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APOSEMATISMO EN LA RANA *PHYLLOBATES VITTATUS* (ANURA: DENDROBATIDAE): INTEGRANDO DEFENSAS, SEÑALES Y RIESGO DE DEPREDACIÓN

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Biología para optar al grado y título de Maestría Académica en Biología

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Dedicatoria

A mis papás: Maurizio y Maritza

A mis hermanos: Arianna y Fabrizzio

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Contenidos

Resumen

El aposematismo es una estrategia defensiva en la cual animales que poseen una defensa lo advierten a los depredadores a través de una señal conspicua. Por ejemplo, algunos animales tóxicos o con poca palatabilidad le advierten a los depredadores sobre esta característica desagradable a través de una coloración brillante. Los depredadores por su parte aprenden a asociar esta defensa con la coloración conspicua, evitando así este tipo de presas en el futuro. Se cree que la rana *Phyllobates vittatus* (Dendrobatidae), una rana venenosa endémica del Pacífico Sur de Costa Rica, presenta esta estrategia debido a su patrón de coloración contrastante de franjas anaranjadas sobre un dorso negro. Sin embargo, para esta especie no se ha demostrado su toxicidad, conspicuidad, ni que los depredadores las eviten. Por lo tanto, mi objetivo principal fue poner a prueba estas predicciones clave del aposematismo en *P. vittatus,* para lo cual trabajé con ranas de tres localidades de la Península de Osa. Primero, evalué la toxicidad de las ranas analizando los alcaloides en su piel y estimando una dosis letal media en ratones con extractos de su piel. A pesar de que no hubo mortalidad en los ratones, por lo que no pude determinar la dosis letal media de toxicidad, si pude registrar la presencia de síntomas de toxicidad que concuerdan con un efecto cardiotóxico. Además, presento evidencia de una respuesta dependiente de la dosis en la intensidad de los síntomas observados en los ratones. A través de análisis con LC/MS, identifiqué la presencia del alcaloide altamente tóxico batracotoxinina A en su piel, así como otros alcaloides tóxicos (DHQ **251A** y Lehm **275A**) que pueden ser los responsables de los síntomas de toxicidad observados en los ratones. Segundo, realicé mediciones espectrométricas de las ranas y sus sustratos más comunes para determinar su conspicuidad para depredadores potenciales. A través de modelaje visual calculé los contrastes de las ranas para la visión de tres posibles depredadores: aves, lagartijas y cangrejos. A pesar de que hubo variación geográfica en el patrón de coloración de las ranas y en su conspicuidad total, todos los depredadores son fuertemente estimulados por el contraste entre las franjas dorsales anaranjadas y las partes de su cuerpo con coloración oscura. Además, estas franjas incrementan la conspicuidad visual para los depredadores sobre una variedad de sustratos. Finalmente, llevé a cabo experimentos en el campo con modelos de plasticina, variando su conspicuidad y patrón de coloración, para determinar si la señal más conspicua presente en *P. vittatus* disuade intentos de depredación por depredadores potenciales. El experimento de depredación mostró que los principales animales que atacaron los modelos fueron cangrejos y lagartijas. Por otra parte, los modelos con un patrón dorsal de franjas anaranjadas, similares a *P. vittatus*, sufrieron una menor depredación que modelos completamente anaranjados. Estos resultados desafían la suposición general de que solamente las aves promueven la evolución de patrones de coloración conspicuos en presas defendidas químicamente. En general, proporciono evidencia de que las ranas *P. vittatus*: (1) están defendidas químicamente contra depredadores, (2) son visualmente conspicuas para tres depredadores potenciales, y (3) que es posible que las lagartijas eviten su coloración, comparado con una coloración completamente anaranjada. Por lo tanto, es probable que la hipótesis de aposematismo para esta especie sea cierta, al menos para depredadores como lagartijas.

Abstract

Aposematism is a defensive strategy where animals that possess a defense advertise it to predators through a warning signal. For instance, some animals that are toxic or unpalatable warn predators about this unpleasant trait with a bright coloration. In turn, predators learn to associate the defense with the bright coloration, thereby avoiding such prey in the future. *Phyllobates vittatus* (Dendrobatidae), an endemic poison frog from the South Pacific of Costa Rica, is thought to display such a strategy, due to its contrasting color pattern of bright orange stripes on a black dorsal background. However, neither toxicity, conspicuousness, nor predator avoidance have been demonstrated for this species. Therefore, my main goal was to test these key predictions of aposematism in *P. vitattus*. I sampled frogs from three localities at the Osa Peninsula. First, I assessed frogs toxicity by analyzing their skin alkaloids and conducting a median lethal dose in mice with extracts of their skin. Even though there was no mice mortality, hence, I was not able to determine a median lethal dose, I was able to record a list of toxicity symptoms in agreement with a cardiotonic effect. Furthermore, I presented evidence of a dose response in toxicity symptoms. Through LC/MS analysis, I identified the presence of the highly toxic alkaloid Batrachotoxinin A in their skin, as well as other alkaloids (DHQ **251A** and Lehm **275A**) that could be responsible for the toxicity symptoms observed in mice. Second, I made spectrometric measurements of frogs and their usual backgrounds in order to determine their conspicuousness for potential predators. I calculated frogs contrasts, through visual modelling, for the view of three potential predators: birds, lizards and crabs. Despite geographic variation in the colour pattern and overall conspicuousness of *P. vittatus*, all predators are strongly stimulated by the contrast between dorsal orange stripes and darker patches of frog colouration. Moreover, these stripes enhances visual conspicuousness for predators against a variety of substrates. Finally, I conducted field experiments with clay models varying in their conspicuousness and color pattern in order to test if the most conspicuous colour signal displayed by *P. vittatus* deters predation attempts by potential assailants. The predation experiment showed that the main assailants were crabs and lizards. Moreover, models with the striped colour pattern resembling *P. vittatus* suffer less lizard predation than entirely orange phenotypes. Thus, challenging the long-held assumption that birds alone drive the evolution of conspicuous colour patterns in chemically defended prey. Overall, I provide evidence that *P. vittatus* frogs: (1) are chemically defended against predators, (2) are visually conspicuous for three potential predators, and (3) lizards appear to avoid their coloration as opposed to a plain orange coloration. Therefore, the aposematism hypothesis for this species is likely to be true, at least for lizard predators.

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Supporting Information

Lista de Abreviaturas

AB	Agua Buena
ANOVA	Análisis de varianza
BTX	Batracotoxinas
CI	Intervalo de confianza
CID	disociación inducida por colisión
CIPRONA	Centro de Investigación en Productos Naturales
ΔL	Contraste de brillo o acromático
ΔS	Contraste de color o cromático
D20	Dosis que representa un 20% de la dosis límite
D50	Dosis que representa un 50% de la dosis límite
D80	Dosis que representa un 80% de la dosis límite
DHQ	Decahydroquinolinas
DNP	Diccionario de Productos Naturales
DO	Dorso
GLM	Modelo lineal generalizado
HL	Patas traseras
IACUC	Comité Institucional para el Cuidado y Uso de Animales
IUCN	Unión Internacional para la Conservación de la Naturaleza
jnd	Solo diferencias notables
LC-MS	Cromatografía líquida acoplada a espectrometría de masas
LC-MS/MS	Cromatografía Líquida acoplada a espectrometría de masas/ espectrometría de masas
LD_{50}	Dosis letal media
LEBi	Laboratorio de Ensayos Biológicos
Lehm	Lehmizidinas
LL	Hojarasca
LT	La Tarde
LWS	Receptores sensibles a longitudes de onda largas

MANOVA	Análisis multivariado de varianza
MS/MS	Espectrometría de masas/espectrometría de masas
MS^1	Fórmula molecular y masa exacta
MWS	Receptores sensibles a longitudes de onda medias
n	Densidad de los fotorreceptores
OECD	Organización para la Cooperación y Desarrollo Económicos
PI	Piro
PTX	Pumiliotoxinas
RO	Roca
SD	Desviación estándar
SE	Error estándar
SO	Tierra, suelo
ST	Franjas dorsales
SVL	Longitud hocico-cloaca
SWS	Receptores sensibles a longitudes de onda cortas
TR	Tronco
Tukey HSD	Diferencia honestamente significativa de Tukey
UCR	Universidad de Costa Rica
UPLC-ESI-QTOF	Cromatografía Líquida de ultra eficiencia-Ionización con Electroespray-Cuadrupolo con tiempo de vuelo
UV	Ultravioleta
UV/VS	Receptores sensibles a longitudes de onda ultravioletas

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Introducción General

La depredación es una relación entre organismos en la cual uno sirve de fuente de alimento para el otro, ya sea parcial o completamente, y es un riesgo que suele estar presente para cualquier organismo (Smith & Smith, 2007). A pesar de este riesgo, las especies han desarrollado una amplia variedad de defensas contra los depredadores, evitando así ser detectados, seleccionados o capturados por éstos (Nonacs & Blumstein, 2010).

En el caso específico de los anfibios, parecen ser muy vulnerables a la depredación debido a su tamaño pequeño en relación con otros vertebrados terrestres, tienen movimiento relativamente lento y carecen de dientes y garras adecuadas para su defensa. Por otro lado, son eficientes en términos de convertir la energía consumida en biomasa, por lo tanto suelen alcanzar densidades poblacionales altas y son una fuente de proteína para animales que se encuentran en estratos superiores de la cadena trófica. Estos factores, unido a que los anfibios no posean materiales indigeribles como pelo, plumas o quitina hacen que sean en particular comida de alta calidad (Scott & Limerick, 1991; Wells, 2007).

La depredación es una presión selectiva que afecta muchos aspectos de la ecología y comportamiento de los anfibios. La presión de la depredación sobre los anfibios ha sido importante en la evolución de defensas contra los depredadores; tales como una piel tóxica o con sabor desagradable, patrones de coloración crípticos y aposemáticos, y una variedad de posturas de defensa y comportamiento (Scott & Limerick, 1991; Wells, 2007).

La variación en los patrones de coloración de algunos anfibios, especialmente anuros, es extraordinaria y ha llamado la atención de muchos investigadores (Rojas, 2016). Esta variabilidad cromática es el resultado de fuerzas evolutivas, como la selección natural y selección sexual actuando simultáneamente sobre ciertos aspectos de su historia natural; como protección contra la radiación solar, comunicación, y estrategia defensiva contra depredadores y parásitos (Endler, 1978; Toledo & Haddad, 2009; Rojas, 2016; Ruxton, Allen, Sherrat, & Speed, 2018). La coloración puede prevenir el ataque de depredadores en múltiples formas ya que puede funcionar evitando la detección de la presa, desviando la atención del depredador y también como una señal de

advertencia para éstos (Merilaita, 1998; Wells, 2007; Ruxont et al., 2018). Por medio de la coloración, el depredador puede recibir información de que la presa es tóxica, con poca palatabilidad o no vale el esfuerzo (Santos, Coloma, & Canatella, 2003; Mappes, Marples, & Endler, 2005). De esta forma, las presas pueden reducir la probabilidad de ser consumidas una vez que se da el encuentro con el depredador, o reducir la probabilidad de ser detectadas o identificadas por este (Wells, 2007).

El aposematismo es una estrategia defensiva en la que organismos presentan una señal conspicua a través de la cual indican a los posibles depredadores de que tiene poca palatabilidad, y por lo tanto no es rentable consumirla ya que puede ser tóxica o peligrosa (Ruxton et al., 2018). Esta estrategia contra depredadores se encuentra ampliamente distribuida entre taxones y hábitats (Rojas, 2016; Ruxton et al., 2018). Puede observarse comúnmente en insectos (abejas, avispas, mariposas), moluscos (nudibranquios), reptiles (serpiente coral), peces (pez globo) y mamíferos (zorrillo) (Santos et al., 2003; Blount, Speed, Ruxton, & Stephens, 2009).

Las señales de advertencia en animales aposemáticos pueden incluir un elevado contraste de color y brillo de la presa contra el fondo en el cual se encuentre, dos o más colores contrastantes dentro del patrón de su cuerpo (Aronsson & Gamberale-Stille, 2013; Arenas & Stevens, 2017; Green, Urquhart, van den Berg, Marshall, & Cheney, 2018; Ruxton et al., 2018) y comportamientos conspicuos tales como forrajeo activo (Speed, Brockhurst, & Ruxton, 2010) y despliegues sexuales exagerados (Rudh, Breed, & Qvarnström, 2013). Estas señales conspicuas advierten las consecuencias desagradables de atacar una presa aposemática. Así mismo, confiere confianza a las presas, que ganan al estar expuestas libremente en su ambiente; direcciona la atención del depredador hacia algún componente aversivo (Ruxton et al., 2018) y, al estar asociados con toxicidad, el costo de ser conspicuo para depredadores solo puede ser soportado por individuos que están bien defendidos (Speed & Ruxton, 2007; Blount et al., 2009).

Las especies terrestres aposemáticas poseen comúnmente coloraciones brillantes para advertir su mal sabor a los potenciales depredadores, entre las más comunes se encuentran las rojas, amarillas y anaranjadas combinadas con negro (Prudic, Skemp, & Papaj, 2007; Lawrence & Noonan, 2018). Estos colores contrastan fuertemente contra sustratos verdes o cafés y hacen que las presas sean fáciles de reconocer y discriminar de las que si son comestibles (Endler & Mappes,

2004; Toledo & Haddad, 2009). La coloración aposemática es ventajosa solamente cuando los depredadores poseen visión a color; es por esta razón que se asume que las aves son el principal agente que favorece los patrones de coloración aposemáticos en animales diurnos, como es el caso de algunos anuros (Wells, 2007).

En conjunto, la coloración y toxicidad funcionan porque los depredadores aprenden a asociar el mal sabor con coloración brillante debido a que las señales más fuertes son más detectables y fáciles de recordar, por lo tanto facilitan la evasión por aprendizaje de manera más rápida (Endler & Mappes, 2004; Mappes et al., 2005; Darst, Cummings, & Cannatella, 2006; Wells, 2007). Las sustancias que confieren un mal sabor a las presas, y por lo tanto pueden funcionar como defensa química contra depredadores, están ampliamente distribuidas en la naturaleza y además de las plantas, pueden encontrarse en grupos como insectos, ácaros, peces, anfibios (Daly, 1995) y aves (Dumbacher, Beehler, Spande, Garrafo, & Daly, 1992; Dumbacher & Pruett-Jones, 1996; Dumbacher, Spande, & Daly, 2000).

Se ha encontrado que la mayoría de anfibios poseen sustancias nocivas e incluso tóxicas en las secreciones de su piel, las cuales varían en su naturaleza química, actividad biológica y origen, ya que pueden ser sintetizadas o provenir de una fuente alimenticia. Las sustancias sintetizadas son aminas, péptidos, proteínas, bufadienolidas y los alcaloides de las salamandras, salamandrinas. El origen de las tetrodotoxinas, que son alcaloides solubles en agua, es poco claro; mientras que los alcaloides lipofílicos provienen de la dieta, fundamentalmente de artrópodos (Daly, Myers, & Whittaker, 1987; Daly, 1995).

Los alcaloides lipofílicos de anfibios actúan sobre los canales iónicos y por lo tanto funcionan efectivamente como defensas químicas (Daly, 1995). La mayoría de alcaloides conocidos provienen de la familia Neotropical de ranas Dendrobatidae; algunos son extraordinariamente tóxicos, mientras que otros son levemente nocivos (Daly et al., 1987). Inicialmente se consideraba que estos alcaloides eran sintetizados por las ranas, sin embargo más adelante se descubrió que son obtenidos de la dieta, principalmente de hormigas y ácaros presentes en la hojarasca de los bosques en que habitan y son el componente principal de su dieta (Toft, 1995; Saporito, Spande, Garraffo, & Donnelly, 2009). Probablemente la especialización en la dieta está relacionado con la evolución del aposematismo en esta familia (Santos et al., 2003). En

la actualidad se han descrito más de 500 alcaloides presentes en la piel de estas ranas (Daly et al., 2005; Saporito et al., 2009; Saporito, Donnelly, Spande, & Garraffo, 2012; Santos, Tarvin, & O'Connell, 2016).

