# Linking Predator Responses to Alkaloid Variability in Poison Frogs

J.P. Lawrence1,2\*, Bibiana Rojas3,4, Annelise Blanchette5, Ralph A. Saporito5, Johanna Mappes3.6, Antoine Fouquet7, and Brice P. Noonan1

1 Department of Biology, University of Mississippi, University, MS 38677, USA

2 Current Address: Lyman Briggs College, Michigan State University, East Lansing, MI 48825, USA

3 University of Jyväskylä, Department of Biology and Environmental Science, P.O. Box 35, 40014, Jyväskylä, Finland

4 Konrad Lorenz Institute of Ethology, Department of Interdisciplinary Life Sciences, University of Veterinary Medicine Vienna, Savoyenstraße 1, 1160, Vienna, Austria

5 Department of Biology, John Carroll University, University Heights, OH 44118, USA

6 Organismal and Evolutionary Biology Research Programme, Faculty of Biological and Environmental Sciences, Helsinki University, Finland

7 Laboratoire Evolution et Diversité Biologique, UMR5174, Université Paul Sabatier, 31062 Toulouse cedex 9, France

\*Corresponding Author: JPLarry@gmail.com

**Abstract-** Many chemically-defended/aposematic species rely on diet for sequestering the toxins with which they defend themselves. This dietary acquisition can lead to variable chemical defenses across space, as the community composition of chemical sources is likely to vary across the range of (an aposematic) species. We characterized the alkaloid content of two populations of the Dyeing Poison Frog (*Dendrobates tinctorius*) in northeastern French Guiana. Additionally, we conducted unpalatability experiments with naive predators, Blue Tits (*Cyanistes caeruleus*), using whole-skin secretion cocktails to assess how a model predator would respond to the defense of individuals from each population. While there was some overlap between the two *D. tinctorius* populations in terms of alkaloid content, our analysis revealed that these two populations are markedly distinct in terms of overall alkaloid profiles. Predator responses to skin secretions differed between the populations. We identified 15 candidate alkaloids (including three previously undescribed) in seven classes that are correlated with predator response in one frog population. We describe alkaloid profile differences between populations for *D. tinctorius* and provide a novel method for assessing unpalatability of skin secretions and identifying which toxins may contribute to the predator response. In one population, our results suggest 15 alkaloids that are implicated in predator aversive response. This method is the first step in identifying the causal link between alkaloids and behavioral response of predators, and thus makes sense of how varying alkaloid combinations are capable of eliciting consistent behavioral responses, and eventually driving evolutionary change in aposematic characters (or characteristics).

**Key Words**: Unpalatability, Dendrobates tinctorius, Alkaloids, Birds, Aposematism.

INTRODUCTION

Aposematism is a ubiquitous defensive strategy in which prey species use warning signals to inform would-be predators of a secondary defense (Poulton 1890), often in the form of defensive chemicals. For a number of aposematic taxa (e.g., nudibranchs, butterflies, and dendrobatid frogs), defensive chemicals are sequestered from food sources rather than being synthesized *de novo* (Proksch 1994; Nishida 2002; Saporito et al. 2011), often resulting in interpopulation variation in defenses because food sources vary spatially and temporally (Saporito et al. 2006, 2007a; Daly et al. 2008; Prates et al. 2019). Consequences of this variation range from automimicry (Speed et al. 2006) to polytypy (interpopulation variation) in aposematic phenotypes (Siddiqi et al. 2004).

Honest signaling occurs when a signal advertises the consequence of a secondary defense (Summers et al. 2015). If a signal is associated with a poor defense, predators will not strongly connect the signal with a defense (Rowland et al. 2007). Conversely, a poor signal that is over-defended may result in predators not strongly associating the signal with the defense (Lindström et al. 1999). Additionally, chemical defenses are often costly (Ojala et al. 2005; Sandre et al. 2007; Zvereva and Kozlov 2015; Burdfield-Steel et al. 2019). Therefore, having a well-matched signal should ensure that energy and resources are not wasted. While some studies suggested the occurrence of honest signaling among populations of aposematic species (e.g., see Vidal-Cordero et al. 2012; Maan and Cummings 2012), these conclusions have been based on analysis of the defense mechanism alone, not the effect this defense has on predators. Furthermore, this is not a universal trend, as ladybird beetles are known to exhibit a positive or negative correlation between signal and chemical defense depending on whether they are raised on low amounts of food or under resource abundance, respectively (Blount et al. 2012). Likewise, some species of poison frogs seem to display a tradeoff between secondary defense and signal (Darst and Cummings 2006; Wang 2011).

Alkaloid variation in dendrobatid poison frogs has been well-characterized in several species (Daly et al. 1987; Bolton et al. 2017). Over 800 different types of diet-based alkaloids have been described from poison frogs (Daly et al. 2005), defenses primarily derived from ants and mites (Saporito et al. 2003, 2007b; McGugan et al. 2016), and some other invertebrates (i.e., millipedes and beetles; Saporito et al. 2007). As the source of these defensive chemicals are arthropods (Darst et al. 2005), alkaloid profiles among frog populations have been shown to vary among conspecific populations (Prates et al. 2019) and over time within populations (Saporito et al. 2006, 2007a). Characterizing the chemical profiles of secondary defenses in aposematic species is important for understanding how these vary among populations and how well these species are defended from predators. Even more important is to identify which chemicals are actually relevant for predator deterrence. Furthermore, because secondary defenses contribute to the efficacy of aposematic signaling, baseline data on alkaloid profiles can provide important insight into why aposematic signals vary among species and populations.

