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Toxicity and Alkaloid Profiling of the Skin of the Golfo Dulcean Poison Frog *Phyllobates vittatus* (Dendrobatidae)

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Abstract

Frogs in the genus *Phyllobates* are known for the presence of batrachotoxin, a highly toxic alkaloid, in their skin. Nevertheless, *Phyllobates* frogs from Costa Rica and Panama (*P. lugubris* and *P. vittatus*) are considered non-toxic, as they have been reported to harbor low concentrations of this alkaloid. However, the potential toxicity of Central American *Phyllobates* has not been assessed experimentally. Our goal was to determine the toxicity of the whole skin of *P. vittatus*, an endemic species from the Southeastern Pacific region of Costa Rica. We performed median lethal dose (LD_{50}) tests in mice to determine general toxicity, and an irritant assay based on the behavioral responses of mice to subcutaneous injection, to determine differences in irritability, as a measure of toxicity, among three study localities. Using UPLC-ESI-QTOF, we obtained chemical profiles of the methanolic extract of frog skins. Due to the absence of mortality at the studied doses, we were unable to estimate LD_{50}. However, we recorded a list of toxicity symptoms in mice that are consistent with cardiotoxic effects, and found that mice presented more symptoms at higher concentrations of skin extracts during the first hour of the LD_{50} assays, recovering completely at all doses by the end of the assay. On the other hand, we did not detect differences in irritability among studied localities. Additionally, we putatively identified three toxic alkaloids (Batrachotoxinin A, DHQ **251A** and Lehm **275A**). This study provides the first experimental data on the toxicity and associated symptoms in mice, as well as the chemical profile of the skin of *P. vittatus*. We suggest that the skin alkaloids of *P. vitattus* may confer a chemical defense towards predators.

Keywords Alkaloids · Batrachotoxin · Chemical defenses · Toxicity assays · Costa Rica · LC-MS/MS · Predation · Aposematism

Introduction

Chemical defenses are widespread in nature (Berenbaum 1995; Mebs 2002). Their evolution is mainly driven by selective pressures related to predation, such as reduced capacity to escape or behaviors and phenotypes that enhance detectability, which correspond to foraging, mating and communication (Speed and Ruxton 2005). In animals, defensive compounds are extraordinarily diverse, exhibiting a broad range of chemical structures, biological activities and origins. These compounds can be synthesized by the animal itself or sequestered from environmental sources (Reviewed by Santos et al. 2016; Saporito et al. 2012; Savitzky et al. 2012).

Sequestration of defensive compounds is an evolved capacity and it confers a selective advantage via the retention of specific compounds within tissues (Savitzky et al. 2012). Sequestration is a novelty among the vertebrate tetrapods and only a few taxa have this ability. Two bird genera, *Pitohui* and *Ifrita*, sequester toxins from prey, but the specific source of such toxins is still unknown (Dumbacher et al. 1992, 2000;

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but Dumbacher et al. 2004 proposed a putative source). Among reptiles, there are two examples: (1) some populations of the common garter snake *Thamnophis sirtalis*, which feed on newts of the genus *Taricha,* sequester the alkaloid Tetrodotoxin (TTX) contained in newt skin (Williams et al. 2012) and (2) the snake *Rhabdophis tigrinus* obtains toxins from predated toads (Hutchinson et al. 2007). Among amphibians, five families of poison frogs have the capacity to accumulate lipophilic alkaloids in their skin from dietary arthropods: Bufonidae, Eleutherdactylidae, Mantellidae, Myobatrachidae, and Dendrobatidae (Reviewed by Daly et al. 2005; Saporito et al. 2012).

Amphibian lipophilic alkaloids act on the ion channels of cells, disrupting neuromuscular function (Daly et al. 2003). Their toxicity varies widely, and even if some alkaloids are non-lethal, they are generally distasteful (Santos et al. 2016), thus acting as effective deterrents against pathogen bacteria, parasites and predators (Daly 1995; Daly et al. 1987, 2005; Hovey et al. 2018; Santos et al. 2016). Most of the natural alkaloids known today occur in the Neotropical frog family Dendrobatidae (Daly et al. 2005; Saporito et al. 2009, 2012). These frogs obtain alkaloids from their diet, which consists primarily of ants and mites present in the forest leaf litter (Saporito et al. 2009; Toft 1995).

Within the family Dendrobatidae, the genus *Phyllobates* is the only one that sequesters the alkaloid batrachotoxin (Albuquerque et al. 1971; Myers 1987; Saporito et al. 2012). This is a steroidal alkaloid and one of the smallest non-proteic molecules with the highest known toxicity in nature (Daly et al. 1980; Myers et al. 1978). The high toxicity of batrachotoxin is the result of a selective permeability of sodium channels in cell membranes. Batrachotoxin keeps them permanently open and causes an irreversible depolarization of nerves and muscles, and in turn produces arrhythmias, fibrillation and cardiac failure (Albuquerque et al. 1971; Daly et al. 1980).

Batrachotoxin content varies widely among *Phyllobates* species. For example, in populations of *P. lugubris* from Panama and *P. vittatus* from Costa Rica, reported amounts of batrachotoxin range from undetectable to 0.8 μg per individual (Daly et al. 1980). In contrast, in *P. aurotaenia*, *P. bicolor* and *P. terribilis,* levels of batrachotoxin are considerably higher, the latter being the most toxic. The skin of an adult *P. terribilis* can contain as much as 1.9 mg batrachotoxin (Daly et al. 1980), which is enough poison to kill up to 20,000 mice of 20 g average weight (Myers et al. 1978).

Because batrachotoxin is almost undetectable in their skin, some authors have suggested that *P. lugubris* and *P. vittatus* might be less protected from predators, compared to other members of *Phyllobates* that contain large quantities of this alkaloid (Daly et al. 1980; Mebs et al. 2014). However, the role of batrachotoxin and/or other alkaloids in the skin of these species in protecting these frogs against predators has not been investigated experimentally. Anecdotal statements by Myers et al. (1978) suggest that at least *P. vittatus* may in fact have some level of toxicity that is effective against snakes and humans. Thus, data regarding the toxicity of *P. vittatus* from chemical and natural history perspectives appear to be at odds with each other.