La familia de ranas Dendrobatidae ha sido ampliamente estudiada debido a su gran diversidad de patrones de coloración y toxicidad; encontrando desde especies crípticas y poco tóxicas, hasta muy conspicuas y altamente tóxicas, rasgos que permiten responder una amplia gama de preguntas ecológicas y evolutivas, ligadas especialmente a la evolución de la asociación entre señales de advertencia y defensas secundarias. En esta familia los géneros considerados aposemáticos corresponden a *Phyllobates*, *Epipedobates, Dendrobates* (sensu Santos et al., 2003; actualmente el género *Dendrobates* se ha dividido en *Minyobates, Oophaga, Dendrobates, Adelphobates, Excidobates, Andinobates*, sensu Grant et al., 2017) y *Ameerega* (Santos & Canatella, 2011). Sin embargo, a pesar de que se cree que la coloración conspicua junto con la presencia de alcaloides tóxicos en algunas especies de dendrobátidos es un ejemplo de aposematismo (Maan & Cummings, 2012), existe poca evidencia experimental que apoye este supuesto.

Saporito y colaboradores (2007) pudieron confirmar que la coloración conspicua en *Oophaga pumilio* funciona como una señal aposemática para depredadores potenciales. Realizaron experimentos de depredación con modelos de plasticina y encontraron que la tasa de depredación fue menor en modelos con coloración conspicua (semejantes a *O. pumilio*) en comparación con modelos de coloración café o críptica. Adicionalmente, de acuerdo a las marcas presentes en la plasticina dejadas por los depredadores, pudieron determinar que hubo un bajo número de ataques de aves sobre los modelos rojos, lo cual indica que las aves son capaces de discriminar presas por sus colores y evitan atacar presas que señalan con coloración conspicua sus defensas tóxicas (Saporito, Zuercher, Roberts, Gerow, & Donnelly, 2007).

La especie mejor estudiada en estos aspectos es *O. pumilio*. Sin embargo, es importante extender estos estudios a otros miembros de la familia, para así poder entender los patrones de coloración y toxicidad a grandes rasgos y tener una visión más general de los aspectos ecológicos y evolutivos que actúan sobre las señales aposemáticas. Por esta razón, es necesario incluir especies que han recibido menos atención, pero que de igual manera pueden aportar mucha información al

conocimiento sobre la evolución del aposematismo. Es por esto que se sugiere incluir a *Phyllobates vittatus* (Dendrobatidae) dentro de esta clase de investigaciones.

El género *Phyllobates* consta de cinco especies: *P. aurotaenia*, *P. bicolor*, *P. terribilis*, *P. lugubris* y *P. vittatus*. Las tres primeras especies se encuentran en Colombia; *P. lugubris* se distribuye en el Caribe de Costa Rica y Panamá, mientras que *P. vittatus* sólo se encuentra en el Pacífico Sur de Costa Rica, por lo que se considera endémica para el país. El grupo de *Phyllobates* se considera monofilético y contiene dos linajes filogenéticos; uno comprende las especies de Centroamérica y el otro las especies de Sur América (Myers, Daly, & Malkin, 1978; Widmer, Lötters, & Jungfer, 2000; Grant et al., 2006, 2017).

La presencia de la batracotoxina es la principal característica que identifica al género *Phyllobates*. La batracotoxina es un alcaloide altamente tóxico que no está presente en el resto de los dendrobátidos (Albuquerque, Daly, & Witkop, 1971; Myers, 1987, Saporito et al., 2012). La batracotoxina es un alcaloide esteroidal complejo y es una de las moléculas pequeñas no proteicas más tóxicas conocidas en la naturaleza. Su toxicidad se debe a una interacción selectiva en la permeabilidad de los canales de sodio, ocasionando la despolarización irreversible de nervios y músculos, produciendo arritmias, fibrilación y fallo cardiaco (Albuquerque et al., 1971; Daly, Myers, Warnick, & Albuquerque, 1980). Los niveles de batracotoxina difieren ampliamente entre las especies de *Phyllobates*. En poblaciones de Panamá de *P. lugubris* y de Costa Rica de *P. vittatus* las cantidades van desde indetectables hasta los 0.8 µg por individuo; mientras que los niveles son mucho mayores en *P. aurotaenia*, *P. bicolor* y *P. terribilis*, siendo esta última la más tóxica: la piel de un adulto contiene hasta 1.9 mg (Daly et al., 1980).

A pesar de que la cantidad de batracotoxina presente en *P. vittatus* es casi indetectable (Daly et al., 1980; Mebs, Vargas, Pogoda, Toennes, & Köhler, 2014), datos anecdóticos indican que la piel de esta especie puede resultar bastante tóxica (Myers et al., 1978). Una serpiente de México, *Rhadinaea taeniata aemula*, después de probar un individuo de *P. vittatus*, tuvo evidente dolor y estuvo inactiva por al menos cuatro horas, hasta que al día siguiente al encuentro se recuperó completamente. En el caso del ser humano, el contacto directo de la lengua con el dorso de *P. vittatus*, provocó entumecimiento de esta y estrechamiento de la garganta (Myers et al., 1978). Al considerarse la batracotoxina como un carácter autopomórfico del género *Phyllobates,* y

debido a su indetectabilidad en las especies de Centroamérica, es que se debe realizar un mayor esfuerzo en su investigación, así como se debe también determinar cuáles son los alcaloides que le confieren la toxicidad percibida de *P. vittatus*.

Phyllobates vittatus posee una coloración contrastante; el dorso y la cabeza son negros, con dos líneas anchas dorsolaterales naranja rojizo que se extienden desde la base del muslo hasta la nariz, la superficie de las extremidades son verdes azuladas, posee una raya blanca desde la base de los brazos hasta debajo de los ojos y una raya clara ventrolateral en los costados (Savage, 2002). Es una especie diurna territorial (Summers, 2000), que habita bosques húmedos cercanos a ríos y se encuentra en simpatría con otras especies de dendrobátidos como *Oophaga granulifera*, *Dendrobates auratus*, *Silverstoneia flotator,* y *Allobates talamancae* (Savage, 2002). Se alimentan principalmente de hormigas y ácaros, pero también pueden hacerlo de otros insectos como termitas, escarabajos y moscas (Toft, 1995; *Mebs et al.,* 2014).

Actualmente hay poca o nula información sobre el papel de la coloración y el poder de sus toxinas para disuadir depredadores en esta especie. De hecho, Mebs y colaboradores (2014) sugieren que *P. vittatus* se beneficia de su coloración conspicua y de la toxicidad que poseen otros dendrobátidos simpátricos para advertir falsa toxicidad. No obstante, no hay ninguna rana simpátrica de *P. vittatus* que comparta características similares a su coloración y que sea tóxica, por lo que esta afirmación resulta cuestionable. Debido a esto es que el objetivo principal de la tesis es determinar si *P. vittatus* es una especie aposemática, es decir, que su coloración y posible toxicidad actúan en conjunto para indicar a los depredadores que su palatabilidad es baja o es tóxica.

Este tipo de información resulta de gran importancia para el conocimiento de la especie, ya que al ser una especie endémica para Costa Rica, la información sobre su ecología e interacciones con otras especies y su ambiente, ayudará a conservarla mejor. Actualmente existe escasa información sobre la identidad de los depredadores de los miembros de la familia Dendrobatidae, especialmente de *P. vittatus* que ha sido poco estudiado, las distintas clases de alcaloides en defensa contra estos depredadores y la habilidad que éstos poseen para detectar las defensas químicas, por lo que es necesario un conocimiento más profundo sobre la relación entre las defensas tóxicas y el aposematismo para entender mejor la ecología y evolución de las

interacciones entre depredador y presa (Saporito et al., 2012). Adicionalmente, los estudios de toxicidad pueden, de una manera más profunda, dar las bases para estudios biomédicos (Savitzky et al., 2012) y la síntesis de compuestos farmacéuticos, ya que hasta el momento la actividad farmacológica de las toxinas de los anfibios ha sido poco estudiada (Daly, Spande, & Garrafo, 2005).

El objetivo general de esta investigación es determinar si la coloración dorsal de *P. vittatus* cumple una función aposemática. Es decir, si su coloración advierte toxicidad a los depredadores y por lo tanto estos evitan atacarlos. Específicamente, evaluaré la toxicidad de *P. vittatus* por medio de ensayos de toxicidad y análisis de los alcaloides presentes en su piel que puedan ser importantes en defensa contra depredadores. Adicionalmente, evaluaré la conspicuidad de *P. vittatus* en términos de coloración inherente y para depredador potenciales. Por último, determinaré si los depredadores evitan la coloración de *P. vittatus* realizando experimentos de depredación en el campo con modelos de plasticina con diferentes patrones y coloraciones.

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CAPÍTULO 1. Toxicity and alkaloid profiling of the skin of the Golfo Dulcean poison frog *Phyllobates vittatus* (Dendrobatidae)

(con formato para *Journal of Chemical Ecology*)

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 a toxicity assay based on the behavioral responses of mice to subcutaneous injection, to determine differences in toxicity among three study locations. 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 toxicity 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 skin alkaloids of *P. vitattus* may function as an effective deterrent for predators that facilitates aversion learning by inducing non-lethal toxicity symptoms.

Key Words: 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; but see Dumbacher et al. 2004). 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, 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, using mice as a proxy model to better understanding the frog's toxicity to natural predators. Additionally, given that previous studies have shown variation in toxicity among populations of dendrobatid poison frogs (e.g. Maan and Cummings 2012; Wang 2011), we aimed to detect whether there are differences in toxicity of *P. vittatus* from different localities in Costa Rica.

MATERIALS AND METHODS

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 dendrobatoidean 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 8ºC 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 (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 37ºC 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 -70ºC 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 22ºC. 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 experiments, before euthanizing by cervical dislocation (Close et al. 1997).

Assay 1: 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 four days after injection until the end of the observation period.

*Assay 2: Variation in Toxicity Among Localities***.** In order to estimate toxicity variation among localities, we followed the methods of Darst et al. (2006), and Maan and Cummings (2012), which provide a proxy of the irritant effects of frog alkaloids on potential predators using mice models. 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 toxicity. A higher latency to sleep is interpreted as a higher toxicity (Darst and Cummings 2006; Darst et al. 2006; 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 toxicity assays (skins were first extracted for the toxicity 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 disolved 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).

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 x 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 (N2) flow, 300 L/h, desolvation temperature, 250°C, cone gas (N2) 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.

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 seconds, 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/). 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) were 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 performed a Kruskal-Wallis test followed by *post hoc* Mann-Whitney pairwise tests.

RESULTS

Assay 1: 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 five 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).

Assay 2: Variation in Toxicity 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 locations 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 among treatments (X^2 =11.171, d.f. = 3, P = 0.011). Mice injected with extracts from the three localities had a significantly higher latency to sleep than control mice, but latency did not differ among the localities (Fig. 3, Table 5).

Alkaloid Identification. Based on the custom database search, we putatively annotated 62 previously reported alkaloids in *P. vittatus* skin extracts at the MS^1 level (molecular formula and exact mass; Table S1). We performed further MS/MS experiments to obtain the fragmentation spectra of all annotated compounds in order to corroborate identification. Due to the low quantity of compounds remaining in skins following initial extraction for toxicity assays, we only obtained suitable MS/MS profiles for the three major compounds.

These three compounds were putatively identified (Fig. 4 and Fig. S1-S3; based on the fragmentation patterns, exact mass and molecular formula) as batrachotoxinin 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 locations.

Batrachotoxinin A and DHQ **251A** were only found in skins from Agua Buena and La Tarde. No Benzocaine was detected in any samples. The rest of the compounds identified to MS^1 level ranged in structural characteristics and included peptides (Deltorphins reported to have high affinity and selectivity as agonist for *δ*-opioid receptors; Kreil et al. 1989), fifteen other *Dendrobates* alkaloid-type compounds and five more analogues of batrachotoxinin A (Table S1).

DISCUSSION

In this study, we provide the first experimental evidence of toxicity of the skin of *Phyllobates vittatus* using mice as a proxy model to better understand toxicity to natural predators. 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 two 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 obvious 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). Here, our experimental data confirm the presence of toxic compounds in the skin of *P. vittatus* that are likely to induce both immediate toxic symptoms and a complete recovery within a few hours.

According to spectral data, *P. vittatus* alkaloids detected were batrachotoxinin A, a batrachotoxin analog; alkaloid **251A**, one decahydroquinoline-type; and alkaloid **275A**, one lehmizidine-type (Fig 4). Among other minor 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).

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, and consequently how toxicity could affect potential natural predators.

Alkaloid content varies temporally as well as spatially in other poison frog species (Saporito et al. 2006, 2007). The alkaloids we found are different from those reported by Mebs et al. (2014) in his

study on alkaloid content in *P. vittatus*. We suggest that there could be a seasonal pattern responsible for this variation on alkaloid content of the skin of *P. vittatus*, given that we collected individuals during the rainy season, while Mebs et al. (2014) collected during the dry season. Alkaloid availability depends on arthropod prey (Saporito et al. 2006, 2007), thus variation in arthropod prey together with foraging patterns could explain the difference in alkaloids found in this study compared to those from Mebs et al. (2014). In general, frogs from the Dendrobatidae family are 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, and consequently could increase alkaloid defenses of the frogs. Also, due to their active courtship behavior, frogs should be more exposed to predators during the rainy season than in the dry season, when their activity lowers significantly (Pröhl 1997). Alternatively, locations studied by Mebs et al. (2014) could be different with respect to their arthropod communities as compared to the sites in the current study. However, the sampling location of Mebs et al. (2014) at Reserva Los Patos is approximately 2 km from our location in La Tarde, and nearby populations tend to have more similar alkaloid composition than distant populations (Saporito et al. 2006, 2007).

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 *Dendrobates* alkaloid **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 among studied localities for *P. vittatus*, although we did find batrachotoxinin A in two of three sites. We presume that arthropod prey are similar among the three localities, given their geographic proximity and habitat similarity. Future research should focus on determine how availability of toxic prey influences chemical defenses, including studies on frog's diet.

The toxicity assay based on sleeplessness in mice (Darst et al. 2006; Maan and Cummings 2012), here used to estimate differences in 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 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, which would provide a more accurate representation of how chemical defenses in poison frogs function in nature.

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 Amezquita 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 an 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 that could deter predators from consuming them, which supports the hypothesis that this species is aposematic. Because we were unable to provoke lethality at the studied doses, we suggest that skin alkaloids may 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). In order to establish whether *P. vittatus* coloration is aposematic, further evidence is needed, such as coloration measurements, visual modeling to assess chromatic and achromatic contrasts for putative predators, and 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 potential predators, 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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TABLES

Table 1. Samples' attributes for the toxicity assays to determine the Median Lethal Dose (LD50) and variation in toxicity 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)
LD_{50} estimation					
Combined skins	2.31 ± 0.13	1.46 ± 0.05	0.16 ± 0.03	0.48	5.00
Variation in toxicity 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

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

Treatment	Dosage (mg/kg)	Injected volume (mL)	Initial weight (g)	Final weight (g)
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
Variation in toxicity 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

		Treatment	D20						D50						D80					
Symptom	Symptom description	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	Paleness								2	2						3	2			

vasoconstriction				
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	

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**

Table 5. Results of the Mann-Whitney pairwise test to assess variation in toxicity among localities. Significant differences are in bold.