While alkaloid defenses have been the subject of scientific inquiry for decades, their relationship to aposematic signal variation is less understood. When examining the polytypy in the Strawberry Poison Frog (*Oophaga pumilio*)in Bocas del Toro, Panama, (Daly and Myers 1967) found no relationship between toxicity and color/pattern. Conversely, Maan and Cummings (2012) suggested that color/pattern of the poison frogs they studied were honest indicators of defense, particularly towards avian predators, and therefore there should be a strong relationship between chemical defense and conspicuousness of warning signals. This apparent contradiction may be the result of differences in methodological techniques. Daly and Myers (1967) assessed toxicity by determining LD50 (lethal dose for 50% of test subjects) of toxin extracts for mice from different populations, while Maan and Cummings (2012) examined the discomfort mice exhibited after injection. Evidence that these two methods of inferring defense provided by integumentary alkaloids are not complementary was provided by Bolton et al. (2017), who found divergent results for LD50 and discomfort for individual samples. Importantly, however, the relationship between palatability (the direct means of predator interaction with chemical defenses and upon which they presumably base decisions) and toxicity appears to be unrelated (Bolton et al. 2017; but toxicity could be predator-dependent), nor is total alkaloid quantity predictive of palatability (Lawrence et al. 2019a). More importantly, not only are mice not relevant predators of poison frogs (and thus, not a selective agent behind the evolution of toxicity), but injections are an unrealistic method of assessing the biological function of defensive alkaloids, as anecdotal evidence on poison frog predation points to ingestion (e.g., by birds) as the mechanism of exposure. While assays with mice may act as a proxy of toxicity, they may not be biologically relevant as these toxins are experienced by the predator in the oral cavity and digestive tract when capturing and consuming the prey species, rather than directly entering the bloodstream, musculature, and/or peritoneal cavity (Holen 2013; Weldon 2017; Saporito and Grant 2018). Therefore, how the quantity and composition of alkaloids present in frog skins relate directly to predator responses presents a glaring knowledge gap in the evolutionary puzzle of signal honesty.

The Dyeing Poison Frog (*Dendrobates tinctorius*) is found throughout the Eastern Guiana Shield region (sensu Vacher et al. 2020) in northern South America. Throughout its range, it shows considerable color and pattern variation among (and sometimes within) populations (Noonan and Gaucher 2006; Rojas and Endler 2013; Lawrence et al. 2019a). *Dendrobates tinctorius* sequester alkaloids (Summers and Clough 2001; Santos et al. 2003), but only four populations have had their alkaloids characterized to date (Daly et al. 1987; Lawrence et al. 2019a). Here, we leverage the between-population warning signal variability in this chemically-defended species to ask how variability in alkaloid defenses may relate to avoidance responses by model predators. First, if the skin alkaloids vary considerably among populations, it suggests that toxin content (or skin secretions) vary along with the environment and that individuals have relatively weak control over their toxic defenses. Second, if alkaloid content is different among populations, we expect predator responses to differ as well. If not, that would suggest weak (or relaxed) selection for alkaloid content - specifically alkaloids responsible for distastefulness. However, if the response by predators does differ between populations, is there a subset of alkaloids that would explain the response?

METHODS AND MATERIALS

*Field Collection* – We collected 18 *Dendrobates tinctorius* in May-June 2013 and August 2014 from two populations in French Guiana: Matoury (4.89°N, 52.34 °W; 10 individuals) and Kaw Mountains (4.57°N, 52.21°W; 8 individuals). For each encountered frog, we recorded individual variation (sex, snout-vent length). Next, frogs were euthanized by cervical transection and pithing in the field immediately after taking measurements. We skinned frogs and placed whole skins in 100% methanol in 4mL vials with PTFE caps, referred to hereafter as the “methanol extract.”

*Alkaloid Extraction*– We followed the protocol outlined by Saporito et al. (2010) to conduct acid-base fractionations of the methanol frog skin extracts. We took 1mL of the methanol extract and added it to a graduated conical vial along with 50μL of HCl and 100μL of an internal nicotine standard (L-Nicotine, 99+%, Acros Organics). Samples were then dried down to 100μL using a gentle flow of N2 and then 200μL of DI H2O was added. Following this, the sample was then extracted three times, each time with 300μL of hexane. The solution was then basified with NaHCO3. The sample was then extracted three times, each time with 300μL of ethyl acetate and then dried with anhydrous NaSO4, and evaporated to dryness under a gentle flow of N2. The sample was reconstituted in 100μL of methanol for alkaloid analysis.

*GC-MS Analysis* - Gas chromatography-mass spectrometry (GC-MS) was performed for each individual sample on a Varian Saturn 2100T ion trap MS instrument, which was coupled to a Varian 3900 GC with a 30m x 0.25mm inside diameter Varian Factor Four VF-5ms fused silica column. GC separation of alkaloids was achieved using a temperature program from 100°C to 280°C at a rate of 10°C per minute with helium as the carrier gas (1 mL/min). Each alkaloid fraction was analyzed in triplicate with electron impact MS and once with chemical ionization (CI) MS with methanol as the ionizing reagent.

Individual alkaloids of *D. tinctorius* were identified based on comparison of mass spectral properties and GC retention times with those of previously reported alkaloids in dendrobatid frogs (Daly et al. 2005; Saporito, unpublished poison frog alkaloid library). Alkaloids in dendrobatid frogs have been assigned a series of code names that consist of a boldfaced number indicating the alkaloids’ nominal mass, and a boldfaced letter to distinguish those alkaloids with the same nominal mass (Daly et al. 2005). Alkaloid quantities for each individual frog were calculated by comparing the average observed peak area of individual alkaloids to the average peak area of the nicotine standard from the triplicate EI-MS analyses using a Varian MS Workstation v.6.9 SPI. Only alkaloids that were present in quantities of ≥ 0.5μg were included in the analyses (Bolton et al. 2017).

*Unpalatability Assays* – We used data from a previously published study (Lawrence et al. 2019b) to investigate the link between amount and composition of skin alkaloids and predator response. Briefly, the unpalatability assays consisted of the following methodology. We took 1mL of the methanol extract and evaporated it to dryness under a gentle stream of N2 and then reconstituted the extract in 0.5mL ethanol to then be used in unpalatability trials with Blue Tits (*Cyanistes caeruleus*). While Blue Tits are a palearctic species and thus would not encounter Neotropical frogs, predators of poison frogs are assumed to be birds (Comeault and Noonan 2011; Chouteau and Angers 2011; Rojas et al. 2014; Paluh et al. 2014) and some anecdotal evidence seems to support this assumption (Master 1999; Alvarado et al. 2013). Bird taste systems are generally conserved across genera (Wang and Zhao 2015), which suggests that the response from Blue Tits to alkaloids will be similar to other birds. The use of Blue Tits serves an additional advantage for this study. Use of sympatric species runs the risks that individuals have previously experienced these toxins and responses may be influenced by prior experience. Thus, using this species allows us to use adult birds that are truly naive to these toxins.