In this study, we tested whether the skin of *P. vittatus* has toxic or irritant properties in mice. Additionally, given that previous studies have shown variation in irritability, as a measure of toxicity, among populations of dendrobatid poison frogs (e.g. Maan and Cummings 2012; Wang 2011), we aimed to detect whether there are differences in irritability of *P. vittatus* from different localities in Costa Rica.

Methods and Materials

Study Species *Phyllobates vittatus* is an endemic poison frog from the Southeastern Pacific of Costa Rica. It is a diurnal, territorial species (Summers 2000), that inhabits rainforests near streams. *P. vittatus* is sympatric with other poison frogs, such as *Oophaga granulifera, Dendrobates auratus, Silverstoneia flotator* and *Allobates talamancae* (Savage 2002). It feeds mainly on ants and mites, but other insects such as termites, beetles, and flies might be included in its diet as well (Mebs et al. 2014; Toft 1995).

Samples Collection and Preparation We performed field sampling during the rainy season in April 2017 at three localities in the Osa Peninsula of Costa Rica: Agua Buena, La Tarde and Piro (Fig. 1). We captured frogs in the field and took them to a laboratory, where we measured Snout-to-Vent Length (SVL) and weighed them (Table 1).

We euthanized frogs by applying two drops of Benzocaine (Anestesión Forte, Laboratorios Bondos S.A, Costa Rica) in the venter (Campos et al. 2016; Maan and Cummings 2012). In order to remove the excess of Benzocaine so that toxicity assays would not be biased by this anesthetic agent (Saporito and Grant 2018), we washed the frogs with distilled water. We then applied cervical transection to confirm death (Campos et al. 2016). Following, we removed the complete skin of the frogs, weighed it and stored it in methanol (Technical grade, J.T. Baker, USA; Table 1) at approximately 8 °C until toxicity assays were conducted. We collected all specimens under the research permit of the Ministry of the Environment ACOSA-INV-017-16 (adendum 003–16). Skinned specimens were individually stored in 70% ethanol and deposited in the Zoology Museum at University of Costa Rica.

Because we performed two different biological assays, we stored skin samples differently for each one. First, we aimed to test toxicity of the skin of *P. vittatus* (regardless of locality of collection), so we stored the skin of one individual from each locality in one vial in order to determine a median lethal dose

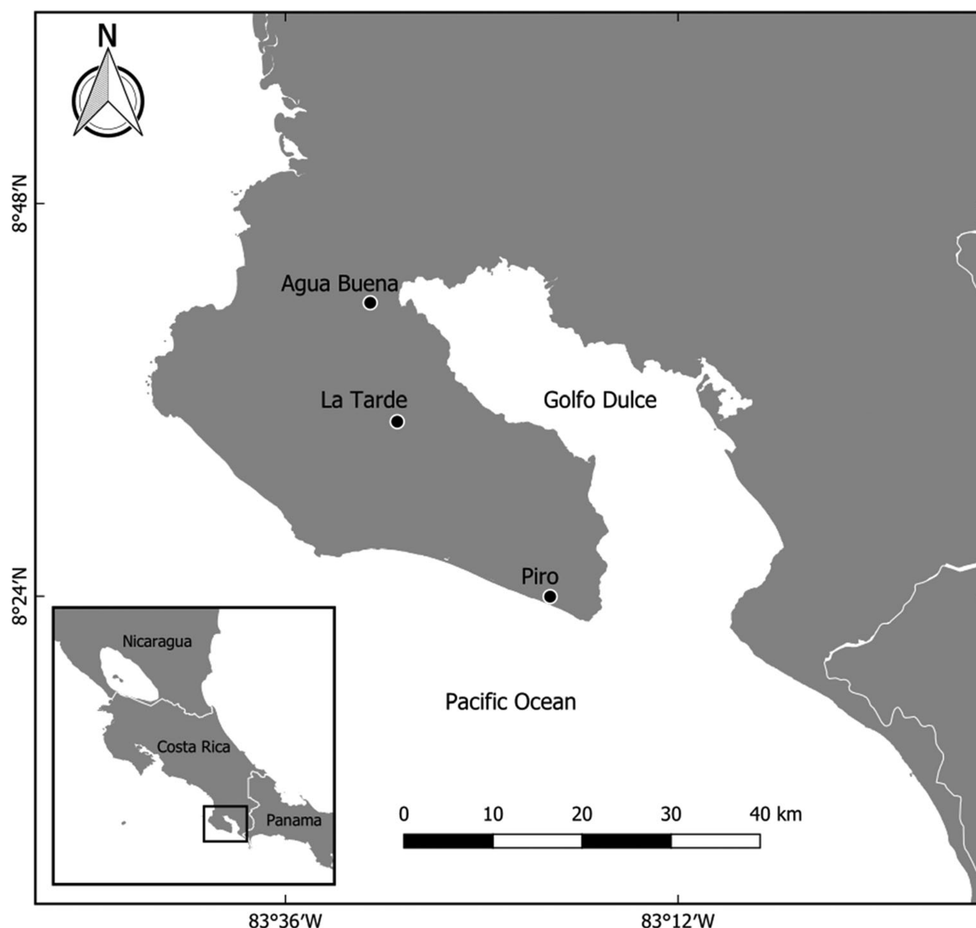

Fig. 1 Sampling localities for *P. vittatus* in the Osa Península, Costa Rica

(LD_{50}). To assess possible differences in toxicity among localities, we stored together the skin of five frogs from each site in one vial. In total we stored 15 skins in three different vials according to the locality of collection.

To concentrate skin extracts, we evaporated methanol in a water bath at 37 °C for approximately 8 h, after which residues were resuspended in a sterile saline solution (Baxter, sodium chloride 0.9%). After toxicity assays, the remaining frog skins from both assays were stored at −70 °C for further chemical analysis.

