic pathways engaged in alkaloid metabolism, highlighting the liver and skin's crucial roles in the rapid absorption and transformation of these compounds, as well as the fat bodies as site of absorption. By mapping the translocation patterns of these alkaloids this research advances our understanding of anuran chemical defense mechanisms, providing novel perspectives on the evolutionary adaptations that facilitates sequestration and modification of alkaloids. The outcomes not only contribute to the field of herpetology and biochemistry but also offer potential implications for pharmacological research. This thesis underscores the complexity of chemical defense strategies in poison frogs, paving the way for future investigations into the ecological and evolutionary significance of alkaloid modification.