We used a protocol developed by (Rojas et al. 2017) that is designed to test the chemical defense efficacy where the target compound is offered to a predator in a novel context. Prior to experiments, Blue Tits (*Cyanistes caeruleus*) were trained to eat oat flakes. We tested a total of 25 birds, eight with extracts from the Kaw population, 10 with extracts from the Matoury population, and seven controls. Each of two oats were soaked with 15µl of the extract of one frog skin and left for 24h at room temperature to ensure that all ethanol had evaporated. Two other oats were soaked each with 15µl of pure ethanol that were used at the beginning and end of the experiment with each bird. Each bird went through four trials. The first trial consisted of a control oat which needed to be consumed entirely by the bird before the experiment could be initiated. Following this, two consecutive extract treatments each consisted of a single oat with extract. The final trial involved the second control oat which was offered to ensure that the birds were not refusing to eat the oats coated with extract out of satiation or lack of motivation to eat in general. Birds in the control treatment received oats soaked with pure ethanol four all four treatments in order to compare directly the response of birds to oats containing frogs’ extracts vs. oats with ethanol only.

Each oat was presented on a hatch that had a visual barrier, which allowed us to identify the exact moment at which the oat was seen which determined the actual beginning of each of the two experimental trials where birds were exposed to toxins. We measured the percentage of the oat eaten as an analog for how distasteful the oat was. Birds were watched for a 2-min period after they finished eating the oats, or for a maximum of 5 min in the cases in which the oat was not fully eaten, to make sure that any delayed response to the oat taste was not going to be missed.

*Data Analysis –* As individuals and populations can be quite variable in alkaloid content, it is unlikely that the entire suite of alkaloids is contributing to the behavioral response of predators. In order to identify the most likely alkaloid candidates driving predator response, we performed an exploratory factor analysis using the quantities of each type of alkaloid. Factor analyses group independent variables into loadings that explain population variation in these variables. Following this, we performed a multiple linear regression to identify which loadings are explanatory for variation in behavioral response. We performed this analysis for each population to determine what, if any, alkaloids are important for behavioral response to the frogs’ chemical defenses. All analyses were conducted using the R platform (R 2016).

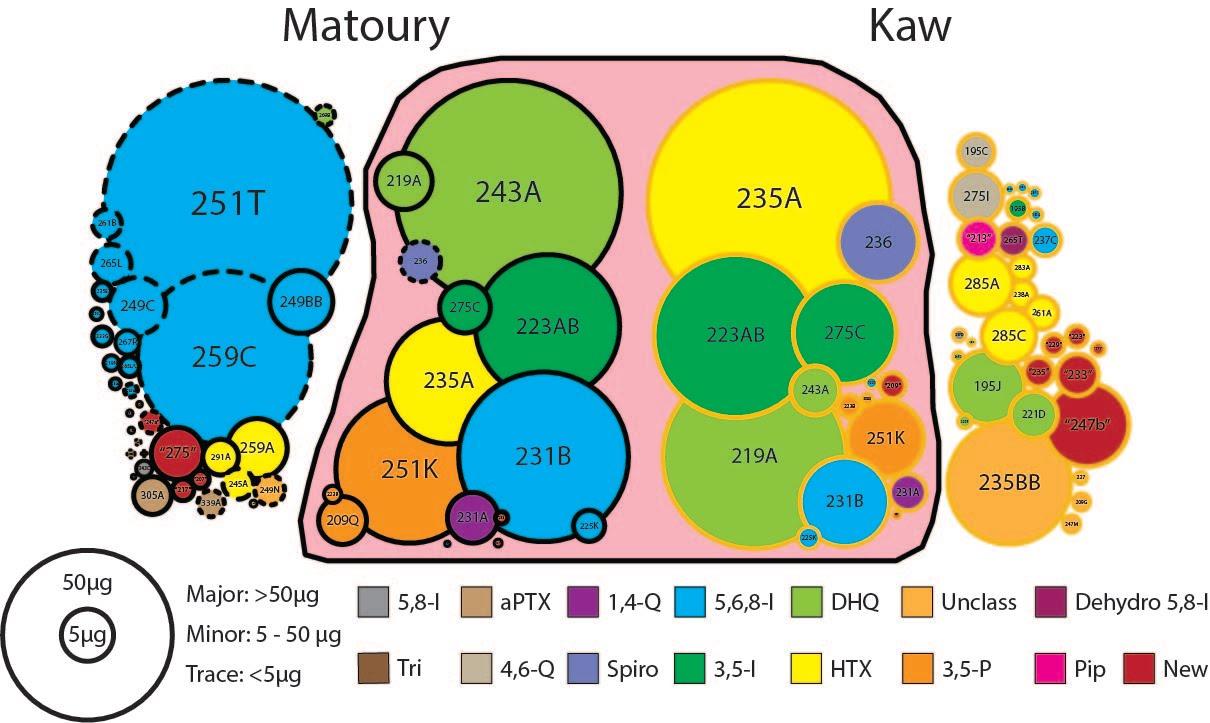
RESULTS

The Matoury and Kaw populations showed similar diversity, though different composition, of alkaloids (49 in 11 classes and 46 in 12 classes, respectively; Table 1). Fifteen alkaloids were found in both populations, albeit in different quantities (Figure 1). Only the Matoury population had significant loadings which implicated 15 alkaloids with behavioral response, all of which were unique to this population (Table 1).