Experimental Conditions and Animals Female mice (outbreed strain Hsd:ICR [Harlan/ENVIGO] produced by the Laboratory of Biological Assays [LEBi-UCR], 4–5 weeks old, $n = 32$) were kept at the LEBi-UCR in May 2017, when we conducted all assays. Mice were kept in individual cages with food and water ad libitum at a mean room temperature of 22 °C. Assays followed the Institutional Animal Care and Use Committee (IACUC) protocols and permits (IACUC-061-16 and IACUC-052-16). We weighed all mice at the beginning and at the end of the

Table 1 Samples' attributes for the assays to determine the Median Lethal Dose (LD_{50}) and variation in irritability among localities of *Phyllobates vittatus* frogs

Samples	Mean frogs' SVL (cm)	Mean frogs' weight (g)	Mean skin sample weight (g)	Combined weight of the skin sample (g)	Total volume of methanol for the sample (mL)
Toxicity assay: LD_{50} estimation					
Combined skins	2.31 ± 0.13	1.46 ± 0.05	0.16 ± 0.03	0.48	5.00
Irritant assay: Variation among localities					
Agua Buena	2.49 ± 0.11	1.38 ± 0.19	0.17 ± 0.02	0.89	5.00
La Tarde	2.50 ± 0.10	1.61 ± 0.35	0.18 ± 0.03	0.92	6.00
Piro	2.33 ± 0.13	1.21 ± 0.20	0.15 ± 0.02	0.79	7.00

experiments, before euthanizing by cervical dislocation (Close et al. 1997).

Toxicity Assay: Median Lethal Dose (LD_{50}) Estimation Because there is no currently available information on the amount of batrachotoxin and other toxic alkaloids in the skin of *P. vittatus*, we used a range of doses in order to approach a LD_{50} for this species. We performed a stepwise procedure following the Organization for Economic Cooperation and Development 423-guidelines on acute toxicity (OECD 2002), adapted to subcutaneous injection and with three mice per dose level. This procedure is reproducible and uses very few animals (OECD 2002). A stepwise procedure also ensures the evaluation of toxicity without the need to dissect many individuals of *P. vittatus*, a species classified as endangered due to its limited occurrence, fragmented populations and ongoing habitat reduction and deterioration (IUCN et al. 2013).

Sample concentration was 215.64 mg/mL of frog skin extract and we based dosages on a limit dose of 2000 mg of frog skin per kg of mouse (OECD 2002). We injected mice with 20%, 50% and 80% of the limit dose: 400 mg/kg, 1000 mg/kg and 1600 mg/kg respectively (hereafter D20, D50 and D80) and used saline solution (Baxter, sodium chloride 0.9%) as a control (Table 2). We observed mice for toxicity symptoms on a five-hour period after injection (at 0.5, 1, 2, 3, 4 and 5 h), and daily for the next 14 days. We listed toxicity symptoms based on published literature (OECD 2000; 2002; Maan and Cummings 2012) and scored their presence or absence at each observation time (Table 3). We weighed mice every 4 days after injection until the end of the observation period.

*Irritant Assay: Variation Among Localities***.** In order to estimate whether skin extracts from different localities vary in their toxic effect in mice, we followed the approach of toxicity of Darst et al. (2006), and Maan and Cummings (2012), and performed an irritant assay. In this assay, sleeping mice are awakened with a subcutaneous injection of the skin extract of the frogs and the time (minutes) it takes the mice to return to sleep is then used as a measure of irritability. A higher latency to sleep is interpreted as a higher irritability, that might be linked to toxicity (Darst and Cummings 2006; Darst et al. 2006; Maan and Cummings 2012). Given that voltage-gated ion channels are basic components of both invertebrate and vertebrate taxa, and that alkaloids target these channels (Daly et al. 1980), it is therefore assumed that their action may be generalized (Maan and Cummings 2012). Yet, even though this method is not a representative approach of how frog alkaloids could function deterring potential predators (Weldon 2017), a more realistic method such as avian palatability assays, had not been developed at the time we made these assays (see Lawrence et al. 2019). Therefore, we consider the assay used in this study as a reasonable approach to assess variation in "toxicity" among populations of poison frogs, as it has been used in several studies (Darst and Cummings 2006; Darst et al. 2006; Wang 2011; Maan and Cummings 2012).

We used five mice for each treatment (locality) and sterile saline solution (Baxter, sodium chloride 0.9%) as a control. We observed all mice for toxicity symptoms as above. Samples concentration were 114.20 mg/mL, 181.13 mg/mL and 233.75 mg/mL for Agua Buena, La Tarde and Piro, respectively. We aimed to inject mice with a dose of 700 mg/kg, but due to low availability of skin extract sample, doses varied slightly (see Table 2).

Samples for Chemical Analysis We obtained samples for Liquid Chromatography-Mass Spectrometry (LC-MS) by profiling fragments of skin of the same individuals used in the irritability assay (skins were first extracted for the assays and then for the chromatographic profiles). Alkaloids were extracted from skin three times in an ultrasonic bath for 30 min using 5 mL of acetonitrile each time. The final volume was reduced to dryness, and the residue was dissolved with 1.5 mL of acetonitrile containing 0.1% formic acid. Prior to injection, we filtered samples using 0.2 μm, GHP ACRODISC, 13 mm (Waters, Milford, USA).

Table 2 Dosage, mean (± SD) injected volume, and mice initial and final weight for each treatment of toxicity and irritant assays with skin extracts from *Phyllobates vittatus* frogs

Treatment	Dosage (mg/kg)	Injected volume (mL)	Initial weight (g)	Final weight (g)
Toxicity assay: LD_{50} estimation				
Control	0	0.10 ± 0.01	24.50 ± 1.44	24.93 ± 1.57
D20	400	0.04 ± 0.00	23.70 ± 1.06	25.37 ± 2.41
D50	1000	0.10 ± 0.01	24.03 ± 1.63	24.47 ± 2.14
D80	1600	0.16 ± 0.01	23.90 ± 1.78	25.23 ± 0.70
Irritant assay: Variation among localities				
Control	0	0.15 ± 0.02	24.20 ± 3.57	23.06 ± 3.16
Agua Buena	701.78	0.15 ± 0.02	25.06 ± 3.02	23.34 ± 3.06
La Tarde	668.58	0.09 ± 0.01	23.84 ± 2.40	22.05 ± 2.51
Piro	684.62	0.07 ± 0.00	23.90 ± 1.03	22.62 ± 0.94

Table 3 Description of symptoms of toxicity and number of mice that presented each symptom according to the treatment and time after injection of frog skin extracts