	Control	Agua Buena	La Tarde
Agua Buena	**0.037**		
La Tarde	**0.012**	0.53	
Piro	**0.012**	0.21	0.14

FIGURES

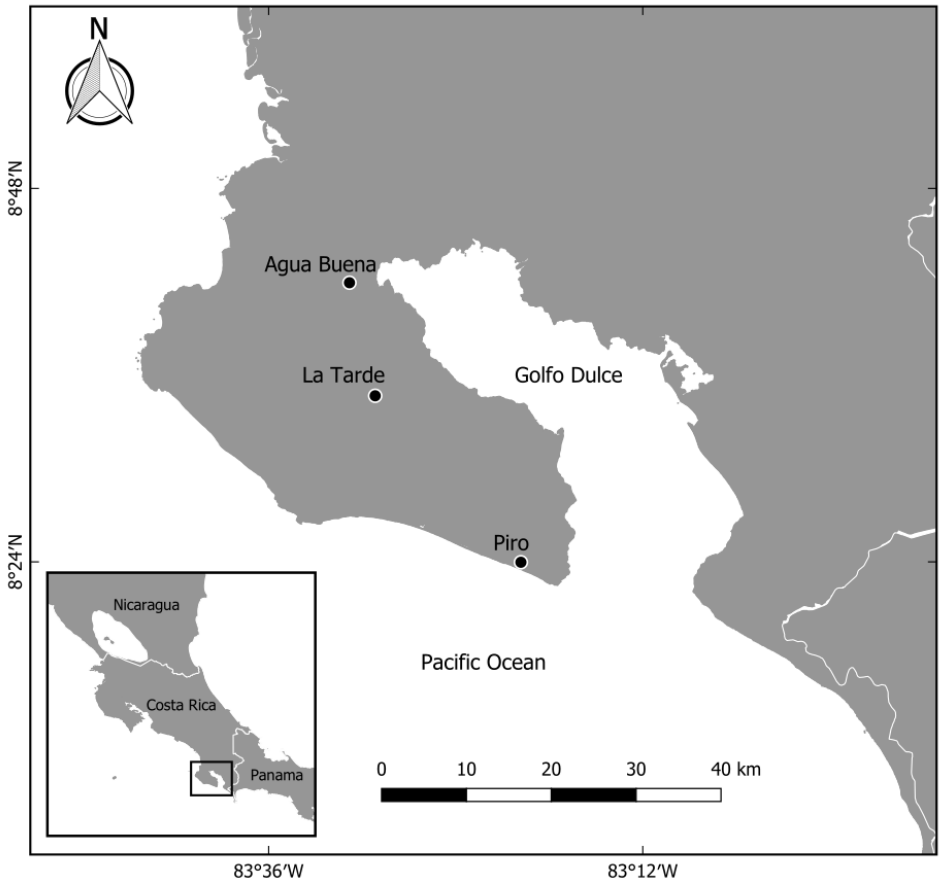

Fig. 1 Sampling localities for *Phyllobates vittatus* in the Osa Península, Costa Rica.

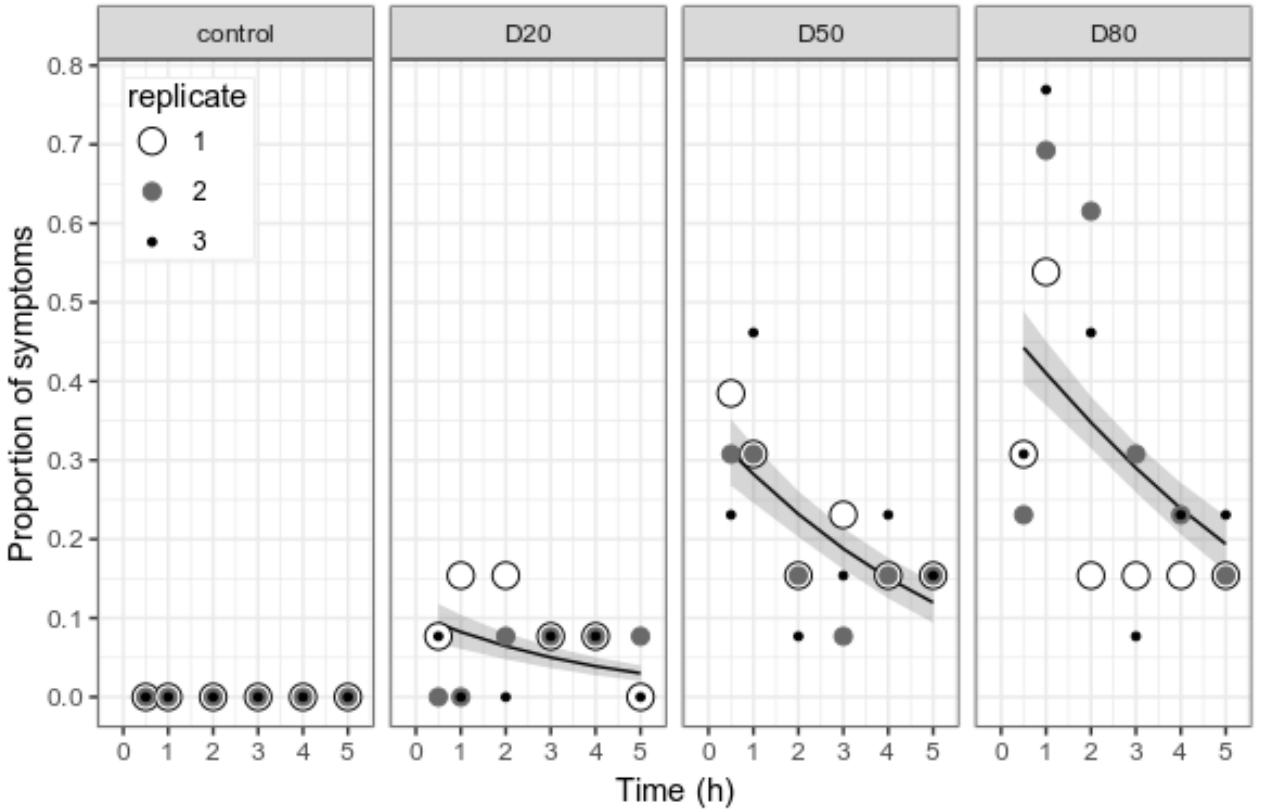

Fig. 2 Proportion of symptoms present in the five-hour observation period after injection, according to treatment. Replicates refer to mice used in each treatment (each mouse was used in only one treatment). Lines represent the model prediction and shades the standard error of the prediction. Given that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

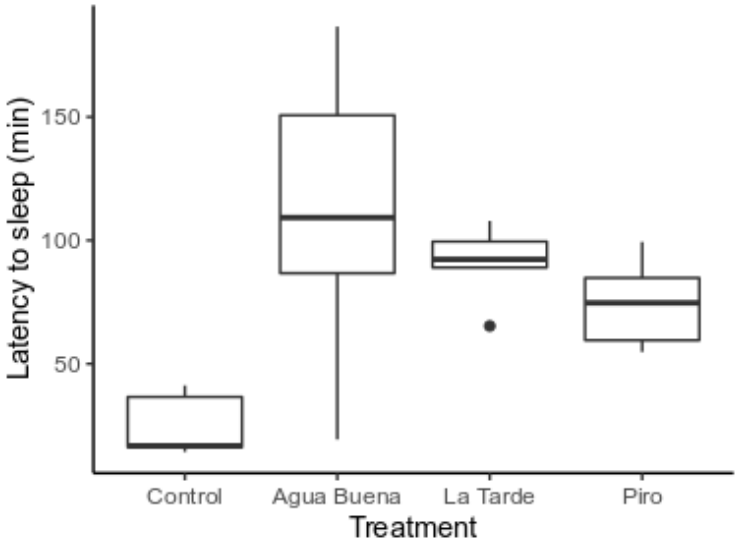

Fig. 3 Median latency to sleep of mice injected with frog skin extracts from different locations.

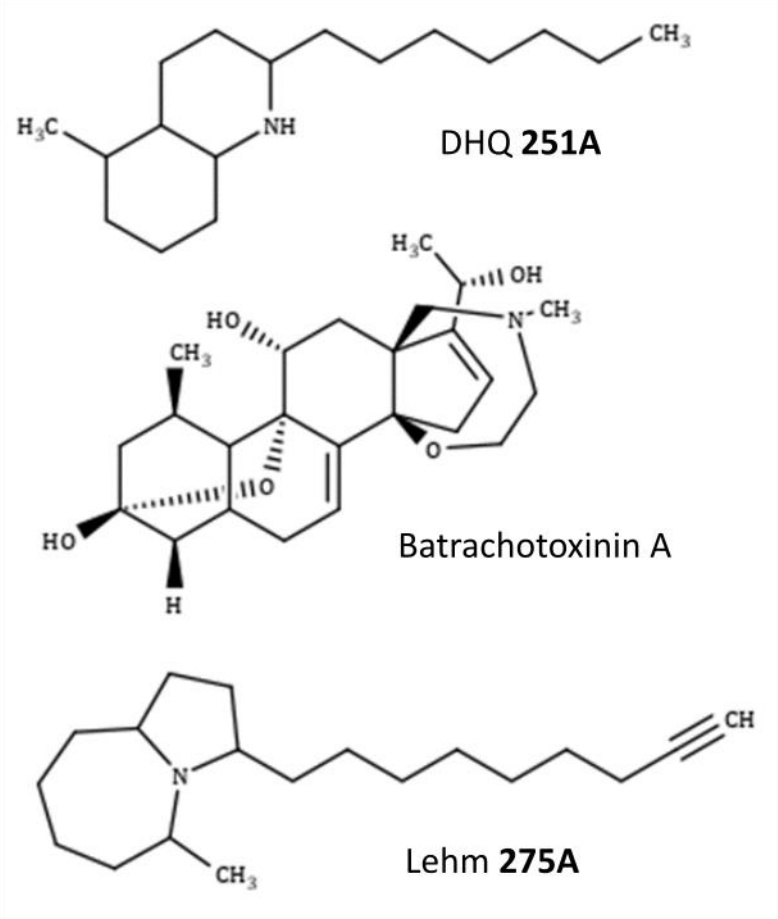

Fig. 4 Chemical structures of putatively identified alkaloids in the skin of *Phyllobates vittatus*.

SUPPORTING INFORMATION

Table S1 Initially identified compounds from the custom database search

[M+H]+	Retention time (min)	Putative identity	Molecular Formula	error (ppm)
399.2665	27.6	12,14-Dihydroxy-11-oxobufa-3,20,22-trienolide- (5β,12β,14β)-form	$C_{24}H_{30}O_5$	4.5
427.2922	26.2	14,16-Dihydroxybufa-5,20,22-trienolide- (14β,16β)-form, 16-Ac	$C_{26}H_{34}O_5$	5.2
196.2088	25.6	2-Butyldecahydroquinoline	$C_{13}H_{25}N$	1.3
671.3741	0.87	3,14-Dihydroxybufa-20,22-dienolide- (3β,5β,14β)-form, 3-(Argininyladipoyl)	$C_{36}H_{54}N_4O_8$	6.2
685.4346	22.1	3,14-Dihydroxybufa-20,22-dienolide-(3β,5β**,**14β)-form, 3-(Argininylpimeloyl)	$C_{37}H_{56}N_4O_8$	7.3
699.4462	21.2	3,14-Dihydroxybufa-20,22-dienolide- (3β,5β,14β)-form, 3-(Argininylsuberoyl)	$C_{38}H_{58}N_4O_8$	1.4
543.3414	0.87	3,14-Dihydroxybufa-20,22-dienolide-(3β,5β,14β)-form, 3-(Hydrogen suberoyl)	$C_{32}H_{47}O_7$	-2.6
531.3664	21.0	3,14-Dihydroxycard-20(22)-enolide-(3β,5β,14β,17β)-form, 3-O--(7-Carboxyheptanoyl)	$C_{31}H_{46}O_7$	-2.9
169.1055	28.4	4-Decen-9-olide; (S-,Z-)-form	$C_{10}H_{16}O_2$	6.1
904.4897	28.9	9-Desarginylbradykinin	$C_{44}H_{61}N_{11}O_{10}$	5.4

240.1668	20.7	Anabasamine; N-De-Me	$C_{37}H_{56}N_3$	-4.6
418.2585	6.01	Batrachotoxinin A	$C_{24}H_{35}NO_5$	-1.9
555.3080	9.86	Batrachotoxinin A; 4β-Hydroxy, O^{20}-(2,4-dimethyl-3-pyrrolecarboxylate)	$C_{31}H_{42}N_2O_7$	2.0
569.3165	28.9	Batrachotoxinin A; 4β-Hydroxy, O^{20}-(2-ethyl-4-methyl-3-pyrrolecarboxylate)	$C_{32}H_{44}N_2O_7$	3.4
539.3464	25.2	Batrachotoxinin A; O^{20}-(2,4-dimethyl-3-pyrrolecarboxylate)	$C_{31}H_{42}N_2O_6$	5.0
553.3552	20.7	Batrachotoxinin A; O^{20}-(2-ethyl-4-methyl-3-pyrrolecarboxylate)	$C_{32}H_{44}N_2O_6$	-3.3
567.3621	0.87	Batrachotoxinin A; O^3-Me, O^{20}-(2-ethyl-4-methyl-3-pyrrolecarboxylate)	$C_{33}H_{46}N_2O_6$	4.3
201.1185	28.9	Bufogargarizanine B	$C_8H_{12}N_2O_4$	4.0
185.0936	28.9	Bufogargarizanine B- Deoxy	$C_8H_{12}N_2O_3$	7.3
459.2404	0.93	Bufogargarizin A	$C_{25}H_{30}O_8$	5.8
385.2488	8.64	Bufogenin	$C_{24}H_{32}O_4$	-7.8
401.2384	28.9	Bufogenin- 20ξ,21ξ-Epoxide	$C_{24}H_{35}O_5$	5.3
697.4295	22.1	Bufogenin- 3-O-(Argininylsuberoyl)	$C_{38}H_{56}N_4O_8$	6.8
205.1280	28.8	Bufotenine	$C_{12}H_{16}N_2O$	6.1
219.1337	28.8	Bufotenine- N^b-Me, inner salt	$C_{13}H_{18}N_2O$	3.0

299.0879	26.4	Bufotenine- N^b-Me, inner salt, O-sulfate	$C_{13}H_{18}N_2O_{4S}$	-2.5
221.1420	28.2	Bufotenine- N^b-oxide	$C_{12}H_{16}N_2O_2$	6.0
347.2032	7.23	Calycanthine; (-)-form	$C_{22}H_{26}N_4$	5.9
469.3799	22.8	Cholestane-3,7,12,25,26,27-hexol; (3α,5α,7α,12α)-form	$C_{27}H_{48}O_6$	2.4
453.3702	6.77	Cholestane-3,7,12,25,26-pentol; (3α,5β,7α,12α,25ξ)-form	$C_{27}H_{48}O_5$	3.3
437.3156	24.9	Cholestane-3,7,12,26-tetrol; (3α,5β,7α,12α,25ξ)-form	$C_{27}H_{48}O_4$	-1.3
203.1021	22.4	Dehydrobufotenine	$C_{12}H_{14}N_2O$	3.0
283.0659	28.0	Dehydrobufotenine; O-Hydrogen sulfate inner salt	$C_{12}H_{14}N_2O_4S$	3.8
937.4721	23.1	Deltorphin; 2-*L*-Isoleucine analogue	$C_{45}H_{64}N_{10}O_{10}S$	6.9
769.4340	22.8	Deltorphin C	$C_{37}H_{52}N_8O_{10}$	7.8
783.4342	7.47	Deltorphin C; 4-Glutamic acid analogue	$C_{38}H_{54}N_8O_{10}$	-7.3
182.2004	25.7	3,5-I **181A**	$C_{12}H_{23}N$	2.0
204.1936	25.4	*Dendrobates* Alkaloid 203	$C_{14}H_{21}N$	2.7
224.2508	11.9	DHQ **219A**; Tetrahydro	$C_{15}H_{29}N$	3.4
254.2201	27.3	PTX **237A**; 7-Hydroxy	$C_{15}H_{27}NO_2$	5.6