**Table 1: Alkaloid variation seen in the Matoury and Kaw populations**

Alkaloids with gray background were found to significantly impact predator behavior in unpalatability experiments. Alkaloids are divided into the following structural classes: 3,5-disubstituted indolizidines (3,5-I), 5,6,8-trisubstituted indolizidines (5,6,8-I), 3,5-disubstituted pyrrolizidines (3,5-P), histrionicotoxins (HTX), decahydroquinolines (DHQ), 1,4-disubstituted quinolizidines (1,4-Q), allopumiliotoxins (aPTX), 5,8-disubstituted indolizidines (5,8-I), spiropyrrolizidine (Spiro), 4,6-disubstituted quinolizidines (4,6-Q), dehyrdo-5,8-disubstituted indolizidines (Dehydro-5,8-I), tricyclics (Tri), unclassified alkaloids (Unclass), piperidine (Pip). The piperidine and fifteen (New) alkaloids have not previously been described but are in quotes as further characterization is needed in order to be documented as new alkaloids. Major alkaloids (\*) are found in quantities greater than 50μg, minor alkaloids (†) are found in quantities between 5μg and 50μg, and trace alkaloids (o) are found in quantities less than 5μg.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Structural Class | Alkaloid | Matoury Population | | | | | | | | | | Matoury Average | Kaw Population | | | | | | | Kaw Average |
| M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 | M9 | M10 | K1 | K2 | K3 | K4 | K5 | K6 | K7 |  |
| 3,5-I | **195B** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | o |  | o |
|  | **223AB** | † | † | † | o | † | \* | \* | † | \* | o | † | † | † | † | \* | † | † | \* | † |
|  | **275C** |  |  | o | † | o | † | o | † |  | † | o | † | † |  | † | † |  | † | † |
| 5,6,8-I | **193G** |  |  |  |  |  |  |  |  |  |  |  |  |  |  | o |  |  |  | o |
|  | **195D** |  |  |  |  |  | o |  |  |  |  | o |  | o |  |  |  |  |  | o |
|  | **205A** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | o |  | o |
|  | **207C** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | o |  |  | o |
|  | **219N** |  |  |  |  |  |  |  | o |  |  | o |  |  |  |  |  |  |  |  |
|  | **223A** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | o |  |  | o |
|  | **225K** |  |  |  |  |  | † | † |  |  |  | o |  |  |  |  | o |  | o | o |
|  | **231B** | \* | † | \* | † | † | \* | \* | † | † | † | \* | o | † | o | † | † | † | † | † |
|  | **233G** |  |  | o |  |  |  | o |  | o |  | o |  |  |  |  |  |  |  |  |
|  | **235E** |  |  |  |  |  |  |  | o | o | o | o |  |  |  |  |  |  |  |  |
|  | **237C** |  |  |  |  |  |  |  |  |  |  |  | o | o |  |  |  |  |  | o |
|  | **245G** |  |  |  |  |  |  |  |  | o |  | o |  |  |  |  |  |  |  |  |
|  | **249C** | † |  | † | o |  |  | † |  |  |  | † |  |  |  |  |  |  |  |  |
|  | **249BB** |  |  |  | † | o |  |  | † | † | o | † |  |  |  |  |  |  |  |  |
|  | **251T** | \* | \* | \* | o | \* | \* | \* | \* | \* | \* | \* |  |  |  |  |  |  |  |  |
|  | **259C** | \* | † | \* | † | † | † |  | † | † | † | \* |  |  |  |  |  |  |  |  |
|  | **261B** | † |  | † |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **263A** | o |  | o |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **265L** | o |  | † |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **265U** | o |  | o |  |  |  |  |  | o |  | o |  |  |  |  |  |  |  |  |
|  | **265L** |  |  |  |  |  |  |  | † |  | † | o |  |  |  |  |  |  |  |  |
|  | **267R** |  |  |  | o | o | o | o | o | o |  | o |  |  |  |  |  |  |  |  |
| 3,5-P | **209Q** |  |  | † |  |  | † | † | o | o | o | o |  |  |  |  | o |  |  | o |
|  | **223B** |  |  |  |  |  |  |  | † |  |  | o | o |  |  | o |  |  |  | o |
|  | **251K** | † | † | † | o | o | \* | \* | † | † | \* | † | o | o |  |  |  |  | † | † |
| HTX | **235A** | † | \* |  | o | † | † | \* | † | † | † | † | \* | \* |  |  | \* |  |  | \* |
|  | **238A** |  |  |  |  |  |  |  |  |  |  |  |  | † |  |  |  |  |  | o |
|  | **245A** |  |  | † |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **259A** |  |  |  |  |  | † | † | † | † |  | o |  |  |  |  |  |  |  |  |
|  | **261A** |  |  |  |  |  |  |  |  |  |  |  |  |  |  | o |  | o | o | o |
|  | **283A** |  |  |  |  |  |  |  |  |  |  |  | † |  |  |  |  |  |  | o |
|  | **285A** |  |  |  |  |  |  |  |  |  |  |  | † |  |  |  |  |  |  | o |
|  | **285C** |  |  |  |  |  |  |  |  |  |  |  |  | † |  |  |  |  |  | o |
|  | **291A** |  |  |  |  |  |  | † |  |  | † | o |  |  |  |  |  |  |  |  |
| DHQ | **195A** |  |  |  |  |  |  |  |  |  |  |  |  | o |  |  |  |  |  | o |
|  | **195J** |  |  |  |  |  |  |  |  |  |  |  | † | † |  | o |  | † | o | † |
|  | **219A** | o | o | † |  |  | † | o |  | o | o | † | † | † | † | \* | † | \* | \* | † |
|  | **221D** |  |  |  |  |  |  |  |  |  |  |  |  |  | o | o | o | o | † | o |
|  | **223F** |  |  |  |  |  |  |  |  |  |  |  |  | o |  |  |  |  |  | o |
|  | **243A** | \* | \* | † | † | † | \* | \* | † | \* | † | \* | † |  |  |  |  | o | † | o |
|  | **245Q** |  |  |  |  |  |  |  |  |  |  |  | o |  |  |  |  |  |  | o |
|  | **269B** | o | o |  |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
| 1,4-Q | **231A** | † | o | o |  |  | † |  | † | o |  | o | o | o | o | o | o | o |  | o |
|  | **235U** |  |  |  |  |  |  |  |  |  | o | o |  |  |  |  |  |  |  |  |
| aPTX | **305A** |  |  |  |  | † | † |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **339A** |  |  | † |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
| 5,8-I | **243C** |  |  |  |  |  | o |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **237D** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | o |  | o | o |
| Spiro | **236** |  | o | † |  |  |  | † |  |  |  | o |  | † | † | † |  | † | o | † |
| 4,6-Q | **195C** |  |  |  |  |  |  |  |  |  |  |  | † | o |  |  | o |  |  | o |
|  | **275I** |  |  |  |  |  |  |  |  |  |  |  | † |  |  |  |  |  | † | o |
| Dehydro-5,8-I | **265T** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | † |  |  | o |
| Tri | **205B** |  |  | o |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **205E** |  |  |  |  |  | o |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **207G** |  |  | o |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
| Unclass | **209G** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | o |  | o |
|  | **209M** |  |  |  |  |  | o |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **227** |  |  |  |  |  |  |  |  |  |  |  |  | o |  |  |  |  | o | o |
|  | **235BB** |  |  |  |  |  |  |  |  |  |  |  |  |  | \* | † |  | † | † | † |
|  | **247M** |  |  |  |  |  |  |  |  |  |  |  | o |  |  |  |  |  |  | o |
|  | **249N** | † | o | o | o | o | o | o | o | o |  | o |  |  |  |  |  |  |  |  |
| Pip | **“213”** |  |  |  |  |  |  |  |  |  |  |  |  |  | o | o | o | † |  | o |
| New | **“171”** |  |  |  |  |  |  |  |  |  |  |  |  | o |  |  |  |  |  | o |
|  | **“193”** |  | o |  |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **“207”** |  | † |  |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **“209”** |  | o |  |  |  |  |  |  |  |  | o |  | † |  |  |  |  |  | o |
|  | **“217”** |  | † |  |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **“223”** |  |  |  |  |  |  |  |  |  |  |  | o | o |  |  |  |  |  | o |
|  | **“229”** |  |  |  |  |  |  |  |  |  |  |  | o | o |  |  |  |  |  | o |
|  | **“233”** |  |  |  |  |  |  |  |  |  |  |  |  |  | † |  |  | † | o | o |
|  | **“235”** |  |  |  |  |  |  |  |  |  |  |  |  |  |  | o |  |  | † | o |
|  | **“237”** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | **“245”** | o |  |  |  |  |  |  |  |  |  | o |  |  |  |  |  |  |  |  |
|  | **“247a”** | o |  | o |  |  | o | o |  | o | o | o |  |  |  |  |  |  |  |  |
|  | **“247b”** |  |  |  |  |  |  |  |  |  |  |  |  |  | † | † | † | † | † | † |
|  | **“253”** |  | o | o |  |  |  |  |  |  |  | o |  | o |  |  |  |  |  | o |
|  | **“275”** |  | o | o | o | † | † | † | o | o | o | o |  |  |  |  |  |  |  |  |
| Total |  | 17 | 18 | 25 | 13 | 13 | 22 | 19 | 19 | 20 | 17 | 49 | 19 | 22 | 10 | 15 | 16 | 16 | 18 | 46 |