Symptom	Symptom description	Treatment	D20						D50						D80					
		Time after injection (h)	0.5	1	2	3	4	5	0.5	1	2	3	4	5	0.5	1	2	3	4	5
Piloerection	Erection of hairs				1	2	2	1	3	3	3	3	3	3	3	3	3	3	3	3
Difficult breathing	Increase or decrease in respiratory rate					1				2		1	2	2		3	3	1	3	3
Salivation	Excess of buccal secretion								3	3		1		1	3	3	3	2		
Dehydration	Robinou test: Pinch the skin, which does not return to its normal position						1			1			2			3	3	1	2	1
Hyperactivity	Increase in motor activity, generally running around the cage		1	1	1						1	1								
Somnolence	Sleepiness								1	3					1	3				
Stimuli reaction	Reduced response to touch or noise			1	1						1					2				
Peripheral vasoconstriction	Paleness								2	2						3	2			
Tremors and convulsions	Spontaneous abnormal muscle contraction		1													2	1			
Reduced motor activity	Decrease in normal activity															3	1			
Diarrhea	Soft stools or aqueous deposition																1			
Ataxia	Loss of balance, erratic walk																1			
Paralysis	Loss of response of any limb															1				

Chromatographic and Mass Spectrometry Analysis We obtained chromatographic profiles on an ACQUITY Ultra Performance LC™ system equipped with an auto sampler and Photodiode array detector hyphenated to a Waters® SYNAPT ESI-QTof system (Waters, Milford, USA). The chromatographic conditions were as follows: column, Waters® ACQUITY™ 1.7 μm BEH C_{18} 50 × 2.1 mm, column temperature 35 °C, Injection volume, 5.0 μL, flow rate, 100 μL/min. A gradient elution was carried out, with a binary system consisting of [A] 0.1% aqueous formic acid (Optima, Fisher Scientific, USA) and [B] 0.1% formic acid (Optima, Fisher Scientific, Waltham, USA) in acetonitrile (Optima, Fisher Scientific, Waltham, USA). An increasing linear gradient (*v*/v) of [B] was used as follows [*t*(min), %B]: 0.00, 2; 1.00, 2; 25.00, 100; 27.00, 100; followed by re-equilibration steps (28.00, 2; 30.00, 2). PDA detector was set from 190 to 600 nm with a resolution of 1.2 nm.

Mass spectrometer parameters were set as follows: desolvation gas (N_2) flow, 300 L/h, desolvation temperature, 250 °C, cone gas (N_2) flow, 10 L/h, source temperature, 100 °C, capillary voltage, 1.1 kV, sampling cone voltage, 35 V., extraction cone voltage 3.5 V. MS/MS experiments were obtained using collision induced dissociation (CID) functions with collision energy from 20 eV to 50 eV for all the molecules. We performed MS/MS experiments to obtain the fragmentation spectra of all annotated compounds in order to corroborate putative MS^1 level identification (see below).

All analyses were conducted using Lock Spray™. Leucine-enkephalin was used as lock mass (V^+: 556.2771; V^-: 554.2615). Data were collected in continuous mode, with a lock spray frequency of 10 s, and data were averaged over 10 scans. The Synapt was calibrated in negative mode with sodium formate (reference mass 860.8467 uma), and in positive mode with sodium iodide (reference mass 922.3552), both for an *m/z* range from 100 to 1000 uma. MassLynx software (version 4.1, Waters) was used for acquisition and data processing. All samples were measured in positive and in negative ionization mode.

MZmine Data Treatment We treated the resulting chromatographic profiles of the skin extracts with MZmine software v2.37 (Pluskal et al. 2010) for data mining. We considered all peaks with an intensity above 100 (ion count), using the "Grid Mass" (Treviño et al. 2015) algorithm with an *m/z* tolerance of 0.01 ppm and a min-max width time of 0.05–1.5 min. Afterwards, we applied deisotoping and filtering procedures to remove all isotopic peaks. Alignment was performed using the "Join Aligner" algorithm with a retention time tolerance of 0.2 min and *m/z* tolerance of 8 ppm. Gap filling was achieved using the "Same RT and *m/z* Range Gap Filler" algorithm with a RT tolerance of 0.2 min and an *m/z* tolerance of 8 ppm.

Dereplication against DNP in-House Database We created a database using all of the compounds reported for amphibians based on the commercial Dictionary of Natural Products (DNP v.27.2, http://dnp.chemnetbase.com/), in order to narrow the possibility for a better match at MS^1 level identification (molecular formula and exact mass). We searched all detected ions from the chromatographic profiles

against the in-home database with a *m/z* tolerance of 8 ppm, using the algorithm "Custom database search" in MZmine. Benzocaine ($[M+H]^+$ at *m/z* 166.0868 as well as adduct $[M+Na]^+$ at *m/z* 189.0766, and $[M-H]^-$ at *m/z* 164.0712) was carefully searched in all samples in order to corroborate that observed toxicity was indeed the product of alkaloids present in the skin rather than the euthanization agent.

Generation of in Silico MS/MS We generated the in silico MS/MS for suspected compounds identified in MZmine using a custom data base search with the SMILES input from each structure in the in silico fragmentation tool CFM-ID v 2.0 (available at http://sourceforge.net/ projects/cfm-id/).

Statistical Analyses We performed a Generalized Linear Model (GLM) with a binomial error distribution in order to determine how both time after injection and treatment affected the proportion of toxicity symptoms exhibited (response variable). None of the control mice displayed any toxicity symptoms. Therefore, we did not include this treatment in the statistical analysis, to avoid violating the homoscedasticity assumption of the statistical model. Statistical significance of predictor variables was assessed with chi-square tests based on log-likelihood ratios, using the function "Anova" of the "car" package (Fox and Weisberg 2011), and pairwise comparisons between treatments were assessed using the function "pairs" of the "emmeans" package (Lenth 2019) in R (R Core Team 2018). To test for differences in toxicity among localities (based on latency to sleep), we used a Cox regression model. In short, a Cox regression evaluates the effect of one or more factors on the rate at which a particular event happens. In this case, we tested the effect of toxins from different localities on the rate at which mice return to sleep, as an irritation assay. We estimated hazard ratios (and SE) for the three frog localities versus the control injection of sterile saline solution, using the R package 'survival' (Therneau and Grambsch 2000), and visualized results with 'survminer' (Alboukadel et al. 2019). Hazard ratios represent the 'risk' that the event (returning to sleep) happens, with ratios smaller than 1 indicating increased latency to sleep relative to the control, and ratios greater than 1 indicating a higher rate of returning to sleep.