252.2689	13.1	DHQ **251A**	$C_{17}H_{33}N$	-1.6
268.2692	23.7	PTX **251D**; 5ξ-Hydroxy	$C_{16}H_{29}NO_2$	6.5
256.2336	27.7	N-Methyl-DHQ **257A**	$C_{18}H_{25}N$	4.3
270.2492	11.4	DHQ **269A**	$C_{19}H_{27}N$	2.3
276.2871	11.6	Lehm **275A**	$C_{19}H_{33}N$	-1.4
282.2705	28.8	PTX **281A**	$C_{17}H_{31}NO_2$	-6.6
284.2854	27.3	Unclass **283B**	$C_{19}H_{25}NO$	8.0
296.2869	20.0	*Dendrobates* Alkaloid 295	$C_{19}H_{37}NO$	-3.7
298.2872	21.5	aPTX **297A**	$C_{17}H_{31}NO3$	7.5
298.3016	23.6	PTX **297B**	$C_{18}H_{35}NO2$	3.7
308.2844	28.7	PTX **307B**	$C_{19}H_{33}NO_2$	5.6
310.2219	26.7	PTX **307D**	$C_{18}H_{31}NO_2$	3.2
310.2999	27.0	PTX **309A**	$C_{19}H_{35}NO_2$	5.3
310.3526	27.2	Unclass **309B**	$C_{20}H_{39}NO$	5.4
326.2989	17.9	*Dendrobates* Alkaloid 325	$C_{19}H_{35}NO_3$	3.6

803.3819	0.87	Dermorphin	$C_{40}H_{50}N_8O_{10}$	7.9
959.3976	26.4	Dermorphin; 4-Tryptophan, 7-asparaginamide analogue	$C_{50}H_{58}N_{10}O_{10}$	-5.6
222.1396	20.6	Dehydrodesmethyl **PTX** 221F	$C_{14}H_{23}NO$	-4.0
262.2631	12.3	Tricyclic **261C**	$C_{18}H_{31}NO$	-2.5
266.1757	8.49	Dehydro-5,8-I **265F**	$C_{16}H_{27}NO_2$	-7.4
385.3657	28.2	DHQ-dimer **384A/B**	$C_{26}H_{44}N_2$	1.9
253.2204	18.7	SpiroP **252B**	$C_{14}H_{24}N_2O_2$	6.5

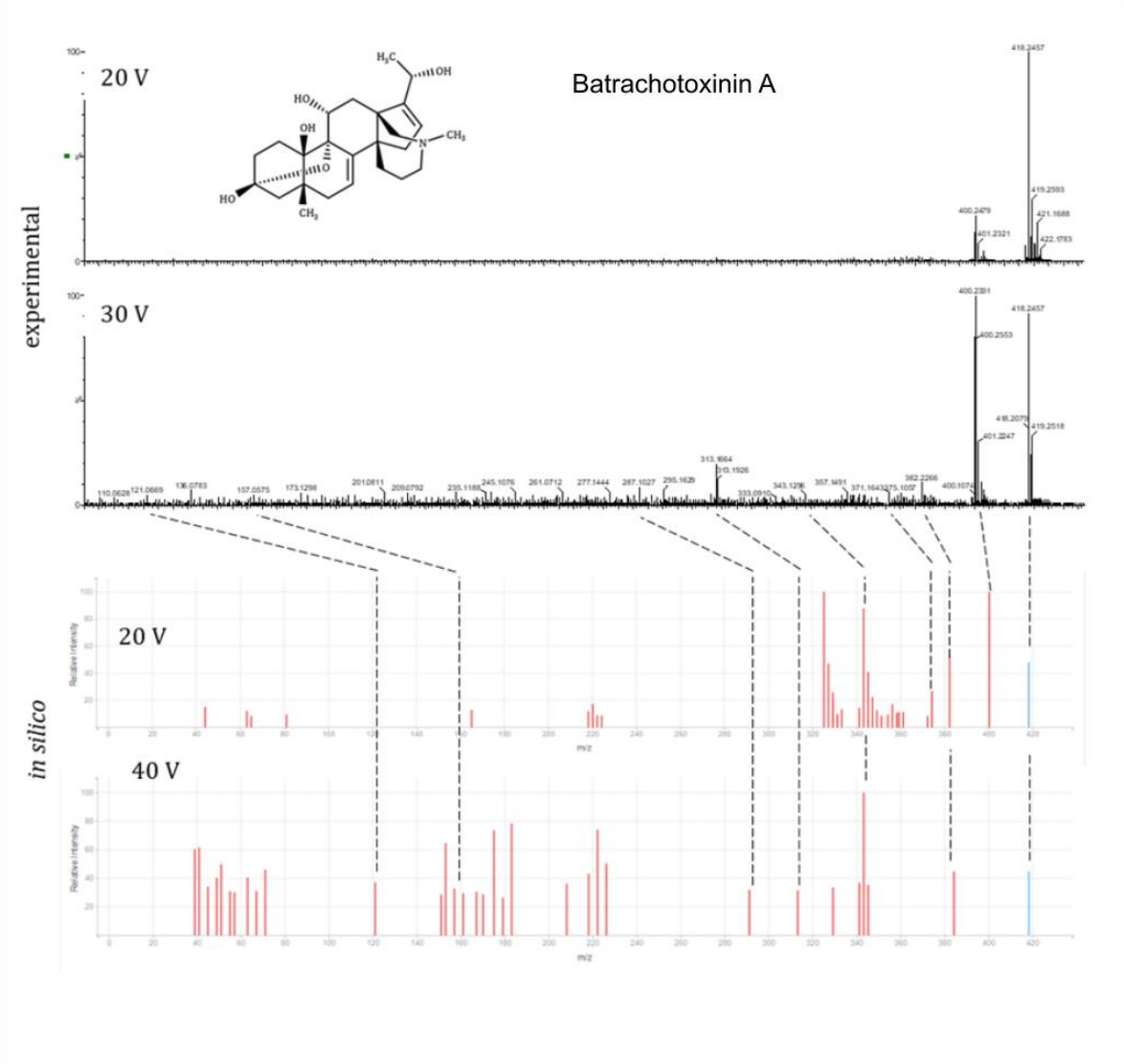

Fig S1 Comparison of experimental and *in-silico* MS/MS spectra for batrachotoxinin A.

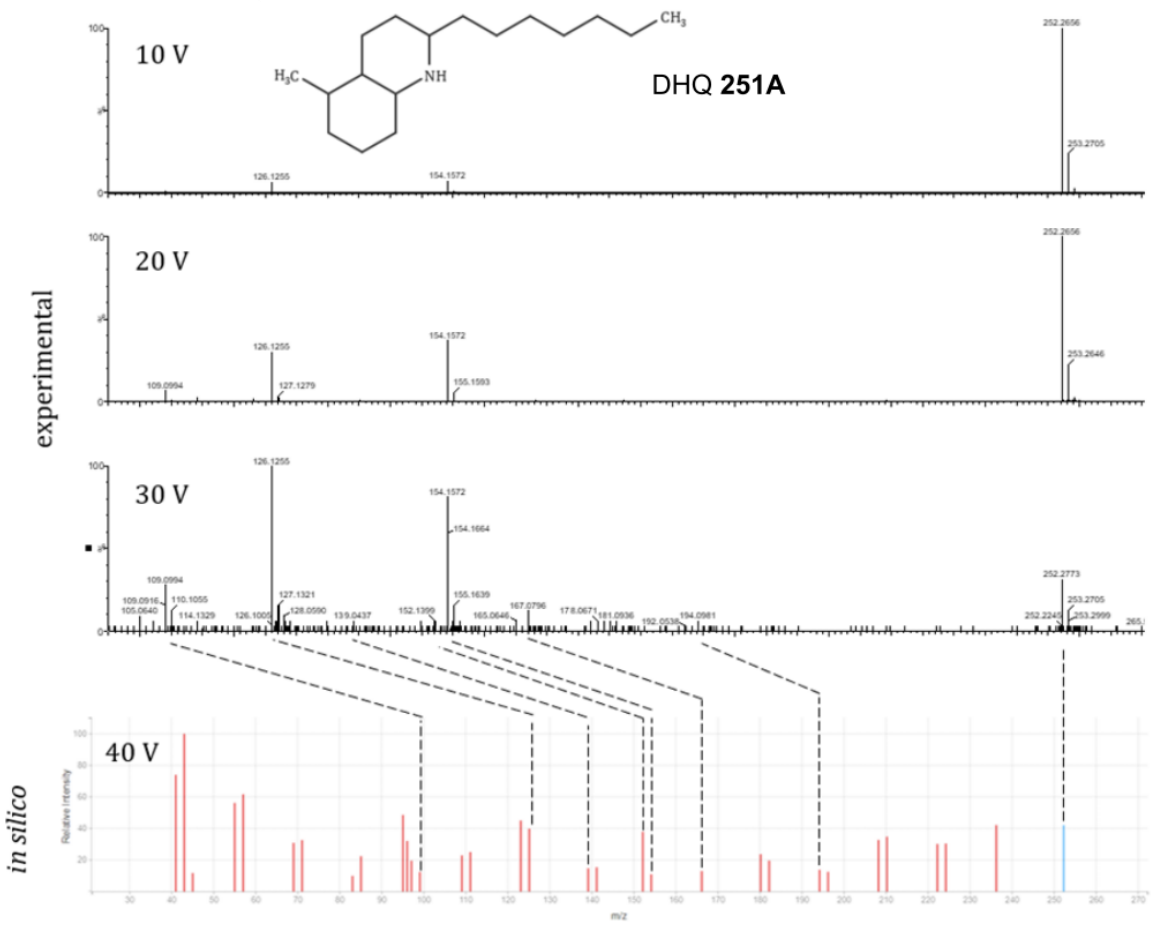

Fig S2 Comparison of experimental and *in-silico* MS/MS spectra for DHQ **251A**.

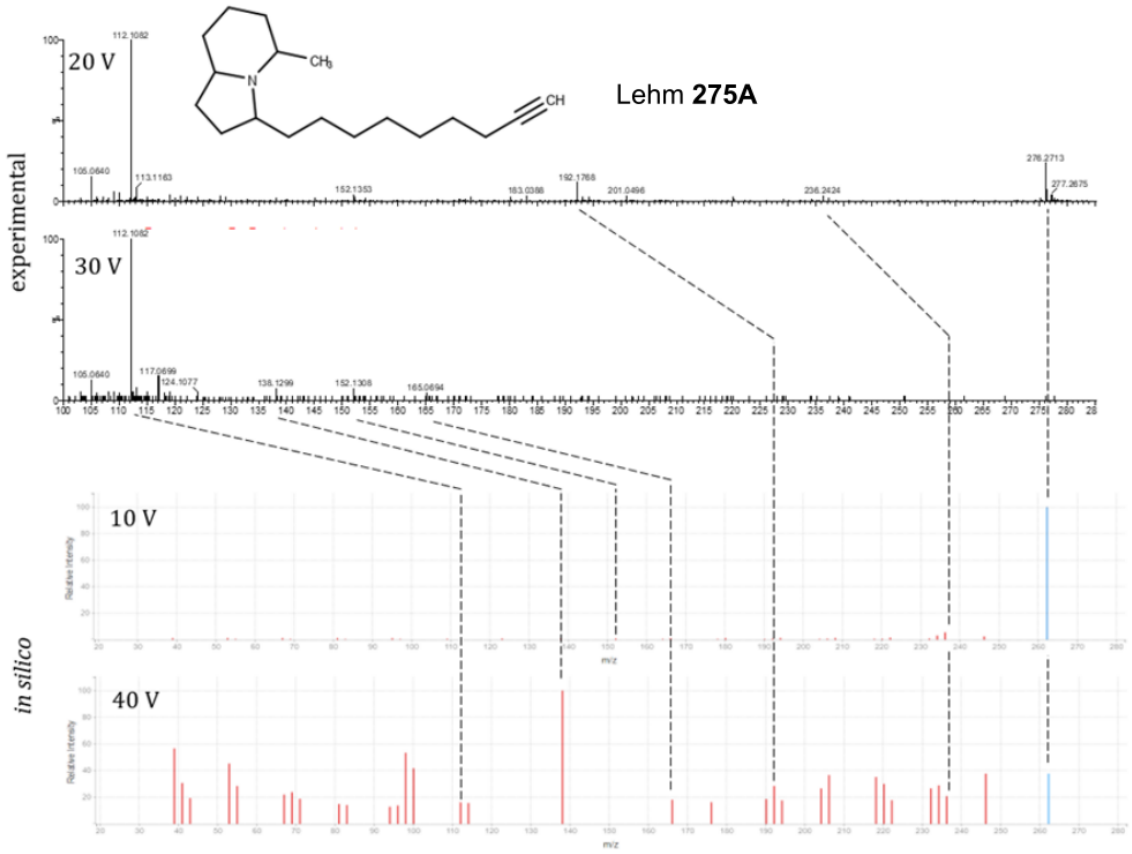

Fig S3 Comparison of experimental and *in-silico* MS/MS spectra for Lehm **275A.**

CAPÍTULO 2. Aposematism in the poison frog *Phyllobates vittatus* (Dendrobatidae): integrating signal detection and predation risk

(con formato para *Journal of Evolutionary Biology*)

ABSTRACT

It is normally assumed that bright and toxic animals are aposematic. However, predator avoidance of warning signals, a key component of aposematism, is rarely assessed for natural predators. We evaluated visual conspicuousness of a mildly toxic poison frog, *Phyllobates vittatus*, to three potential predators: birds, lizards and crabs. Then, we conducted field experiments with clay models to test if the most conspicuous colour signal displayed by *P. vittatus* deters predation attempts by these potential assailants. Despite geographic variation in the colour pattern and overall conspicuousness of *P. vittatus*, all predators are stimulated by the contrast between dorsal orange stripes and darker patches of frog colouration. Moreover, these stripes enhance visual conspicuousness for predators against a variety of substrates. Our predation experiment suggests that frogs with the striped colour pattern suffer less lizard predation than entirely orange phenotypes. This challenges the long-held assumption that birds alone drive the evolution of colour patterns in chemically defended prey.

Keywords: Golfo Dulcean poison frog, warning colouration, clay models, predation, predator avoidance, visual modelling

INTRODUCTION

Aposematism is a defensive strategy whereby prey organisms advertise their secondary defence to predators (Ruxton, Allen, Sherrat, & Speed, 2018). For an animal to be considered aposematic, a warning signal must have evolved in tandem with the defence, and it must discourage predators to initiate an attack (Ruxton et al., 2018). Aposematism is widespread in nature (Rojas, Valkonen, & Nokelainen, 2015; Ruxton et al., 2018), as examples range from invertebrates such as molluscs and insects to vertebrates such as fish, amphibians, reptiles, and mammals (Ruxton et al., 2018). However, the complete suite of requirements for aposematism is rarely assessed, as the study of avoidance of warning signals by natural predators has been neglected (Dell'Aglio, Stevens, & Jiggins, 2016).

Warning signals typically involve a conspicuous colouration attained by a high contrasts between colour patches within an organism, or against a substrate background (Stevens & Ruxton, 2011). Colour patterns combining black with red, yellow, or orange are usually displayed by terrestrial

animals to advertise their toxicity, unpalatability or unprofitability (Endler & Mappes, 2004). The contrast of these colours against green and brown backgrounds is strong, making defended prey easily detected and discriminated from edible ones (Toledo & Haddad, 2009). Conspicuous coloration and unprofitability work together because predators learn to associate both traits, as stronger signals are easier to remember, thereby favouring avoidance learning (Endler & Mappes, 2004; Mappes, Marples, & Endler, 2005; Darst, Cummings, & Cannatella, 2006). Aposematic colouration is thus advantageous when predators can detect these conspicuous colours, and have the cognitive capacities to associate a conspicuous signal with unpalatability. Because birds have both acute visual and cognitive abilities, it has been long assumed that birds are the main agents favouring the evolution of aposematic colouration in diurnal animals, including anurans (Wells, 2010).

Among anurans, the Neotropical poison frog family Dendrobatidae has been a model group for the study of aposematism (reviewed by Rojas, 2016). Dendrobatids exhibit striking variation in both colouration and toxicity (Summers & Clough, 2001), which is derived of alkaloids sequestered from their diet (Daly, Spande, & Garrafo, 2005). It has been hypothesized that chemical defences in dendrobatid frogs evolved as a result of diurnal activity, followed by the evolution of conspicuous colouration (Summers & Clough, 2001; Darst, Menéndez-Guerrero, Coloma, & Cannatella, 2005; Santos & Grant, 2011). Therefore, dendrobatids meet one of the criteria for aposematism: the evolutionary association of chemical defences and conspicuous colour patterns. Despite the considerable research interest into dendrobatid aposematism (Rojas, 2016), experimental evidence to support predator avoidance, the final criterion for this defensive strategy, is scarce (Summers & Clough, 2001).