****

**Figure 1: Distribution of alkaloids between the two populations**Alkaloids common to both populations of *D. tinctorius* are surrounded by the pink shape. Text in the circles represent the type of alkaloid. Size of circles represents relative proportions of the population average of the alkaloid. Circles with dashed lines denote alkaloids that were implicated in predator response (See Table 1 for complete list). Color of the circle represents the structural class in which the alkaloid is found. Alkaloids are divided into the following structural classes: 3,5-disubstituted indolizidines (3,5-I), 5,6,8-trisubstituted indolizidines (5,6,8-I), 3,5-disubstituted pyrrolizidines (3,5-P), histrionicotoxins (HTX), decahydroquinolines (DHQ), 1,4-disubstituted quinolizidines (1,4-Q), allopumiliotoxins (aPTX), 5,8-disubstituted indolizidines (5,8-I), spiropyrrolizidine (Spiro), 4,6-disubstituted quinolizidines (4,6-Q), dehyrdo-5,8-disubstituted indolizidines.

We detected 49 different alkaloids representing 11 structural classes (Table 1; Figure 1) from frogs in the Matoury population. Twelve of the 49 alkaloids were represented in single individuals. Of the 49 alkaloids, 14 (two 3,5-disubstituted indolizidines (3,5-I) [**223AB** and **275C**], four 5,6,8-trisubstituted indolizidines (5,6,8-I) [**231B**, **251T**, **259C**, and **267R**], two 3,5-disubstituted pyrrolizidines (3,5-P) [**209Q** and **251K**], one histrionicotoxin (HTX) [**235A**], two decahydroquinolines (DHQ) [**219A** and **243A**], one 1,4-disubstituted quinolizidines (1,4-Q) [**231A** and **249N**], and two new alkaloids of molecular weight **247** and **275** were found in most individuals. The new alkaloids could not be assigned a structural class, and will be further characterized in another publication.

The factor analysis revealed five loadings that explained 76% of the variation among alkaloid profiles in the Matoury population. A subsequent multiple linear regression (multiple R2 = 0.97, F5,4 = 34.07, *p* = 0.002) revealed two loadings that significantly deviated from the null when examining the proportion of oats eaten by blue tits. Loading 1 (t = -11.47, *p* = 0.0003), which explains 18% of the population variation, contained the alkaloids Tri (tricyclic) **205B**, Tri **207GH**, spiropyrrolizidine (Spiro) **236**, cyclopentaquinazoline (CPQ) **245A**, 5,6,8-I **265L**, and allopumiliotoxin (aPTX) **339A**. Loading 3 (t = -3.113, *p* = 0.0350), which explains 17% of the population variation, contained the alkaloids new **245**, new **247**, 5,6,8-I **249C**, Unclassified (Unclass) **249N**, 5,6,8-I **251T**, 5,6,8-I **259C**, 5,6,8-I **261B**, 5,6,8-I **263A**, and DHQ **269B**.