Results

Toxicity Assay: LD_{50} Estimation Control mice did not present symptoms of discomfort or abnormal behavior at any time during the 14 days of observation. The different doses of frog's skin extract we used did not lead to any mouse mortality; consequently, it was not possible to estimate an LD_{50} of the skin extracts. However, we did observe toxicity symptoms at all applied doses (Table 3).

In general, all mice injected with the different doses of skin concentrations exhibited discomfort symptoms immediately after injection, including intense grooming of the injected area. We recorded a total of 13 toxicity symptoms during the observation period, and the number of mice that presented those symptoms varied with time and treatment (Table 3). The most frequent symptoms were piloerection, salivation, dehydration and difficult breathing (Table 3). It should be noted that mice injected with the D80 dose experienced the most severe symptoms, such as paralysis, ataxia, tremors and seizures. Moreover, salivation in D80 mice was extreme, as saliva was running from the mouth down the forelimbs. However, mice in all treatment groups recovered almost completely at the end of the five-hour observation time, except for mice in the D80 group, which exhibited piloerection until 5 days after injection. Also, all mice from all treatments gained weight by the end of the observation period (Table 2).

Both time ($X^2 = 17.34$, d.f. = 1, $P < 0.001$, Fig. 2) and treatment ($X^2 = 57.57$, d.f. = 2, $P < 0.001$, Fig. 2) significantly affected the number of symptoms present. Most toxicity symptoms appeared in the first hour after injection and decreased with time (Fig. 2), with a general pattern of higher doses causing more symptoms (Table 4, Fig. 2).

Irritant Assay: Variation among Localities After injection, mice returned to sleep with a latency of 14.3–186.6 min, ranging from a low of (mean ± SD) 25.02 ± 12.80 min for saline controls to a high of 110.51 ± 63.81 min for Agua Buena extracts. Only mice that were injected with extracts from the different localities exhibited toxicity symptoms. Once these were injected, they started grooming excessively in the injection area. The common symptoms were similar to those described in the LD_{50} estimation assay, including excessive salivation, slow and forced abdominal breathing, convulsions and tremors, decreased motor activity, loss of strength and balance, piloerection, eyes half-closed and a hunched posture. Latency to sleep differed between treatments with skin extracts and controls, as shown by greater hazard ratios for extracts from all three localities versus the control (Fig. 3, Fig. S2). However, the effects of alkaloids from different localities were statistically indistinguishable (Fig. 3).

Alkaloid Identification Based on a custom database search, we putatively annotated 62 alkaloids in *P. vittatus* skin extracts at the MS^1 level (molecular formula and exact mass; Table S1). Due to the low quantity of compounds remaining (and possible loss of some compounds) in skins following initial extraction for the assays, we only obtained suitable MS/MS profiles for three compounds. Putative identity confirmation of the rest of compounds could not be assessed in terms of structural information.

These three compounds were putatively identified (Fig. 4 and Fig. S1; based on the fragmentation patterns, exact mass

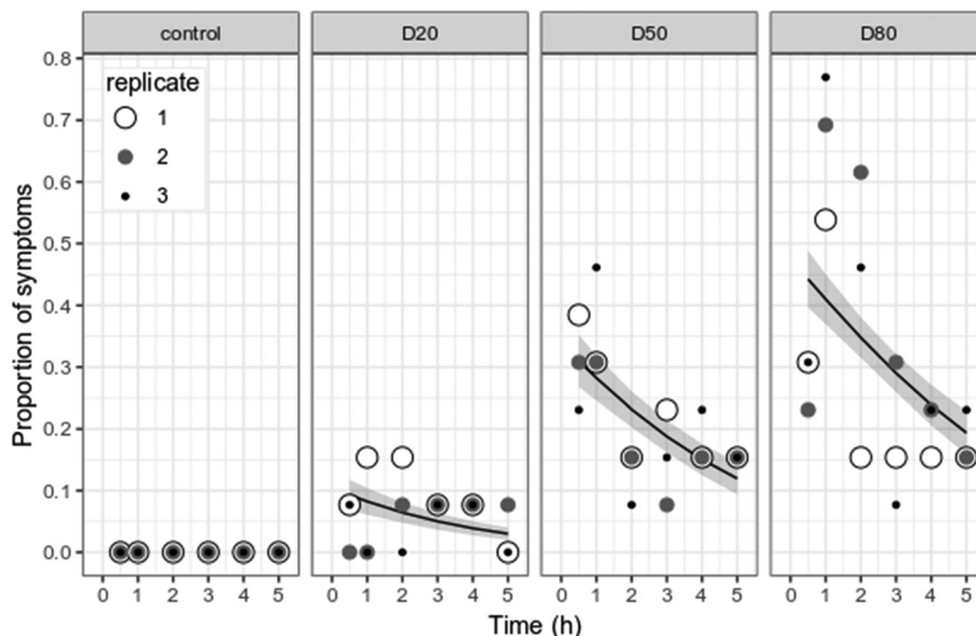

Fig. 2 Proportion of symptoms present in the five-hour observation period after injection, according to treatment. Replicates refer to mice used in each treatment (each mouse was used in only one treatment). Lines represent the model prediction and shades the standard error of the prediction. Given that the control was not included in the model, no predictions are presented for this treatment

and molecular formula) as BTX A at 6.15 min ($[M+H]^+$ at *m/z* 418.2585, for $C_{24}H_{35}NO_5$, −1.9 ppm error), DHQ **251A** known as 2-heptyl-5-methyl-decahydroquinoline at 12.89 min ($[M+H]^+$ at *m/z* 251.2686, for $C_{17}H_{33}N$, −1.6 ppm error) and Lehm **275A** named as 5-methyl-10-(8-nonynyl)lehmizidine at 11.77 min ($[M+H]^+$ at *m/z* 276.2687, for $C_{19}H_{33}N$, −1.4 ppm error). Lehm **275A** was found in samples from all study localities (Table S2). BTX A and DHQ **251A** were only found in skins from Agua Buena and La Tarde (Table S2). No Benzocaine was detected in any sample. The rest of the compounds identified to MS^1 level ranged in structural characteristics and included peptides, such as deltorphins, 15 other alkaloids common to poison frogs, such as pumiliotoxins, five more analogues of batrachotoxinin A, and bufo-compounds such as bufotenin (Table S1, S2).