In poison frogs, conspicuous colouration as a predator deterrent has been typically tested by conducting field experiments with clay models (Rößler, Pröhl, & Lötters, 2018). This method has been used to asses predation risk in the poison frogs *Oophaga pumilio* (Saporito, Zuercher, Roberts, Gerow, & Donnelly, 2007; Hegna, Saporito, Gerow, & Donnelly, 2011; Hegna, Saporito, & Donelly, 2013; Stuart, Dappen, & Losin, 2012; Paluh, Hantak, & Saporito, 2014; Dreher, Cummings, & Pröhl, 2015; Preißler & Pröhl, 2017), *O. granulifera* (Willink, García-Rodríguez, Bolaños, & Pröhl, 2014), *Dendrobates tinctorius* (Noonan & Comeault, 2009; Rojas, Rautiala, & Mappes, 2014), and *Ranitomeya imitator* (Chouteau & Angers, 2011). In almost all of these studies, birds have been

regarded as the main predators of clay models, hence supporting the hypothesis that bird predation selects conspicuous coloration. Yet, little is known about the main predators of adult dendrobatids in nature (Rojas, 2016).

Among the poison frogs, the genus *Phyllobates* is particularly puzzling. *Phyllobates* frogs are brightly coloured and possess in their skin the highly toxic alkaloid batrachotoxin (Myers, Daly, & Malkin, 1978), hence typically are considered aposematic (Santos et al., 2003). Nevertheless, there is no experimental evidence that demonstrates that predators avoid frogs in this genus. Furthermore, it has been suggested that *P. vittatus*, an endemic species from the South Pacific of Costa Rica, is not toxic, and instead takes advantage of its bright colouration (Fig. 1) and co-occurrence with other toxic dendrobatids to feign toxicity (Mebs, Vargas, Pogoda, Toennes, & Köhler, 2014). However, recent evidence suggests that *P. vittatus* might be at least mildly toxic to potential predators, as its skin extracts contain toxic alkaloids that can cause toxicity symptoms in mice (Protti-Sánchez et al., unpublished).

Here, we aimed to determine whether the dorsal colouration of *P. vittatus* acts as a warning signal by testing two key assumptions about aposematism. First, we quantified colour pattern variation among three localities, and evaluated frog conspicuousness to three potential predators. Second, we tested whether the colour pattern of *P. vittatus* grants protection against these predators. To do this, we conducted field experiments with clay models varying in their colour and pattern. If the colouration of *P. vittatus* is aposematic, we predict that (1) frogs will be conspicuous for the view of potential predators; and (2) predators will avoid the colour pattern of *P. vittatus*. Thus, predation attempts should be lower in clay models that accurately resemble *P. vittatus*, compared to other conspicuous colour patterns and to cryptic clay models.

MATERIALS AND METHODS

Frog sampling

We sampled frogs from three localities in the Osa Peninsula, Costa Rica: Agua Buena, La Tarde and Piro (Fig. 2), during the early rainy season of 2017 (April-May). We captured frogs in the field, recording the type of substrate where they were found. Frogs were transported to a laboratory

facility, where we measured their snout-to-vent length (SVL) prior to spectrometric measurements. Frogs from these localities differed in SVL, as frogs from Piro were significantly smaller than frogs from Agua Buena and La Tarde (Table S1, Fig. S1). Given that there is neither sexual dichromatism nor dimorphism in this species (Savage, 2002), we were unable to determine sex.

Reflectance measurements

We gently dried the frogs with a paper towel before measuring their reflectance spectra, as their skin moisture could otherwise create additional reflectance, particularly in darker sections of the dorsum. For each individual, we made four reflectance measurements from each of three body parts: dorsum, dorsal stripes, and hind limbs. Additionally, we took samples of the most common substrates where the frogs were located and made four reflectance measurements from each.

We used an Ocean Optics USB2000+ spectrometer, attached to a PX-2 lamp and an Ocean Optics bifurcal optic fiber R-200-2-UV-Vis. To account for lamp drift, we calibrated with a white reflection standard (PN:WS-1) every time we measured a new frog or substrate. We conducted all measurements at a 90º angle and with the probe at a distance of 2 mm from the frog skin. After spectrometric measurements, we released all frogs in the exact location where they were collected. Due to the territorial behaviour of *P. vittatus* (Summers, 2000), we avoided resampling by not collecting frogs from sites where we already sampled.

We treated all spectrometric data in R (R Core Development Team, 2018) with the package “pavo” (Maia, Eliason, Bitton, Doucet, & Shawkey, 2013). First, we trimmed the reflectance data to the 300-800 nm range. We then corrected negative values, by adding the absolute value of the maximally negative measurement of each spectra to the reflectance at all other wavelengths (setting the minimum value to zero, but scaling other values accordingly; Maia et al., 2013). All four spectrometric measurements from each body region or substrate were averaged to obtain our final data.

To describe colouration of the body regions in terms of their inherent characteristics, we followed Endler (1990) segment classification analysis, which divides the 300-800 nm spectrum into four equally spaced regions, and aims to classify colours based on common traits of many vertebrate visual systems. We measured the relative signal in each region, and calculated three colourimetric

variables: brightness (B1), hue (H4) and chroma (S5). Brightness refers to the sum of reflectance values over all wavelengths, and it is equivalent to the height of the curve on a reflectance spectrum (Endler, 1990). Hue is the dominant wavelength of reflected light given by the angle in the colour space (Endler, 1990). Chroma is equivalent to the saturation of colour, and is given by the distance from the centre of the colour space (Endler, 1990).

Visual modelling

We performed visual modelling, as developed by Vorobyev, Osorio, Bennet, Marshall, & Cuthill (1998) and modified by Stuart-Fox, Moussalli, Marshall, & Owens (2003) and Siddiqi, Cronin, Loew, Vorobyev, & Summers (2004), to evaluate frog conspicuousness for the visual systems of three types of potential predators. We calculated colour (ΔS, chromatic) and brightness (ΔL, achromatic) contrast within the body regions of frogs (stripes vs. dorsum, stripes vs. hind limbs, and dorsum vs. hind limbs). In addition, we estimated the overall conspicuousness of frogs on each substrate (leaf litter, rock, soil, and trunk), by combining both colour and brightness contrasts of all body regions for each individual frog (Maan & Cummings, 2012). Overall conspicuousness is calculated as the Euclidean distance between the colour and brightness contrast (Maan & Cummings, 2012).

We calculated frog conspicuousness for the visual system of birds, lizards and crabs. Birds have been regarded as the main drivers of the evolution of aposematic colours in diurnal animals, because of their acute visual system (Wells, 2010). Moreover, these predators have been reported as making the most predation attempts on clay models of frogs, in the South Pacific region of Costa Rica (Willink et al., 2014), whereas lizards and crabs were potential predators revealed in this study and a previous study (Willink et al., 2014; see Results).

Colour contrast is the perceived difference between two colours. Brightness contrast is assumed to be mediated by the long-wave-sensitive cone class (LWS). The bird LWS is important for prey detection and seems to be essential for detection of small targets (Osorio, Miklósi, & Gonda, 1999; Théry, Debut, Gomez, & Casas, 2004). Both colour and brightness contrasts are measured in units called "just noticeable differences" (jnd) where a value greater than 1 indicates that the difference is discernible by a viewer according to the properties of its visual system (Siddiqi et al., 2004). Detailed descriptions of colour and brightness contrast calculations can be found in Pröhl & Ostrowski (2011), and Maan & Cummings (2012).

Visual systems vary among taxa. For the bird visual model, we used spectral sensitivity data from the tetrachromatic visual system, with UV sensitivity, of the blue tit *Cyanistes caeruleus* (Paridae). This species is a model predatory bird, previously used for conspicuousness estimation of poison frogs to bird predators (e.g. Pröhl & Ostrowski, 2011; Richards-Zawacki, Yeager, & Bart, 2013; Willink, Brenes-Mora, Bolaños, & Pröhl, 2013). Additionally, there is no spectral sensitivity data for the only bird species known to predate on poison frogs, the Rufous Motmot *Baryphthengus martii* (Master, 1999; Alvarado, Álvarez, & Saporito, 2013). Given that Coraciiformes and Passeriformes are closely related (Prum et al., 2015), and that there is little variation in spectral sensitivities of 26 bird species so far investigated (Hart, 2001), we considered the use of *C. caeruleus* as an appropriate approximation of frog conspicuousness to potential bird predators.

We used data from the tetrachromatic visual system of the brown anole *Anolis sagrei* (Dactyloidae) as a lizard model predator because spectral sensitivity data is available (Loew, Fleishman Foster, & Provencio, 2002; Fleishman et al., 2016), but without using the UV sensitivity cones (as in Fleishman et al., 2016). Anoles are closely related to *Basiliscus* spp. lizards (family Corytophanidae; Pyron, Burbrink, & Wiens, 2013), which are common in the areas inhabited by *P. vittatus* (F. Protti, personal observation), and have been reported to attack poison-frog clay models (Willink et al., 2014).

For the crab visual model, we followed the approach of Cummings, Jordão, Cronin, & Oliveira, (2008) and Maan & Cummings (2012) and modelled a dichromatic crab visual system based on spectral sensitivity of *Uca tangeri* (Jordão, Cronin, & Oliveira, 2007) and *U. thayeri* fiddler crabs (Ocypodidae). Even though these are European shore-dwelling species, it is the only data available of crab visual sensitivity. Here, we use this visual system as an initial approach to understand how freshwater crabs see poison frogs. This method has been previously used to evaluate poison frog conspicuousness to crab dichromatic systems (e.g. Crothers & Cummings, 2013; Richards-Zawacki et al., 2013; Dreher et al., 2015).

For all visual models in “pavo” (Maia et al., 2013) we used frog reflectance spectra ranging from 300-700 nm. We assumed a forest shade illumination (Fig. S2), as we always found *P. vittatus* on the ground (F. Protti, pers. obs.). Additionally, we applied the von Kries colour correction transformation (Maan & Cummings, 2012), and used the visual sensitivity data specific for each of the three predators (Table S2).

Predation experiment

We conducted field experiments using clay models to determine whether predators avoid the colouration of *P. vittatus*. We used models with four different colour patterns: (1) resembling *P. vittatus,* with a black dorsum and orange dorsal stripes, (2) plain orange, to test the effect of bright colouration alone on predation attempts, (3) plain brown, similar to sympatric cryptic frogs such as *Craugastor* spp., and (4) brown with cream-coloured dorsal stripes, similar to other sympatric non-toxic frogs, such as *Silverstoneia flotator.* These model types are hereafter referred to as "*P. vittatus*", "orange", "brown" and "striped brown", respectively. We constructed clay models following Willink et al. (2014, Fig. S3.).

We conducted the experiments on November 2017, in Agua Buena and La Tarde, and on May 2018 in Piro (Fig. 2). All trials occurred during the rainy season, when most frogs are reproductively active (Savage, 2002). For each site, we selected a stream with a local population of *P. vittatus*. We placed groups of four models with different colour patterns on the ground on both sides of the stream. Models within the group were separated by approximately 50 cm, and were set in a random order. Each group of four models was separated by 3 m. We placed all models on an experimental brown substrate (hereafter "experimental substrate", size 6 x 5.5 cm), resembling the colour of the leaf litter (Fig. S3F). This way we standardized the contrasts between models and their background. We used a total of 222 replicates in Agua Buena, and 300 in each La Tarde and Piro, for a total of 3288 clay models. We set up the experiment in three days, each day placing 100 four-model replicates. After 72 h we recorded whether a predation attempt occurred or whether a model was missing due to heavy rain or other unknown causes. We photographed predation marks, described them, and stored the attacked clay models for further diagnosis of potential predators. Then, we classified marks in the clay models according to the shape of the damage (following Willink et al., 2014) into six categories: (1) crab, (2) lizard, (3) bird, (4) insect, (5) mammal, and (6) unknown.

Statistical analysis

We conducted all statistical analysis in R (R Core Development Team, 2018). We used a G-test to determine whether frog substrate use differed among localities. Because only a single frog was observed on a rock, we did not include this substrate in the analysis.

We used multivariate analyses of variance (MANOVA) to compare colour elements (brightness, hue and chroma) of frog body regions among localities, as brightness is strongly correlated with both hue and chroma (Fig. S4). To facilitate the interpretation of overall significant multivariate effects, we then performed two-ways ANOVAs, followed by *post-hoc* Tukey HSD tests on each colour element. For both MANOVA and two-ways ANOVAs, we used the locality and body region as factors in a fully factorial design.

To determine whether colour patches within the body of *P. vittatus* created visual contrasts to potential predators, we performed mixed effect models for colour and brightness contrasts (as the response variables), and for each predator separately. We used the contrast type (stripes vs. dorsum, stripes vs. hind limbs, and dorsum vs. hind limbs) and locality (Agua Buena, La Tarde and Piro) as fixed categorical effects. The identity of each frog was treated as a random effect, to account for the non-independence of multiple contrast types measured on the same individual. To determine whether overall conspicuousness (response variable) varied among substrates or localities (fixed effects), we performed another mixed effect model for each predator, again using frog identity as a random effect.

We tested whether the probability of predation (response variable) depends upon model type and locality (factors). We performed a Generalized Lineal Model (GLM) using the pooled predation data from lizard, crab and bird predators, and a binomial error distribution. Because the main predators in this study were lizards and crabs (see results), we followed up this analysis using a binomial mixed effect model, including predation attempts of only these two taxa. Here, we tested if the probability of predation (response variable) differed among model types and between predators. We included the locality as a random effect on the variance of predation risk, and also accounted in the random effect structure for the fact that predation attempts by lizards and crabs were recorded on the same models. After modelling predation risk with a fully factorial design, we excluded non-significant interactions from the models.

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 & Weisberg, 2011). Pairwise comparisons between treatments were assessed using the function “pairs” of the “emmeans” package (Lenth, 2019).

RESULTS

Substrates used by frogs

We sampled 51 frogs, 17 from Agua Buena, 16 from La Tarde and 18 from Piro. In Agua Buena and Piro, frogs used leaf litter, bare soil and trunks as substrates. In La Tarde, we did not find frogs on soil. Leaf litter was the most common substrate used in La Tarde and Piro, whereas frogs from Agua Buena used trunks more frequently, but nearly equally as leaf litter and soil (G-test=11.72, df=4, *P*=0.020; Fig. 3). Reflectance curves of substrates are available in Fig S5.

Spatial variation in frog colour patterns

Frog colour elements differed significantly among localities and body regions. The interaction between both factors had an effect on colour elements (Table 1). The dorsum of frogs from La Tarde had higher values of hue than the dorsum of frogs from Agua Buena and Piro (Table 2, Fig. 4). Both the dorsal orange stripes and the hind limbs of frogs from Piro had smaller values of brightness and chroma than those of frogs from Agua Buena and La Tarde (Table 2, Fig. 4). Brightness and chroma of the dorsum and hind limbs were lower than for the stripes in all the three localities (Table 3, Fig. 4).

Conspicuousness to potential predators

According to visual modelling, birds, lizards and crabs could perceive the brightness contrasts within body regions of *P. vittatus*, as well as the overall conspicuousness of frogs against all backgrounds (Fig. 5, all contrast estimations above the 1 jnd threshold). For all predators, brightness contrasts within frogs depended on which body regions were compared, the locality of frogs, and the interaction between these two factors (Table 4). In two of the localities, Agua Buena and La Tarde, brightness contrasts between the stripes and dorsum were the most conspicuous, while contrasts between the dorsum and hind limbs were less conspicuous (Table 5, Fig. 5 B, E, H). Conspicuousness in Piro was instead mostly due to the contrast created by the stripes and the particularly dark hind limbs of frogs in this locality (Table 5, Fig. 4, Fig. 5 B, E, H).