We detected 46 different alkaloids representing 12 different structural classes from frogs in the Kaw population (Table 1; Figure 1). Notably, of the eight Kaw individuals examined, one individual showed approximately ten times the quantity of alkaloids as compared to other individuals in the population and was thus considered an outlier and removed from analysis. Of the 46 different alkaloids, eighteen were found in single individuals as opposed to being found repeatedly in the population. Eleven alkaloids are represented in a majority of individuals in this population including one 3,5-I (**223AB** and **275C**), one 5,6,8-I (**231B**), DHQs (**195J**, **219A**, and **221D**), one 1,4-Q (**231A**), one Spiro (**236**), one Unclass (**235BB**), one new piperidine (PIP) of molecular weight **213**, and one new alkaloid of molecular weight **247**. Both the new piperidine and alkaloid of molecular weight 247 (also present in Matoury population), will be further characterized in another publication.

The factor analysis revealed four loadings that explained 70% of the variation observed in toxin profiles in the Kaw population. After factor analysis, however, multiple linear regression revealed no differences among loadings when examining either proportion of oats eaten (multiple R2 = 0.07, F4,3 = 0.05, *p* = 0.99) or beak wiping.

DISCUSSION

Dendrobatid frogs are characterized by their impressive alkaloid diversity used as secondary defenses combined with their conspicuous color signals. Whether this diversity is necessary for effective defenses or a subset of important alkaloids primarily drives predator responses has not been previously tested. We report the diversity of alkaloids from two populations of *D. tinctorius* (sampled punctually) and infer which alkaloids may be correlated with predator behavior. These populations show similar richness in alkaloids (numbers and structural classes), but the composition of the alkaloid cocktails is different between the two populations (Lawrence et al. 2019a). Only three alkaloids are well-represented in both populations (**223AB**, **231B**, and **219A**). Mites are known sources for both **223AB** and **231B** (Saporito et al. 2007b; McGugan et al. 2016) and, while no source of **219A** has been identified, as it is a decahydroquinoline, the source for that alkaloid is likely ants (Saporito et al. 2007a). These overlapping and common alkaloids suggest that some prey may be common to both populations, which is not surprising as the two populations are less than 50 km from one another. Matoury shows a large diversity of 5,6,8-trisubstituted indolizidines, six of which (**249C**, **251T**, **259C**, **261B**, **263A**, and **265L**) are implicated in predator aversive response. Allopumiliotoxins and tricyclics were found only in Matoury while dehydro-5,8-indolizidines, 4,6-quinolizidines, and piperidines were found only in Kaw. These classes are known to come from ants, mites, and beetles (Saporito et al. 2007b) which, given the population specificity of these alkaloid classes, suggests differential availability of these sources between the two populations.

With over 800 alkaloid toxins known from poison frogs (Daly et al. 2005), it is likely that palatability varies widely across populations and species. Whole toxin profile examination provides insight into this chemical diversity, it lacks the ability to identify the specific alkaloid components of the diverse toxin cocktail that elicit aversion. Our unpalatability assay offers a new perspective on the linkage between chemical defense and predator response (Lawrence et al. 2019a), which together drive the evolution of aposematic phenotypes. Coupling predator responses with individual alkaloid profiles offers the opportunity to isolate which individual alkaloids influence predator aversive responses. Given the large variety of alkaloids present in toxin profiles of *D. tinctorius* and the small sample size of this study, we acknowledge that the interpretation of our results is limited. However, our study does provide a novel approach for future studies to tease out the contributions of individual alkaloids in driving predator response. Chemical identification alone does not explain how these defensive compounds function in antipredator behavior, and likewise, predator assays do not address the question why predators respond as they do. By using this comprehensive approach of both chemical identification and predator assay, we are able to tease apart the function of defensive compounds.

The proximate causes of predator behavior remain unknown and unstudied, with prior studies focusing on the effects of alkaloid defenses on predators (e.g., Daly and Myers 1967; Maan and Cummings 2012; Bolton et al. 2017) or identification of alkaloids (e.g., Daly et al. 1987; Saporito et al. 2006; McGugan et al. 2016). Given the variability of alkaloids within and among populations, a subset of alkaloids that may be common to most individuals in a population may be responsible for predator responses. Consistency in predator response, despite varied alkaloid profiles, would be important for aposematism to evolve. In this study, we identify 15 alkaloids implicated in predator response for the Matoury population. Interestingly, 14 of the 15 alkaloids are unique to this population, with only the **236** spiropyrrolizidine being common to both. Six of these fifteen alkaloids are 5,6,8-trisubstituted indolizidines. This alkaloid class has the highest representation, both in terms of quantity and diversity, of the fifteen implicated alkaloids, perhaps suggesting the importance of this class in eliciting aversive responses in avian predators. Our approach narrows this large variation into a subset of alkaloids for future investigation, allowing for investigations of the proximate causes of predator behavior.

As *Dendrobates tinctorius* is highly polytypic throughout its distribution (Noonan and Gaucher 2006; Wollenberg et al. 2008; Lawrence et al. 2019a), future research should focus on further characterizing alkaloid diversity among populations and across time. Given that 16 of the 81 (19.7%) alkaloids described here have not previously been described in the literature, we speculate that a large number of undescribed alkaloids are present in the other populations of *D. tinctorius*. Further, our research represents the first study that seeks to understand the drivers of predator response. Future research should expand upon this to determine if there is a subset of toxins that are primarily responsible for predator response. Doing so will give predictive power to future alkaloid characterization studies in how predators will respond to toxins. Importantly, while our study is not able to tease apart which alkaloids may be driving response, our approach narrows down diverse alkaloid profiles into potentially functional alkaloids important for predator responses.

Aposematism is a complex interplay between a warning signal (i.e., coloration) and a secondary defense mechanism (e.g., toxins). While a large amount of research has focused on the warning signal and the psychology of learned avoidance, considerably less research has focused on understanding intraspecific variation in secondary defense and its consequences for predator response, despite being more common than previously thought (Speed et al. 2012). By examining which toxins may be important in driving predator response, we can now begin to predict how predators will respond to highly varied secondary defenses both within and among populations of aposematic prey. This will allow us to better address concepts such as automimicry and honest signaling that are hypothesized drivers of diversification in aposematic signals (Speed et al. 2006, 2010; Maan and Cummings 2012). Diversity within and among populations likely varies over space and time. It is this variation that provides the greatest source of insight into aposematic color evolution as it likely evolves under specific conditions of alkaloid availability and predator community.