Discussion

In this study, we provide the first experimental evidence of toxicity of the complete skin of *Phyllobates vittatus* in mice. Mice injected with increasing dosages of skin extracts exhibited elevating symptoms of toxicity, followed by a complete recovery. In addition, we detected the presence of the highly toxic alkaloid batrachotoxinin A, and other alkaloids that likely explain why mice responded to injections of *P. vittatus* skin extract with behavioral symptoms of discomfort and intoxication.

Anecdotal evidence had previously suggested that *P. vittatus* harbors toxic compounds. Myers et al. (1978) offered an individual *P. vittatus* to a captive *Rhadinaea taeniata aemula* (Colubridae) snake and watched for symptoms of toxicity. Almost immediately, the snake began gaping and rubbing its mouth on the substrate. In subsequent hours, mouth gaping, expansion of the thoracic region and slow body contortion were observed. All symptoms suggested distress. Similarly, a human who licked an individual *P. vittatus* suffered numbing of the tongue followed by tightening of the throat (Myers et al. 1978). Both the snake and the person completely recovered within hours from the initial contact with the frog (Myers et al. 1978). Consistent with these two observations of toxicity symptoms after direct contact through the mouth, our experimental data, through subcutaneous injection in mice, confirm the presence of toxic compounds in the skin of *P. vittatus*. Although symptoms produced by attempted ingestion and subcutaneous injection are not directly comparable, we note than in both anecdotal observations and our experimental results, toxicity symptoms were immediate and complete recovery was attained within a few hours.

According to spectral data, *P. vittatus* alkaloids detected were BTX A, a batrachotoxin analog; DHQ **251A**, one

Table 4 Pairwise comparisons between dose treatments on the proportion of toxicity symptoms observed in injected mice. Effect estimates and standard errors (SE) are given in the log-odds ratio scale of linear predictor. Significance testing based on z statistics requires that the degrees of freedom are set to "Inf", and therefore error is estimated using an asymptotic approximation. Significant differences ($\alpha < 0.05$) are highlighted in bold

Contrast	estimate	SE	d.f.	z ratio	*P* value
D20 - D50	−1.48	0.321	Inf	−4.603	**<0.0001**
D20 - D80	−2.05	0.312	Inf	−6.557	**<0.0001**
D50 - D80	−0.57	0.216	Inf	−2.639	**0.023**

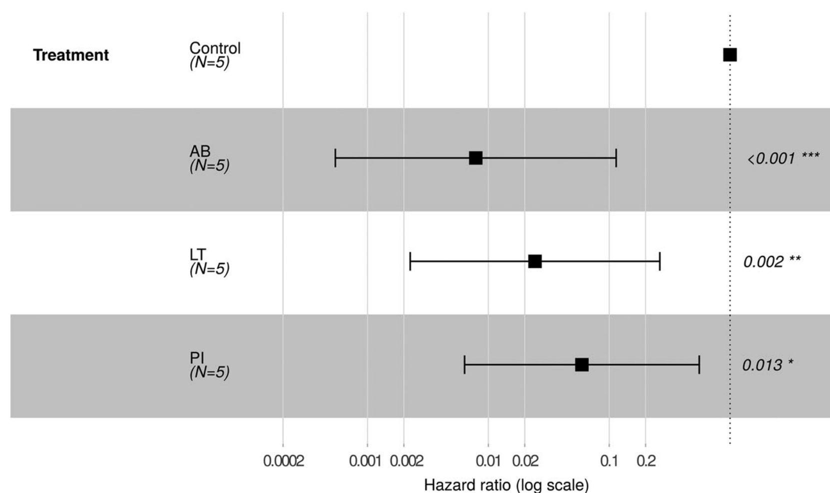

Fig. 3 Hazard ratios from Cox regression, indicating the latency to sleep of mice with injected skin extracts from three frog localities (AB = Agua Buena, LT = La Tarde, PI = Piro) versus saline controls (reference). Squares and whiskers, represent hazard ratio estimates and standard errors, respectively. Hazard ratios smaller than 1 imply higher latency to sleep than controls. *P*-values are shown on the right side of the plot and sample sizes are shown in parenthesis. Latency-to-sleep curves are shown in Fig. S2

decahydroquinoline-type; and Lehm **275A**, one lehmizidine-type (Fig. 4). Among other alkaloids, these compounds may be responsible for the described toxicity symptoms. Batrachotoxinin A is a highly toxic alkaloid that has an LD_{50} of 1 mg/kg in mice (Tokuyama et al. 1969). According to Albuquerque et al. (1971) and Myers et al. (1978), batrachotoxins are cardiotoxins that elicit symptoms such as ataxia, difficulty breathing, convulsions and salivations, all of which we observed in experimental mice. Decahydroquinolines are less toxic; the LD_{50} in mice is higher than 400 μg/kg. However, doses higher than 125 mg/kg can cause locomotor difficulties and convulsions (Daly and Spande 1986), symptoms exhibited by mice injected with the D80 treatment of *P. vittatus* extracts. On the other hand, lehmizidine-type alkaloids are not considered toxic, but may be unpalatable, conferring some protection against predators (reviewed by Santos et al. 2016). Furthermore, we found several pumiliotoxins (Table S1, S2), which could have caused some of the observed symptoms, such as convulsions, paralysis and locomotor difficulties (Daly and Spande 1986). Pumiliotoxins are ranked as having medium to high toxicity, with LD_{50} in mice ranging from 40 to 200 μg/kg (reviewed by Santos et al. 2016).