Birds could discriminate among all body regions of frogs (Fig. 5 A) by their colour contrast, whereas lizards and crabs could not distinguish between the dark dorsum and the turquoise hind limbs (Fig. 5 D, G). There were no locality differences in colour contrast for any predator (Table 4). For the three predators, the colour contrasts between stripes and dorsum, and stripes and hind limbs, were significantly higher than between dorsum and hind limbs (Table 5, Fig. 5A, D, G).

Overall conspicuousness of frogs to all potential predators was significantly affected by the viewing substrate, the locality and their interaction (Table 6, Fig. 5 C, F, I). For all predators, frogs from Agua Buena and La Tarde were significantly more conspicuous on rocks and trunks (Table 7, Fig. 5 C, F, I). Conversely, frogs from Piro displayed higher overall contrast on leaf litter and soil (Table 7, Fig. 5 C, F, I).

Predator avoidance

Out of the 3288 clay models used in the field experiments, 7.8% exhibited some kind of damage, including disappearance over the 72 h trial (48 models). Excluding missing models, 6.3% were recorded as being attacked, of which 43% of the attacks corresponded to crabs, 32% to lizards, 11% to insects, 6% to mammals, 5% to birds and 2% were categorized as unknown (Fig. 6, Fig. S6). The probability of being attacked by any of the three most likely poison frog predators (birds, crabs, and lizards) depended on the colour of the model (X^2=56.12, *df*=3, *P*<0.001), the locality (X^2=7.80, *df*=2, *P*=0.02) and the interaction between both factors (X^2=22.91, *df*=6, *P*<0.001). “Orange” models had a higher probability of being attacked than “*P. vittatus*”, “striped brown” and “brown” models for Agua Buena and Piro, whereas all models from La Tarde had the same probability of being attacked (Fig. 7A). When considering only predation by the main predators, crabs and lizards, colour (X^2=25.82, *df*=3, *P*<0.001) and the interaction between colour and the identity of the predator (X^2=17.20, *df*=3, *P*<0.001) significantly affected predation probability, whereas predator taxa showed a marginally non-significant effect (X^2=3.38, *df*=1, *P*=0.06). The probability of crab predation attempts was similar for all model types whereas lizards were more likely to attack “orange” models than any other colour pattern (Fig. 7B).

DISCUSSION

In this study, we tested whether the dorsal colouration of *Phyllobates vittatus* acts as a warning signal for predators. We show that geographic variation in the colour pattern of *P. vittatus* does not undermine visual detection by potential predators (Fig. 5). The orange stripes in the dorsum of these frogs constitute the colour element of greatest conspicuousness to potential predators (Fig. 5A, D, G). Frogs are visually conspicuous against all natural backgrounds and in all three localities, for the eyes of taxa currently known or suspected to prey upon them (Maan & Cummings, 2012; Willink et al., 2014; Fig. 5). Our results also suggest that local predators, particularly lizards, are not deterred by unknown and conspicuous colour signals. Yet, while entirely orange models suffered the most attacks, models resembling *P. vittatus* seem similarly protected as cryptic prey.

Spatial colour variation in *P. vittatus* was largely caused by differences in brightness and chroma among localities. Both colour characteristics are positively correlated in all *P. vittatus* body regions (Fig. S4). Colour production in animals is achieved by different pigment molecules, nanostructures, or a combination of both (Shawkey, & D'Alba, 2017). In fish, amphibians and reptiles, the dermal chromatophore unit (Bagnara, Taylor, & Hadley, 1968), consists on specialized cells containing pigments arranged in contiguous layers (Grether, Kolluru, & Nersissian, 2004). Changes in the physical structure of some of these pigment-containing cell layers, such as the iridiophores, could simultaneously influence the brightness and chroma of a colour patch (Grether et al., 2004). Thus by modifying the internal structure of the chromatophore unit non-adaptive processes such as drift may cause differences between localities in multiple correlated colour elements (Chouteau & Angers, 2012).

Alternatively, variation in the inherent colour characteristics of frogs could be due to sexual selection. Sexual selection can act simultaneously over different colour traits affecting fitness in an interactive way, through correlational selection (Sinervo & Svensson, 2002; Cole & Endler, 2015). For instance, female guppies (*Poecillia reticulata*) display preferences over groups of colours of males rather than individual colours on different environmental conditions, thus selecting entire colour patterns based on overall visual contrasts (Cole & Endler, 2015). Moreover, it has been demonstrated for *Oophaga pumilio*, another species of poison frog, that females prefer brighter males (Maan & Cummings, 2009). Together with assortative mating (Reynolds & Fitzpatrick, 2007), this preference could lead to geographic variation in hue (Maan & Cummings, 2009) and chroma

(Crothers & Cummings, 2013), as these colour traits may also be correlated (Maan & Cummings, 2009).

We suspect that natural selection through predation is unlikely to drive this colour divergence across localities, because we found no evidence that these colour differences substantially influence detection by potential predators. Thus, there is no indication that colour patterns are locally adapted to variable predator communities, as was suggested for *O. pumilio* (Dreher et al., 2015) and *O. granulifera* (Willink et al., 2014). Even though the spatial variation in *P. vittatus* colour pattern might be discernible for predators, it is not as evident as the variation in colour patterns across populations, and even across small geographic scales, of other poison frogs species such as *O. pumilio* (Summers, Cronin, & Kennedy, 2003; Pröhl, Willink, & Hauswaldt, 2013), *O. granulifera* (Brusa, Bellati, Meuche, Mundy, & Pröhl, 2013; Pröhl et al., 2013; Willink et al., 2013), and *Dendrobates tinctorius* (Rojas & Endler, 2013).

Through visual modelling, we found that contrasts between the orange dorsal stripes and other body regions exacerbate the overall conspicuousness of *P. vittatus*. Hence, it is possible that these stripes, when viewed against the frog background or hind limbs colouration, act as an effective warning signal. Striped patterns within an animal's body have been shown to enhance predator avoidance learning for fish (Green, Urquhart, van den Berg, Marshall, & Cheney, 2018) and bird predators (Aronsson & Gamberale-Stille, 2013). Bird predators learn faster to avoid striped patterns within a prey's body than plain colours (Aronsson & Gamberale-Stille, 2013).

Even though lizards and crabs cannot distinguish the colour contrast between the dorsum and hind limbs of *P. vittatus*, brightness contrasts between all body regions are readily distinguishable for all the three predators. For birds, brightness contrast is important for prey detection (Pröhl & Ostrowski, 2011), especially for detecting small prey over long distances (Osorio et al., 1999, Théry et al., 2004). Brightness contrast heightens prey detection, enables predator aversion learning and increases predator memory retention (Prudic, Skemp, & Papaj, 2007). Thereby, brightness contrast is an important component of aposematic colouration (Prudic et al., 2007, Maan & Cummings, 2009), and it can also be an effective signal to colour blind predators (Prudic et al., 2007).

The particularly dark hind limbs of frogs from Piro enhance the brightness contrast between stripes and hind limbs for the visual system of the predators here studied. In turn, this high

brightness contrast explains why the overall conspicuousness is higher against leaf litter and soil in Piro than in Agua Buena and La Tarde. Pröhl & Ostrowski (2011) showed that calling males of *O. pumilio* exhibit some degree of substrate or background selection. Aposematic red frogs used substrates that enhanced both their colour and brightness contrast towards the view of a bird predator and conspecifics, and cryptic green males used calling places that decreased their colour and brightness contrast (Pröhl & Ostrowski, 2011). Unlike *O. pumilio*, *P. vittatus* appears highly conspicuous in all localities and substrates. This suggests that differences in substrate use among localities likely reflects variation in substrate availability, and not frog selection of backgrounds to enhance warning signals.

Background selection might be superfluous for *P. vittatus*, as visual contrasts between colour patches within the body already make these frogs highly conspicuous. Moreover, *P. vittatus* is a relatively mobile poison frog, which usually hops quickly and for several meters if disturbed (Savage, 2002, F. Protti personal observation). It is possible that individuals *P. vittatus* combine both, aposematic and disruptive elements, in their anti-predator strategies. Even though the colour pattern of *P. vittatus* is visually conspicuous for all potential predators, the dorsal stripes may break the outline of moving frogs, hence acting as a disruptive pattern that difficults predator recognition (Webster, Hassall, Herdman, Godin, & Sherratt, 2013; Rojas, Devillechabrolle, & Endler, 2014; Ruxton et al., 2018). Our behavioural observations of frogs in the field are at odds with typical behaviours expected of aposematic animals, such as high exposure and active foraging (Pröhl & Ostrowski, 2011). *P. vittatus* is usually hidden, difficult to detect if they are not calling, and they escape immediately after being disturbed (F. Protti, personal observation). Therefore, systematic behavioural observations, as well as measurement of behavioural elements, are crucial to better understand the defensive strategy, or the whole array of defensive strategies, that *P. vittatus* frogs may display in different contexts.

The pattern of predation attempts in this study critically differs from previous work based on clay models of poison frogs. Birds have been reported as the main attackers of poison frog models in all previous studies (Saporito et al., 2007; Noonan & Comeault, 2009; Chouteau & Angers, 2011; Comeault & Noonan, 2011; Hegna et al., 2011, 2013; Stuart et al., 2012; Paluh et al., 2014; Rojas et al., 2014; Willink et al., 2014; Dreher et al., 2015; Preißler & Pröhl, 2017), whereas for *P. vittatus* crabs and lizards may play a larger role as predators (Figs. 7, S6). While most bird predators can

probably forage by streams as well as further away in the forest, large lizards, such as the semiaquatic basilisks (*Basiliscus* spp.) and fresh water crabs, are bound to habitats near the water. Fresh water crabs in the Pseudothelphusidae family are the only crabs that could have attacked our frog models (Wehrtmann, Hernández-Díaz, & Cumberlidge, 2019), whereas basilisks are the only lizards so far known to attack clay models of frogs (Willink et al. 2014; see below). Of all poison frogs for which predation experiments have been conducted, *P. vittatus* is the only one living exclusively in association to streams (Savage, 2002), and in our study no individuals were seen anywhere more than 100 m from a stream (F. Protti, personal observation). *O. granulifera*, for which the three predator groups were also detected, usually inhabits areas near fast-moving streams in mature forests (Savage, 2002; Willink et al., 2013). Other poison frogs with only birds identified as potential predators in clay-model experiments, such as *O. pumilio* (Saporito et al., 2007; Paluh et al., 2014; Dreher et al., 2015; Preißler & Pröhl, 2017), maintain territories in various habitats and are not necessarily linked to streams (Savage, 2002). Thus, the unique habitat characteristics of *P. vittatus* explains why our results markedly contrast previous studies.

The results of the predation experiments also show that a conspicuous orange colouration itself does not grant protection against potential predators co-occurring with *P. vittatu*s. The plain orange models suffered the highest rate of predation, both in Agua Buena and Piro, and mainly by lizards. The higher predation rate on orange models, which are a novel phenotype, is consistent with both theory (Endler, 1998) and empirical studies where predators attack novel conspicuous prey more than they do attack local aposematic prey (Noonan & Comeault, 2009; Chateau & Angers, 2011).

We expected that models with better resemblance to *P. vittatus* would suffer less predation than cryptic models, which was not the case in any locality. One possible explanation is that the visual warning signal of *P. vittatus* has actually evolved mainly in response to bird predators, but due to recent habitat degradation, hunting and other environmental threats, most land-dwelling omnivorous birds that may attack *P. vittatus* have been locally extirpated. In this scenario, lizards and crabs identify clay models with resemblance to *P. vittatus* as prey as much as they do so for cryptic clay models. Even if these predators are physiologically capable of detecting the inferred visual contrasts, they may lack sensitivity to the frogs' toxins or the cognitive machinery to generate avoidance learning (Endler & Mappes, 2004). Alternatively, the warning signal in clay

models of *P. vittatus* may be incomplete without the fast-moving behaviour of actual frogs. Experimental evidence shows that movement is important for prey selection by visual predators, as it can amplify the aposematic signal, hence affecting clay model experiments (Paluh et al., 2014). While these scenarios are not mutually exclusive, they point to the daunting challenges of inferring the strength and form of predation, as a selective pressure in the wild.

Lizards are known to rely on both chemical and visual cues when detecting prey (Cooper, 1995) and assessing their palatability (Sword, Simpson, El Hadi, & Wilps, 2000). Recently, it has been shown that some lizards discriminate aposematic prey and avoid them based only on visual cues, such as colour, pattern, and shape (Baruch, Manger, & Stynoski, 2016; Beneš & Veselý, 2017; Lee et al., 2018). Furthermore, experimental evidence suggests that lizards are capable of learning to avoid unprofitable prey (Boyden, 1976; Sword et al., 2000; Tseng, Lin, Hsu, Pike, & Huang, 2014). Therefore, it is not implausible that lizards have learnt to associate the toxicity of *P. vittatus* (Protti-Sánchez et al., unpublished) with their conspicuous dorsal pattern, and that, in combination with behavioural traits, the dorsal colouration of *P. vittatus* acts as a warning signal (e.g. Amézquita, Castro, Arias, González, & Esquivel, 2013). Finally, we cannot rule out that the low predation on cryptic models could be due to enhanced crypsis against the experimental background, so that they would be too difficult to detect. According to visual modelling, however, this is unlikely because overall conspicuousness of clay models on the experimental substrate could be in principle detected by the three types of predators (Fig. S7).

As a preliminary attempt to identify lizard marks on clay models, we imprinted the bite of a preserved individual of the basilisk *B. basilicus* from the Museum of Zoology at the University of Costa Rica, and compared it with marks from the field experiment assigned to predation by lizards. Through a rough comparison by eye, marks on clay models seemed similar than those from the preserved specimen (Fig. S8). Hence, *B. basiliscus* or *B. plumifrons* individuals could have attacked our clay models. We believe that this method could help standardize the identification of predation marks on clay models, thereby reducing observer bias (Rößler et al., 2018). Moreover, it could be easily done with vertebrate specimens from museums. Yet, it should be systematized in order to be replicable.

Because of their habits, *B. basiliscus* lizards are a potential candidate predator of *P. vittatus* and other poison frog species associated to streams. *B. basiliscus* lizards are diurnal, semiaquatic and

are associated with streams most of the time, either basking, foraging or resting (Savage, 2002). They are omnivorous, but mainly feed on active prey, such as insects, freshwater shrimp, fish, other lizards, frogs, snakes, birds and mammals (Fleet & Fitch, 1974; Savage, 2002). For instance, camera traps recorded individuals *B. basiliscus* attacking two poison frog clay models of *O. granulifera*, a sympatric frog of *P. vittatus*, in the South Pacific of Costa Rica (Willink et al., 2014). In addition, their size is large enough to handle an individual *P. vittatus* when compared with other sympatric lizards such as *Anolis* spp. and *Holcosus* spp. Furthermore, lizards had been demonstrated to possess acute colour vision, and photoreceptors sensitive to UV light (Loew et al., 2002, Fleishman et al., 2016), such as some birds.

In our study, crabs were the main assailants of clay models, attacking 43% of the 207 attacked models. Crabs attacked all model types, independently of their colour pattern (Fig. 7B). This result is consistent with a study on predation on *O. granulifera* (Willink et al*.,* 2014), and it could be explained by the nocturnal foraging habit of freshwater crabs (Yeo et al., 2008). *P. vittatus* is not active at night (Savage, 2002), when warning colourations are probably less distinguishable. Therefore, crabs may be opportunistic predators of poison frogs (Wehrtmann et al., 2019). To date we know only of one recorded observation of a freshwater crab attacking a poison frog (Rojas, 2016). While it has been suggested that the more toxic morphs of *O. pumilio* have higher dorsal conspicuousness to crabs (Maan & Cummings, 2012), it should be reminded that the crab visual model developed until today, is based on a European shore-dwelling species (Cummings et al., 2008; Maan & Cummings, 2012). Thus, the selection pressure imposed by crabs on poison-frog colouration should be taken with caution (Willink et al., 2014). We encourage research related with visual ecology of freshwater crabs sympatric with poison frogs, in order to aid the understanding of how they perceive and assess warning signals.