ACKNOWLEDGMENTS

This study benefited from an “Investissement d’Avenir” grant managed by the Agence Nationale de la Recherche (CEBA, ref. ANR-10- LABX-25-01. B.R. and J.M were funded by the Finnish Centre of Excellence in Biological Interactions (Project 28466, to J.M.). B.R. is supported by funding from the Academy of Finland (Academy Research Fellowship No. 318404). We highly value equity, diversity, and inclusion in science (EDI) and thus took into account EDI best practice. Our team includes researchers from (4) different countries (United States, Colombia, Finland and France, and), and diverse backgrounds, and career stages, all of which significantly contributed to the fulfillment and quality of our study.

REFERENCES

Alvarado JB, Alvarez A, Saporito RA (2013) Oophaga pumilio (strawberry poison frog). Predation. Herpetol Rev 44:298

Blount JD, Rowland HM, Drijfhout FP, et al (2012) How the ladybird got its spots: Effects of resource limitation on the honesty of aposematic signals. Funct Ecol 26:334–342. https://doi.org/10.1111/j.1365-2435.2012.01961.x

Bolton SK, Dickerson K, Saporito RA (2017) Variable Alkaloid Defenses in the Dendrobatid Poison Frog Oophaga pumilio are Perceived as Differences in Palatability to Arthropods. J Chem Ecol 43:273–289. https://doi.org/10.1007/s10886-017-0827-y

Burdfield-Steel E, Brain M, Rojas B, Mappes J (2019) The price of safety: food deprivation in early life influences the efficacy of chemical defence in an aposematic moth. Oikos 128:245–253. https://doi.org/10.1111/oik.05420

Chouteau M, Angers B (2011) The role of predators in maintaining the geographic organization of aposematic signals. Am Nat 178:810–817. https://doi.org/10.1086/662667

Comeault AA, Noonan BP (2011) Spatial variation in the fitness of divergent aposematic phenotypes of the poison frog, Dendrobates tinctorius. J Evol Biol 24:1374–1379. https://doi.org/10.1111/j.1420-9101.2011.02258.x

Daly JW, Garraffo HM, Spande TF, et al (2008) Individual and Geographic Variation of Skin Alkaloids in Three Swamp-Forest Species of Madagascan Poison Frogs (Mantella). J Chem Ecol 34:252–279. https://doi.org/10.1007/s10886-015-0616-4

Daly JW, Myers CW (1967) Toxicity of Panamanian poison frogs (Dendrobates): some biological and chemical aspects. Science 156:970–3

Daly JW, Myers CW, Whittaker N (1987) Further classification of skin alkaloids from Neotropical poison frogs (Dendrobatidae), with a general survey of toxic/noxious substances in the Amphibia. Toxicon 25:1023–1095

Daly JW, Spande TF, Garraffo HM (2005) Alkaloids from Amphibian Skin: A Tabulation of Over Eight-Hundred Compounds. J Nat Prod 68:1556–1575

Darst CR, Cummings ME (2006) Predator learning favours mimicry of a less-toxic model in poison frogs. Nature 440:208–211

Darst CR, Menendez-Guerrero PA, Coloma LA, Cannatella DC (2005) Evolution of dietary specialization and chemical defense in poison frogs (Dendrobatidae): A comparative analysis. Am Nat 165:56–69

Holen OH (2013) Disentangling taste and toxicity in aposematic prey. Proc Biol Sci 280:20122588. https://doi.org/10.1098/rspb.2012.2588

Lawrence JP, Rojas B, Fouquet A, et al (2019a) Weak warning signals can persist in the absense of gene flow. Proc Natl Acad Sci 116:19037–19045. https://doi.org/10.1073/pnas.1901872116

Lawrence JP, Rojas B, Fouquet A, et al (2019b) Original data for article: Weak warning signals can persist in the absence of gene flow - JYX, University of Jyväskylä. https://doi.org/10.17011/jyx/dataset/65263

Lindström L, Alvtalo RV, Mappes J, et al (1999) Can aposematic signals evolve by gradual change? Nature 397:249–251

Maan ME, Cummings ME (2012) Poison frog colors are honest signals of toxicity, particularly for bird predators. Am Nat 179:E1–E14. https://doi.org/10.1086/663197

Master TL (1999) Predation by Rufous Motmot on Black-and-Green Poison Dart Frog. Wilson Bull 111:439–440

McGugan JR, Byrd GD, Roland AB, et al (2016) Ant And Mite Diversity Drives Toxin Variation In The Little Devil Poison Frog. J Chem Ecol 42:537–551. https://doi.org/10.1007/s10886-016-0715-x

Nishida R (2002) Sequestration of defensive substances from plants by Lepidoptera. Annu Rev Entomol 47:57–92. https://doi.org/10.1146/annurev.ento.47.091201.145121

Noonan BP, Gaucher P (2006) Refugial isolation and secondary contact in the dyeing poison frog Dendrobates tinctorius. Mol Ecol 15:4425–35. https://doi.org/10.1111/j.1365-294X.2006.03074.x

Ojala K, Julkunen-Tiitto R, Lindström L, Mappes J (2005) Diet affects the immune defence and life-history traits of an Arctiid moth Parasemia plantaginis. Evol Ecol Res 7:1153–1170

Paluh DJ, Hantak MM, Saporito RA (2014) A test of aposematism in the dendrobatid poison frog Oophaga pumilio: The importance of movement in clay model experiments. J Herpetol 48:249–254. https://doi.org/10.1670/13-027

Poulton EB (1890) The Colour of Animals: Their meaning and use, especially considered in the case of insects. D. Appleton

Prates I, Paz A, Brown JL, Carnaval AC (2019) Links between prey assemblages and poison frog toxins : A landscape ecology approach to assess how biotic interactions affect species phenotypes. 14317–14329. https://doi.org/10.1002/ece3.5867

Proksch P (1994) Defensive roles for secondary metabolites from marine sponges and sponge-feeding nudibranchs. Toxicon 32:639–655. https://doi.org/10.1016/0041-0101(94)90334-4

R CT (2016) R: A language and environment for statistical computing.