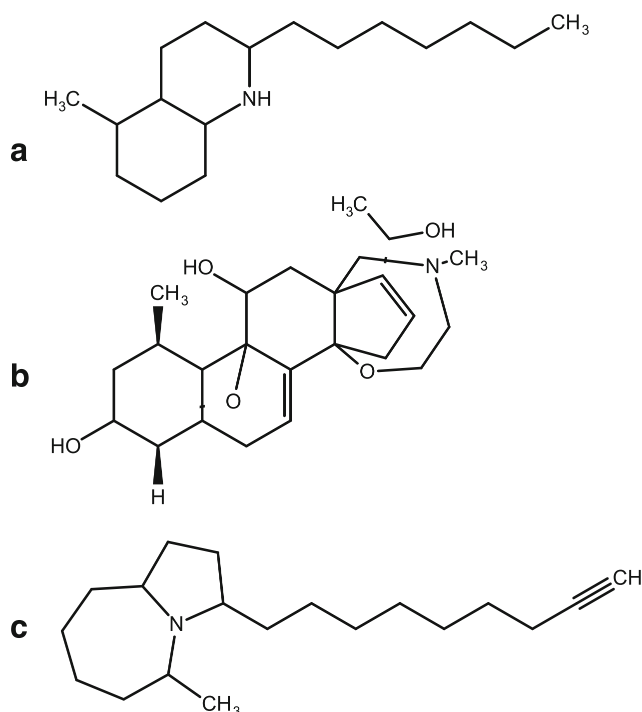

Fig. 4 Putative identified alkaloids in the Phyllobates vitattus skin extract according to spectral match between the experimental and in silico MS2 spectra: **a** DHQ 251A, **b** BTX A (Batrachotoxinin A), **c** Lehm 275A

In addition to the alkaloids mentioned above, we putatively identified the compounds bufotenine and deltorphins in the skin of *P. vittatus* (Table S1, S2). Bufotenine is common among toads (Daly et al. 1987). However, among poison frogs, it has only be found in *Melanophryniscus moreirae* (Bufonidae), which synthesizes this indolealkylamine (Jeckel et al. 2015). To date, bufotenine in *M. moreirae* and the pseudophrynamine alkaloids in members of the genus *Pseudophryne* (Myobatrachidae; Smith et al. 2002), are the only known alkaloids to be produced by poison frogs. Bufotenine had been reported to be toxic in mouse (Erspamer 1994) and could cause hallucinations (McBride 2000). Deltorphins are peptides, but not many peptides have been identified in poison frogs in comparison with frogs in general (Daly et al. 1978). This is probably because peptides are non-volatile compounds that cannot be detected through GC-MS, which is the main method used to analyze poison frog alkaloids. Deltorphins are reported to have high affinity and selectivity as agonist for δ-opioid receptors (Kreil et al. 1989), acting as analgesics (Broccardo et al. 1981). Therefore, given the importance of finding both, bufotenine and deltorphins, further studies analyzing fresh skin samples of *P. vittatus* should be carried in order to confirm their presence and deepen into the ecological implications of these findings.

When injected subcutaneously, batrachotoxinin A's minimal lethal dose in 20 g mice is approximately 20 μg (Myers et al.

1978). Daly et al. (1980) stated that levels of batrachotoxin in *P. vittatus* ranged from undetectable to a maximum of 0.8 μg per individual, but made no distinction about the type of batrachotoxin. For instance, the minimal lethal dose of batrachotoxin-homobatrachotoxin is approximately 0.05 μg when injected subcutaneously in 20 g mice (Myers et al. 1978). We have no information on the relative amount of each of the alkaloids present in the skin of *P. vittatus,* because we extracted the same samples for both biological assays and chemical analysis. Yet, it is possible that the most lethal toxin, batrachotoxinin A, is present only in a very low concentration in the skin of the studied frogs, as none of the tested doses caused mortality in mice. The presence of batrachotoxin in *P. vittatus* is a promising area for future research, as the environmental source of this potent alkaloid for the *Phyllobates* genus has not yet been identified (Dumbacher et al. 2000, 2004).

It is important to note that most reports in the literature of the effects of frog alkaloids and lethal dose assays have tested the delivery of isolated compounds in mice (e.g. Albuquerque et al. 1971; Myers et al. 1978). Although this approach provides important information, it does not necessarily reflect the set of symptoms that potential predators may experience upon contact with a complex array of mixed alkaloids in their prey. The method employed here of injecting extracts of the whole skin of individual frogs in mice may offer a more realistic perspective of the effectiveness of frog skin alkaloids as defenses.

Alkaloid content varies temporally as well as spatially in other poison frog species (Saporito et al. 2006, 2007). We cannot discard that this same pattern could occur with *P. vittatus*. For instance, Mebs et al. (2014) studied alkaloid content of *P. vittatus* skin collected during the dry season and from different localities than those studied here and failed to detect any batrachotoxin on skin extracts. In contrast, we sampled during the rainy season and analyzed alkaloids through LC-MS. Despite these differences in sampling methods, our studies share three alkaloids (DHQ **219A**, PTX **251D** and PTX **309A**; Table S2 from this study, Table 1 from Mebs et al. 2014) out of the ten reported by them (Mebs et al. 2014). Because alkaloid availability depends on arthropod prey (Saporito et al. 2006, 2007), variation in arthropod prey together with foraging patterns could lead to differences in alkaloid content. Frogs from the Dendrobatidae family are, in general, active throughout the year, but their activity peaks during the rainy season, when reproduction occurs (Savage 2002). Given the energetic demands related to reproduction such as in territoriality, courtship and parental care (Pröhl and Willink 2015), foraging should be more active during this period, consequently increasing exposure to potential predators and alkaloid requirements for chemical defense. Further studies should address whether and how ecological factors affect chemical defenses in *P. vittatus* in a seasonal and/or geographic pattern.

In spite of anecdotal evidence regarding the toxicity of *P. vittatus* (Myers et al. 1978), it has recently been speculated that *P. vittatus* is not toxic, but rather benefits from the presence of sympatric dendrobatids that are both toxic and conspicuous, such as *Oophaga granulifera* and *Dendrobates auratus* (Mebs et al. 2014). Co-occurrence with these aposematic species may indeed grant some protection to *P. vittatus*, if experienced predators fail to distinguish its color pattern from those of the brightly colored species they have learned to avoid (Mebs et al. 2014). However, our results do not support this idea, given that we found toxic alkaloids (batrachotoxinin A and DHQ **251A**) in the skin of *P. vittatus,* and their skin extracts caused symptoms of irritation in mice.