CONCLUSIONS

Aposematism should not be always assumed for brightly coloured and defended species, as predator avoidance mediated by the colour pattern is not guaranteed. We found that the dorsal colouration of *P. vittatus* does not increase predation compared to cryptic frogs and a conspicuous novel phenotype, nor does it unequivocally advertise unprofitability to local predators. Here, we

challenge the assumption that birds are the main predators of all poison frogs, and the drivers of aposematic colouration. We propose that lizards are also potential agents selecting over conspicuous colouration in poison frogs. As aposematism grants adaptive benefits to both, predators and preys, effective communication is crucial in this strategy.

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TABLES

Table 1. Multivariate analysis of variance (MANOVA) to test for locality differences in the colouration of Phyllobates vittatus. To facilitate the interpretation of significant overall multivariate effects, a two-way analysis of variance (ANOVA) for each colour variable is presented. Significant effects ($P \leq 0.05$) are highlighted in bold.

	Factor	*Pillai*	*F*	*df*	*P*-value
MANOVA					
	Locality	0.253	6.931	2,286	**<0.001**
	Body region	1.028	50.468	2,286	**<0.001**
	Locality:Body region	0.201	2.587	4,432	**0.002**
ANOVA					
Brightness	Locality		14.75	2	**<0.001**
	Body region		180.11	2	**<0.001**
	Locality:Body region		2.35	4	**0.051**
Hue	Locality		8.76	2	**<0.001**
	Body region		17.92	2	**<0.001**
	Locality:Body region		3.20	4	**0.012**
Chroma	Locality		10.89	2	**<0.001**
	Body region		439.81	2	**<0.001**
	Locality:Body region		2.40	4	**0.05**

Table 2. *P*-values of post-hoc Tukey HSD comparisons of colourimetric variables among the three localities of *Phyllobates vittatus*. Significant effects (*P*≤ 0.05) are highlighted in bold.

		Dorsum		Stripes		Hind limbs	
Colour variables	Locality	AB	LT	AB	LT	AB	LT
Brightness	LT	0.997		0.999		0.376	
	PI	0.738	0.790	**0.003**	**0.003**	**0.003**	**<0.0001**
Hue	LT	**0.001**		0.966		0.936	
	PI	0.483	**<0.0001**	0.997	0.943	0.200	0.105
Chroma	LT	0.995		0.624		0.795	
	PI	0.917	0.878	**<0.0001**	**0.002**	0.061	**0.012**

AB= Agua Buena, LT= La Tarde, PI= Piro

Table 3. *P*-values of post-hoc Tukey HSD comparisons of colourimetric variables among the body regions of *Phyllobates vittatus*. Significant effects (*P*≤ 0.05) are highlighted in bold.

		Agua Buena		La Tarde		Piro	
Colour variables	Body region	Dorsum	Stripes	Dorsum	Stripes	Dorsum	Stripes
Brightness	Stripes	**<0.0001**		**<0.0001**		**<0.0001**	
	Hind limbs	0.254	**<0.0001**	**0.011**	**<0.0001**	0.556	**<0.0001**
Hue	Stripes	0.835		**0.015**		0.211	
	Hind limbs	**0.0002**	**0.0012**	0.715	**0.001**	**0.0008**	0.104
Chroma	Stripes	**<0.0001**		**<0.0001**		**<0.0001**	
	Hind limbs	0.183	**<0.0001**	0.065	**<0.0001**	0.995	**<0.0001**

Table 4. Mixed effect model results for colour (ΔS) and brightness (ΔL) contrast within frogs, and between colour patches. Significant effects ($P \leq 0.05$) are highlighted in bold.

	Factors	Predator	X^2	*d.f.*	*P*
ΔS	Contrast type	Bird	684.99	2	**<0.001**
		Lizard	824.55	2	**<0.001**
		Crab	588.33	2	**<0.001**
	Locality	Bird	0.51	2	0.78
		Lizard	0.91	2	0.63
		Crab	2.58	2	0.27
ΔL	Contrast type	Bird	25.67	2	**<0.001**
		Lizard	24.16	2	**<0.001**
		Crab	39.80	2	**<0.001**
	Locality	Bird	13.12	2	**0.001**
		Lizard	13.05	2	**0.001**
		Crab	13.45	2	**0.001**
	Contrast type:Locality	Bird	37.06	4	**<0.001**
		Lizard	36.846	4	**<0.001**
		Crab	37.97	4	**<0.001**

Table 5. *P*-values of pairwise comparisons between body regions and between localities, for colour (ΔS) and brightness (ΔL) contrasts, under the visual models of three potential predators. Significant effects ($P \leq 0.05$) are highlighted in bold.

	Predator	Bird		Lizard		Crab	
	Comparison	ΔS	ΔL	ΔS	ΔL	ΔS	ΔL
Body region							
Dorsum.Hind limbs	AB - LT	0.86	0.859	0.703	0.854	0.484	0.860
	AB - PI	0.777	0.113	0.675	0.107	0.279	0.100
	LT - PI	0.99	0.319	0.999	0.309	0.935	0.291
Stripes.Dorsum	AB - LT	0.86	0.902	0.703	0.899	0.484	0.925
	AB - PI	0.777	0.995	0.675	0.996	0.279	0.999
	LT - PI	0.99	0.934	0.999	0.929	0.935	0.928
Stripes.Hind limbs	AB - LT	0.86	0.759	0.703	0.765	0.484	0.596
	AB - PI	0.777	**<0.0001**	0.675	**<0.0001**	0.279	**<0.0001**
	LT - PI	0.99	**<0.0001**	0.999	**<0.0001**	0.935	**<0.0001**
Locality							
Agua Buena	DO.HL - ST.DO	**<0.0001**	**0.004**	**<0.0001**	**0.005**	**<0.0001**	**0.000**
	DO.HL - ST.HL	**<0.0001**	0.189	**<0.0001**	0.203	**<0.0001**	**0.033**
	ST.DO - ST.HL	0.2	0.292	0.361	0.312	0.114	0.324
La Tarde	DO.HL - ST.DO	**<0.0001**	**0.0081**	**<0.0001**	**0.0104**	**<0.0001**	**0.0011**
	DO.HL - ST.HL	**<0.0001**	0.984	**<0.0001**	0.991	**<0.0001**	0.809
	ST.DO - ST.HL	0.2	**0.013**	0.361	**0.0151**	0.114	**0.008**

Piro	DO.HL - ST.DO	**<0.0001**	0.645	**<0.0001**	0.727	**<0.0001**	0.303
	DO.HL - ST.HL	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	ST.DO - ST.HL	0.2	**<0.0001**	0.361	**<0.0001**	0.114	**<0.0001**

AB= Agua Buena, LT= La Tarde, PI= Piro, ST= stripes, DO= dorsum, HL= hind limbs

Table 6. Mixed effect model results for frogs overall conspicuousness. Significant effects ($P \leq 0.05$) are highlighted in bold.

Factors	Predator	X^2	*d.f.*	*P*
Substrate	Bird	78.66	3	**<0.001**
	Lizard	99.85	3	**<0.001**
	Crab	104.15	3	**<0.001**
Locality	Bird	9.05	2	**0.011**
	Lizard	7.32	2	**0.026**
	Crab	9.16	2	**0.010**
Substrate:Locality	Bird	123.42	6	**<0.001**
	Lizard	127.60	6	**<0.001**
	Crab	124.70	6	**<0.001**

Table 7. *P*-values of pairwise comparisons between substrates and between localities for the overall conspicuousness of *Phyllobates vittatus*, under the visual models of three potential predators. Significant effects ($P \leq 0.05$) are highlighted in bold.

	Comparison	Bird	Lizard	Crab
Substrate				
Leaf litter	AB - LT	0.999	0.981	0.999
	AB - PI	**<0.0001**	**<0.0001**	**<0.0001**
	LT - PI	**<0.0001**	**<0.0001**	**<0.0001**
Rock	AB - LT	0.698	0.772	0.787
	AB - PI	0.921	0.999	0.905
	LT - PI	0.899	0.781	0.966
Soil	AB - LT	0.998	0.985	0.989
	AB - PI	**<0.0001**	**0.0001**	**<0.0001**
	LT - PI	**<0.0001**	**<0.0001**	**<0.0001**
Trunk	AB - LT	0.661	0.741	0.736
	AB - PI	0.987	0.879	0.954
	LT - PI	0.559	0.441	0.551
Locality				
Agua Buena	LL - RO	**<.0001**	**<.0001**	**<.0001**
	LL - SO	0.472	1	0.989
	LL - TR	**<.0001**	**<.0001**	**<.0001**
	RO - SO	**0.0007**	**<.0001**	**<.0001**
	RO - TR	0.131	0.358	0.058
	SO - TR	**<.0001**	**<.0001**	**<.0001**
La Tarde	LL - RO	**<.0001**	**<.0001**	**<.0001**
	LL - SO	0.589	1	0.999
	LL - TR	**<.0001**	**<.0001**	**<.0001**
	RO - SO	**<.0001**	**<.0001**	**<.0001**
	RO - TR	0.119	0.333	**0.048**
	SO - TR	**<.0001**	**<.0001**	**<.0001**

Piro	LL - RO	**0.013**	**0.032**	**0.038**
	LL - SO	0.839	0.993	0.977
	LL - TR	0.262	0.185	0.482
	RO - SO	**0.001**	0.064	**0.012**
	RO - TR	0.601	0.879	0.585
	SO - TR	**0.042**	0.299	0.260

AB= Agua Buena, LT= La Tarde, PI= Piro, LL= leaf litter, RO= rock, SO=soil, TR= trunk

FIGURES

Fig. 1 Individuals *Phyllobates vittatus* from Piro, South Pacific of Costa Rica. Photo credit: Manuel Sánchez.

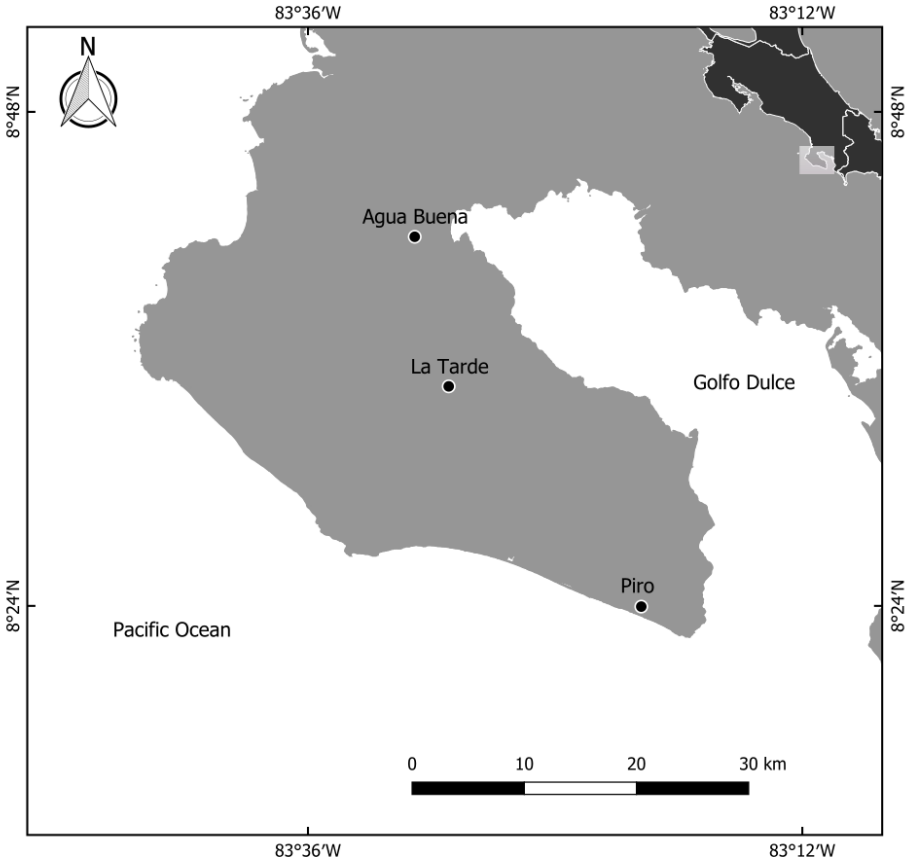

Fig. 2 Sampling localities of *Phyllobates vittatus* in the South Pacific of Costa Rica.

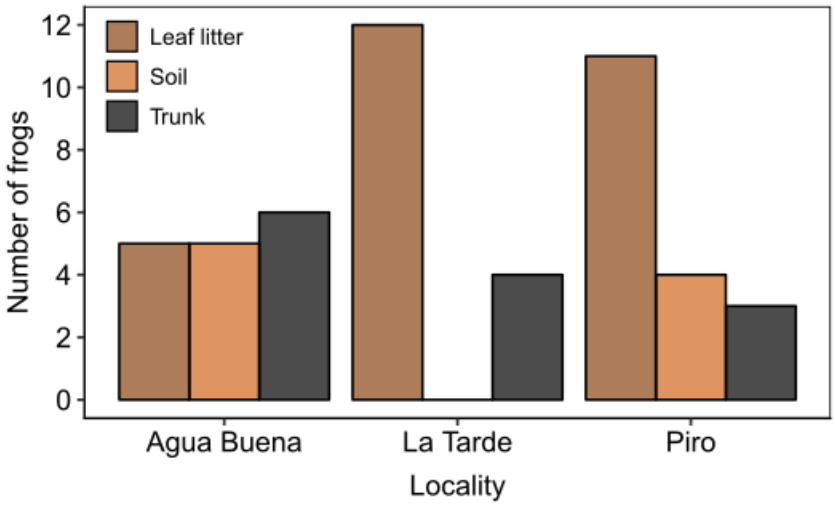

Fig. 3 Number of individuals *Phyllobates vittatus* found on each substrate and sampling locality.

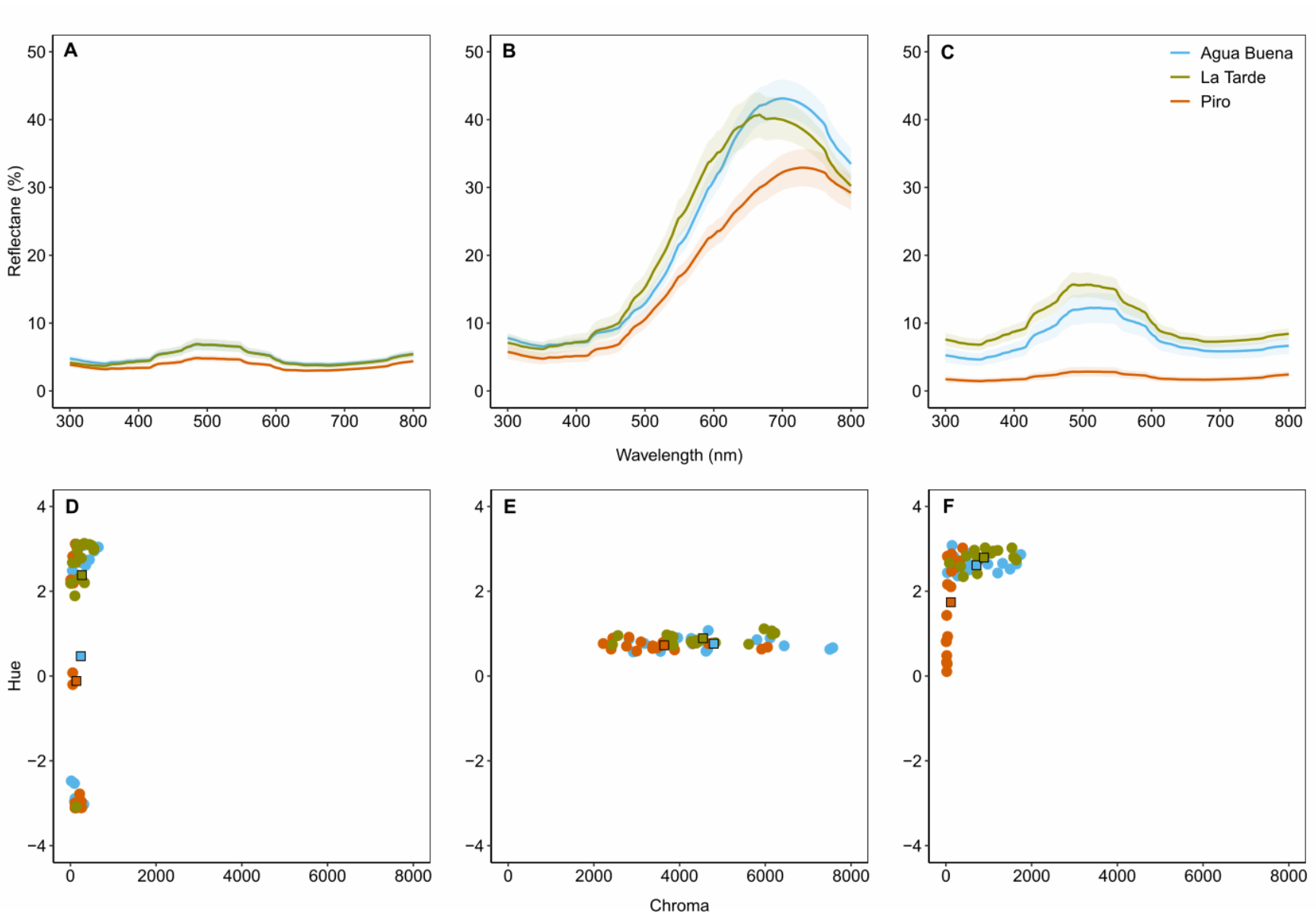
A
B
C
D
E
F
Reflectane (%)
Wavelength (nm)
Hue
Chroma
Agua Buena
La Tarde
Piro

Fig. 4 Reflectance spectra (**A, B, C**) and colourimetric variables (**D, E, F**) of dorsum (**A, D**), stripes (**B, E**) and hind limbs (**C, F**) of *Phyllobates vittatus* from three localities (colours). Solid lines in the reflectance spectra represents the mean of frogs' body region measured in a specific locality, while the shaded area represents the standard error. In **D-F**, circles represent the individuals, and squares represent the mean of sampled individuals at each locality.