Rojas B, Burdfield-steel E, Pakkanen H, et al (2017) How to fight multiple enemies: target-specific chemical defences in an aposematic moth. Proc R Soc B-Biol Sci 284:20171424

Rojas B, Endler JA (2013) Sexual dimorphism and intra-populational colour pattern variation in the aposematic frog Dendrobates tinctorius. Evol Ecol 27:739–753. https://doi.org/10.1007/s10682-013-9640-4

Rojas B, Rautiala P, Mappes J (2014) Differential detectability of polymorphic warning signals under varying light environments. Behav Processes 109:164–172. https://doi.org/10.1016/j.beproc.2014.08.014

Rowland HM, Speed MP, Ruxton GD, et al (2007) Countershading enhances cryptic protection: an experiment with wild birds and artificial prey. Anim Behav 74:1249–1258. https://doi.org/10.1016/j.anbehav.2007.01.030

Sandre SL, Tammaru T, Esperk T, et al (2007) Carotenoid-based colour polyphenism in a moth species: Search for fitness correlates. Entomol Exp Appl 124:269–277. https://doi.org/10.1111/j.1570-7458.2007.00579.x

Santos JC, Coloma LA, Cannatella DC (2003) Multiple, recurring origins of aposematism and diet specialization in poison frogs. Proc Natl Acad Sci U S A 100:12792–12797. https://doi.org/10.1073/pnas.2133521100

Saporito RA, Donnelly MA, Garraffo MA, et al (2006) Geographic and seasonal variation in alkaloid-based chemical defenses of Dendrobates pumilio from Bocas del Toro, Panama. J Chem Ecol 32:795–814

Saporito RA, Donnelly MA, Hoffman RL, et al (2003) A siphonotid millipede (Rhinotus) as the source of spiropyrrolizidine oximes of dendrobatid frogs. J Chem Ecol 29:2781–2786

Saporito RA, Donnelly MA, Jain P, et al (2007a) Spatial and temporal patterns of alkaloid variation in the poison frog Oophaga pumilio in Costa Rica and Panama over 30 years. Toxicon 50:757–778

Saporito RA, Donnelly MA, Madden A a, et al (2010) Sex-related differences in alkaloid chemical defenses of the dendrobatid frog Oophaga pumilio from Cayo Nancy, Bocas del Toro, Panama. J Nat Prod 73:317–21. https://doi.org/10.1021/np900702d

Saporito RA, Donnelly MA, Norton RA, et al (2007b) Oribatid mites as a major dietary source for alkaloids in poison frogs. Proc Natl Acad Sci U S A 104:8885–8890

Saporito RA, Donnelly MA, Spande TF, Garraffo HM (2011) A review of chemical ecology in poison frogs. Chemoecology 22:159–168. https://doi.org/10.1007/s00049-011-0088-0

Saporito RA, Grant T (2018) Comment on Amézquita et al. (2017) “Conspicuousness, color resemblance, and toxicity in geographically diverging mimicry: The pan-Amazonian frog Allobates femoralis.” Evolution 1009–1014. https://doi.org/10.1111/evo.13468

Siddiqi A, Cronin TW, Loew ER, et al (2004) Interspecific and intraspecific views of color signals in the strawberry poison frog Dendrobates pumilio. J Exp Biol 207:2471–2485

Speed MP, Ruxton GD, Blount JD, Stephens PA (2010) Diversification of honest signals in a predator-prey system. Ecol Lett 13:744–753. https://doi.org/10.1111/j.1461-0248.2010.01469.x

Speed MP, Ruxton GD, Broom M (2006) Automimicry and the evolution of discrete prey defences. Biol J Linn Soc 87:393–402. https://doi.org/10.1111/j.1095-8312.2006.00577.x

Speed MP, Ruxton GD, Mappes J, Sherratt TN (2012) Why are defensive toxins so variable? An evolutionary perspective. Biol Rev 87:874–884. https://doi.org/10.1111/j.1469-185X.2012.00228.x

Summers K, Clough ME (2001) The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). Proc Natl Acad Sci U S A 98:6227–6232. https://doi.org/10.1073/pnas.101134898

Summers K, Speed MP, Blount JD, Stuckert AMM (2015) Are aposematic signals honest? A review. J Evol Biol 28:1583–1599. https://doi.org/10.1111/jeb.12676

Vacher J-P, Chave J, Ficetola FG, et al (2020) Large-scale DNA-based survey of frogs in Amazonia suggests a vast underestimation of species richness and endemism. J Biogeogr 47:1781–1791. https://doi.org/10.1111/jbi.13847

Vidal-Cordero JM, Moreno-Rueda G, López-Orta A, et al (2012) Brighter-colored paper wasps (Polistes dominula) have larger poison glands. Front Zool 9:1–5

Wang IJ (2011) Inversely related aposematic traits: reduced conspicuousness evolves with increased toxicity in a polymorphic poison-dart frog. Evolution 65:1637–1649. https://doi.org/10.1111/j.1558-5646.2011.01257.x

Wang K, Zhao H (2015) Birds generally carry a small repertoire of bitter taste receptor genes. Genome Biol Evol 7:2705–2715. https://doi.org/10.1093/gbe/evv180

Weldon PJ (2017) Poison frogs, defensive alkaloids, and sleepless mice: critique of a toxicity bioassay. Chemoecology 27:123–126. https://doi.org/10.1007/s00049-017-0238-0

Wollenberg KC, Lötters S, Mora-Ferrer C, Veith M (2008) Disentangling composite colour patterns in a poison frog species. Biol J Linn Soc 93:433–444

Zvereva EL, Kozlov MV (2015) The costs and effectiveness of chemical defenses in herbivorous insects: a meta-analysis. Ecol Monogr 86:107–124

**AUTHOR CONTRIBUTIONS**

All authors contributed to the study conception and design. Material collection, data preparation, and analysis were performed by J.P. Lawrence, Bibiana Rojas, Johanna Mappes, Annelise Blanchette, and Ralph Saporito. Samples were collected by Antoine Fouquet and Bibiana Rojas. J.P. Lawrence and Brice Noonan wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.