Previous studies have shown variation in toxicity among populations of dendrobatid poison frogs (e.g. Maan and Cummings 2012; Wang 2011), which has been attributed to the heterogeneity of arthropod communities from which alkaloids are sequestered (Maan and Cummings 2012; Rojas 2017; Wang 2011), and to different predation pressures (Wang 2011; Willink et al. 2014). In contrast, we did not find significant differences in toxicity, assessed through an irritant assay, among studied localities for *P. vittatus*. Although we found batrachotoxinin A and DHQ **251A** in two of the three sites (Table S2), other toxic alkaloids such as pumiliotoxins were present in samples from the three localities (Table S2). Pumiliotoxins, batrachotoxins and decahydroquinoles share similar effects of toxicity in mice (Albuquerque et al. 1971; Daly and Spande 1986; Myers et al. 1978; Santos et al. 2016), hence this could explain the lack of differences in irritability among sites. We presume that arthropod prey are similar among the three localities, given their geographic proximity and habitat similarity, but future research should focus on determine how availability of toxic prey influences chemical defenses, including studies on frog diet.

The irritant assay based on sleeplessness in mice (Darst et al. 2006; Maan and Cummings 2012), here used to estimate differences in irritability, as a measure of toxicity, among localities, was developed as a proxy of the relative irritant effect that frog skin alkaloids could have on predators (Darst et al. 2006). Nevertheless, it has been recently criticized by Weldon (2017), for three major reasons: (1) the method of injecting mice with skin extracts does not correspond to the frogs' natural defense mechanism via predator ingestion, (2) it is uncertain how prolonging the time that a predator remains awake will influence frog survivorship, and (3) toxicity and unpalatability are not necessarily related, as was recently demonstrated for the poison frogs *Oophaga pumilio* (Bolton et al. 2017) and *Dendrobates tinctorius* (Lawrence et al. 2019), and for a mimicry ring of nudibranch mollusks (Winters et al. 2018). As described by Myers et al. (1978), it is difficult to estimate the oral potency of batrachotoxin. Compared to subcutaneous injection, batrachotoxin toxicity is lower when introduced directly into the stomach of mice (Myers et al. 1978). Moreover,

it appears to be easily absorbed by buccal and esophageal mucosa, probably leading to death by asphyxiation at lower doses than would occur by gastric absorption (Myers et al. 1978). Given that the toxicity of batrachotoxin depends upon the delivery method, we assume that in the absence of a validated oral assay, injecting skin extracts from frogs subcutaneously should lead to an accurate estimation of toxicity. Yet, we agree with Wang (2011), about the need for the development of a validated oral avian assay (as in Lawrence et al. 2019), which would provide a more accurate representation of how chemical defenses in poison frogs function in nature. For instance, the first palatability assay was recently developed by Lawrence et al. (2019) to test distastefulness of anuran skin alkaloids to avian predators. With this new approach, it is possible not only to assess whether different morphs or populations of frogs vary in their unpalatability to model bird predators, but also to determine variation in unpalatability regardless of alkaloid content in frog skin secretions and without using live frogs (Lawrence et al. 2019). Our assessment of toxicity in mice does not provide direct evidence of how frog alkaloids would affect natural predators, and this is a question that currently remains unclear, limiting our understanding on how chemical defenses act in nature. Further research should aim to test whether and how toxicity and unpalatability are related (Winters et al. 2018; Lawrence et al. 2019) in this group of frogs, and if other compounds in the frogs skin, different than alkaloids, could be distasteful to natural predators, therefore acting as effective deterrents (Bolton et al. 2017).

Saporito and Grant (2018) criticized the use of the anesthetic Benzocaine to euthanize frogs in studies of skin alkaloid toxicity, as Benzocaine and frog alkaloids have similar modes of action at the molecular level. When Benzocaine is administered directly into the oral cavity of frogs (as in Amézquita et al. 2017), it is rapidly accumulated in the skin, which can lead to biased toxicity estimates (Saporito and Grant 2018). We applied the anesthetic Benzocaine to frogs' ventral skin surface, and then euthanized them by cervical transection, following previous protocols (Campos et al. 2016; Maan and Cummings 2012). Once we anesthetized the frogs, we immediately washed them with distilled water in order to remove excess Benzocaine. Moreover, we did not detect Benzocaine in the chemical profiles of frog skin extracts by mass spectrometry, which suggests that the anesthetic was adequately removed prior to the toxicity assays. Based on these confirmations at the chemical level, the symptoms of toxicity observed in mice were likely indeed caused by the alkaloids present in the skin of the frogs.

Among the dendrobatid frog family, the genus *Phyllobates* has been considered aposematic (Santos et al. 2003; Rojas 2017), meaning that the conspicuous coloration of the individuals is a warning signal of unpalatability or toxicity to potential predators (Ruxton et al. 2004; Skelhorn et al. 2016). The combination of conspicuous coloration and toxicity is an effective defense mechanism because predators learn to associate unpalatability with bright color patterns (Mappes et al. 2005). Such aversion learning is achieved at a faster rate when aposematic signals are more conspicuous and are thereby easier to detect and remember (Darst et al. 2006; Endler and Mappes 2004; Mappes et al. 2005; Rojas et al. 2014, 2015). When viewed dorsally, *P. vittatus* has a contrasting color pattern. Two reddish-orange stripes extend from the base of the thigh to the snout over a black background, while the limbs are green-blue (Savage 2002). We provide evidence that the skin of *P. vittatus* contains toxic compounds to mice, which provides support for aposematism in this species. Because we were unable to provoke lethality at the studied doses, we suggest that skin alkaloids might function as a non-lethal deterrent for predators, in accordance with the theory that lethal toxin doses are ineffective because dead predators do not learn or pass wariness to offspring (Longson and Joss 2006). However, further evidence is needed to support this conjecture, such as direct evidence of toxicity towards natural predators. In addition, to establish whether *P. vittatus* coloration is aposematic, conspicuousness for potential predators should be assessed through visual modeling, as well as predator avoidance and learning experiments.

In conclusion, our results provide the first experimental evidence that the complete array of skin alkaloids found in *P. vittatus* does confer toxicity to mice, even though the level of toxicity is lower than that of *Phyllobates* from Colombia (i.e. *P. aurotaenia, P. terribilis* and *P. bicolor*; Daly et al. 1987). We establish a list of symptoms for ranking non-lethal toxicity and cardiotonic effects of alkaloids in mouse models, and provide the basis for future research on the chemical ecology of this Costa Rican endemic poison frog.

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Compliance with Ethical Standards

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

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