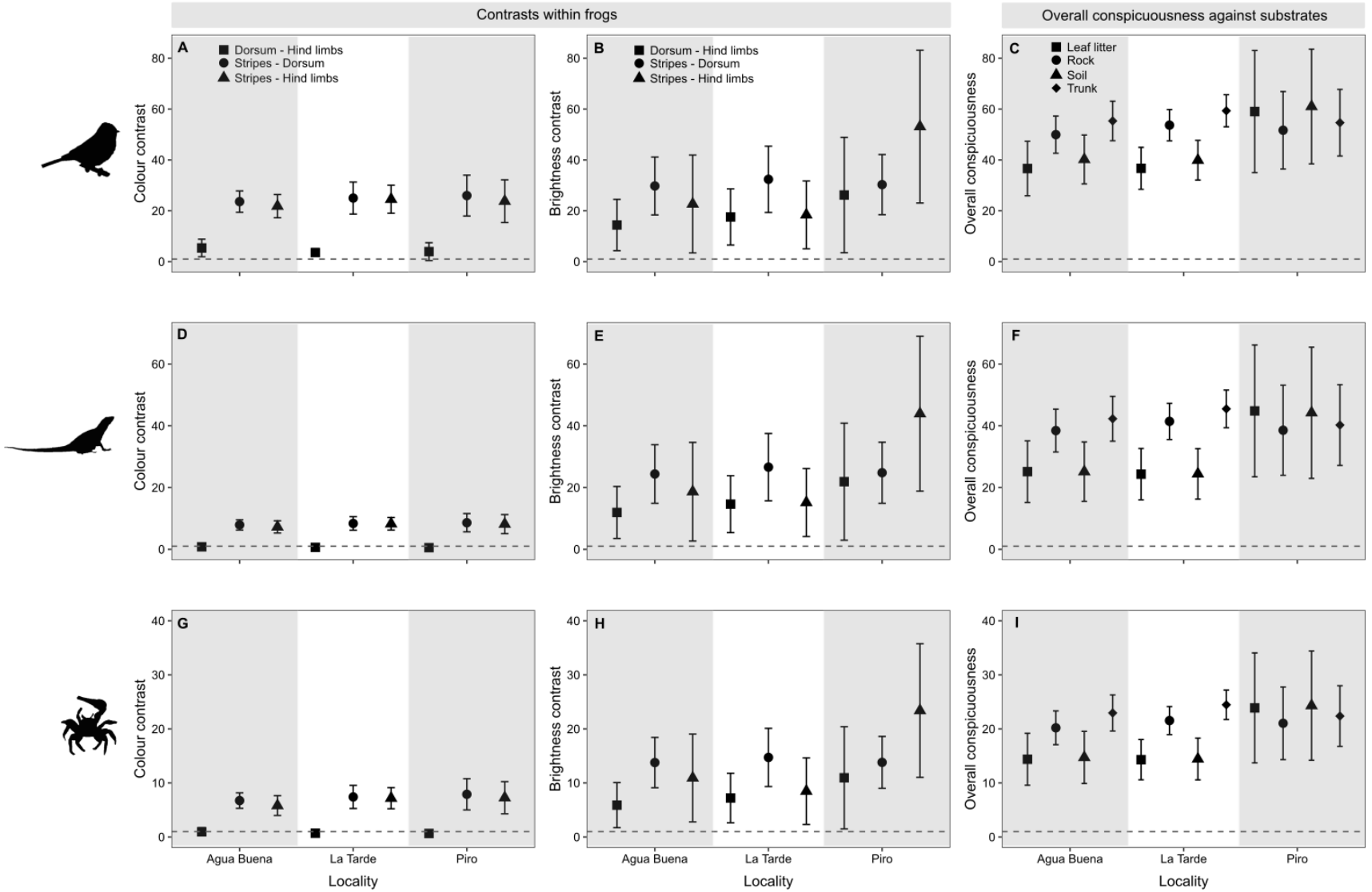
Contrasts within frogs
Overall conspicuousness against substrates
A
B
C
D
E
F
G
H
I
Dorsum - Hind limbs
Stripes - Dorsum
Stripes - Hind limbs
Leaf litter
Rock
Soil
Trunk
Colour contrast
Brightness contrast
Overall conspicuousness
Agua Buena
La Tarde
Piro
Locality

Fig. 5 Conspicuousness of *Phyllobates vittatus* from three localities for the visual systems of potential predators: birds (**A, B, C**), lizards (**D, E, F**) and crabs (**G, H, I**). The shapes represent means and the bars show the standard deviation. Colour and brightness contrasts (**A, B, D, E, G, H**) were estimated within the frogs, between body regions (shapes). Overall conspicuousness (**C, F, I)** was estimated against the most common natural backgrounds (shapes). The units of the model are just noticeable differences (jnd), where values above 1 (dashed horizontal line) indicate that the two colours are distinguishable for the viewer; the higher the value, a more rapid discrimination is achieved (Siddiqi et al., 2004).

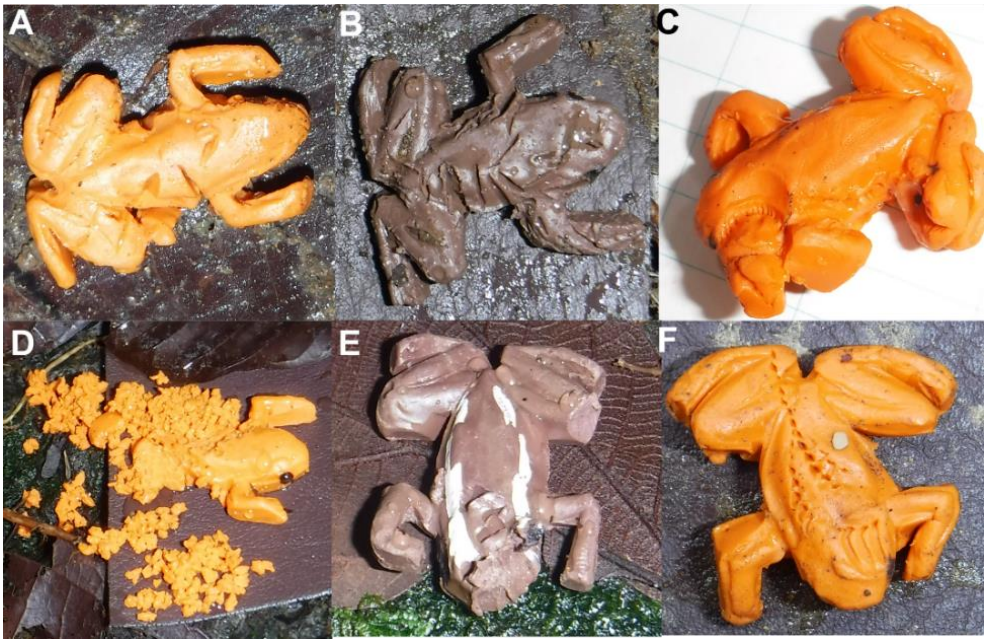

Fig. 6 Examples of marks made in clay models by the different assailants. **A.** Bird. **B.** Crab. **C.** Lizard. **D.** Insect. **E.** Mammal (rodent). **F.** Unknown.

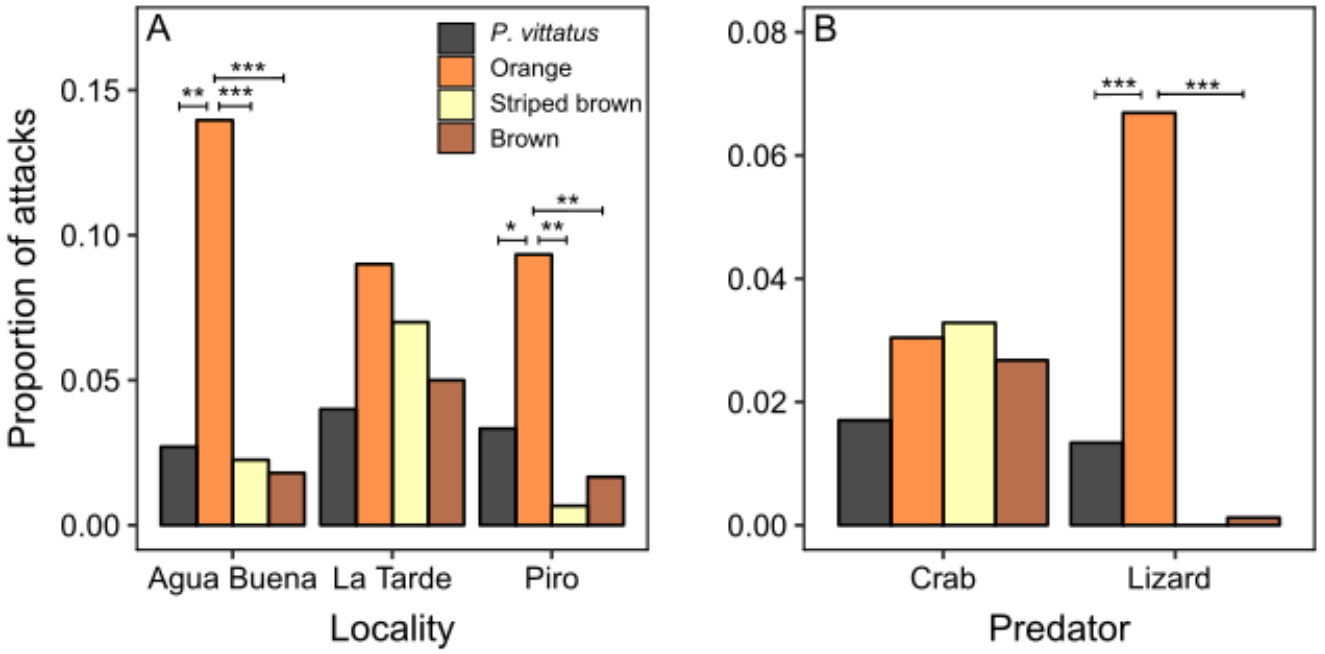

Fig. 7 Proportion of predation attempts on four types of frog clay models. **A.** Proportion of predation attempts by all predators combined (birds, lizards and crabs) on clay models at three localities. **B.** Proportion of predation attempts by the two main predators (crabs and lizards). Lines above the bars represent statistical significant differences based on pairwise comparisons from the statistical models: **P*<0.05, ***P*<0.01, ****P*<0.001. For **B**, the "striped brown" model was excluded from pair-wise comparisons within reptile attacks, given that there were no predation attempts, and hence no associated variance estimates.

SUPPORTING INFORMATION

Table S1 *Post-hoc* Tukey HSD comparisons between localities means in snout-to-vent length (SVL, mm). Significant effects (*P*≤ 0.05) are highlighted in bold.

Comparison	Differences between means of the groups	Lower CI 95%	Upper CI 95 %	Adjusted *P*-value
La Tarde - Agua Buena	-0.059	-1.453	1.335	0.994
Piro - Agua Buena	-1.745	-3.099	-0.391	**0.008**
Piro - La Tarde	-1.686	-3.061	-0.311	**0.012**

Table S2 Visual sensitivity data of the predators used for the visual modelling

Predator		UV/VS	SWS	MWS	LWS
[1]Bird					
Cyanistes caeruleus	n	1	1.171	2.14	1.89
	weber				0.05
	cone sensitivity	372	449	502	563
[2]Lizard					
Anolis sagrei	n		1	1	3
	weber				0.06
	cone sensitivity	365	460	495	567
[3]Crab					
Uca tangeri / U. thayeri	n		1		1
	weber*				0.12
	cone sensitivity		430		590

UV/VS= UV-wavelength-sensitive cone, SWS=short-wavelength-sensitivity cone, MWS= medium-wavelength-sensitivity cone, LWS= long-wavelength-sensitivity cone.

n= photoreceptor densities.

weber=weber.achro= Weber fraction.

Cone sensitivity=peak cone sensitivity (nm).

*For *Apis mellifera* (Vorobyev, Brandt, Peitsch, Laughlin, & Menzel 2001) following Cummings, Jordão, Cronin, & Oliveira (2008).

References: [1]Hart (2001); [2]Loew, Fleishman, Foster, & Provencio (2002), Fleishman, Perez, Yeo, Cummings, Dick, & Almonte (2016); [3]Cummings et al. (2008).

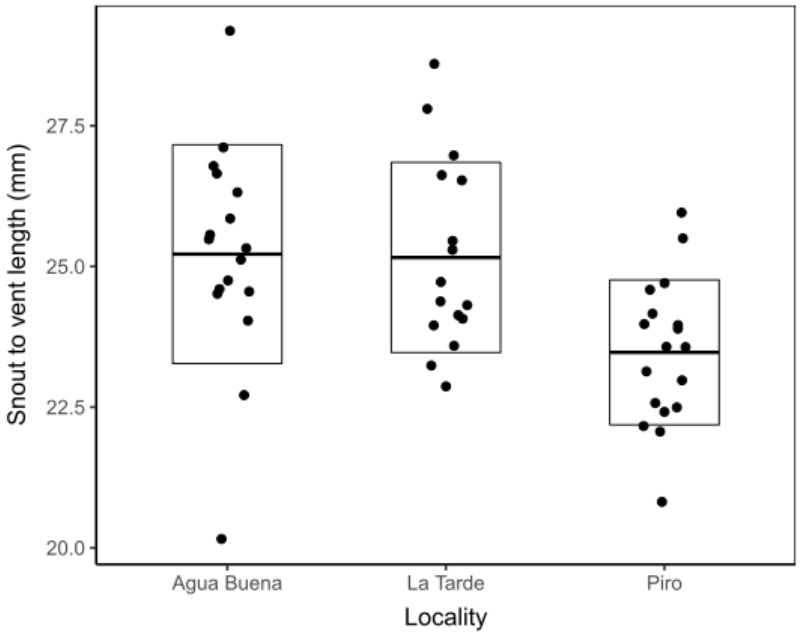

Fig. S1 Snout-to-vent length (SVL, mean and standard deviation) of *Phyllobates vittatus* in three localities. Points represent each individual. SVL was different among localities ($F_{2,48}$= 6.269, P= 0.003). Frogs from Piro were significantly smaller than frogs from Agua Buena and La Tarde (Table S1).

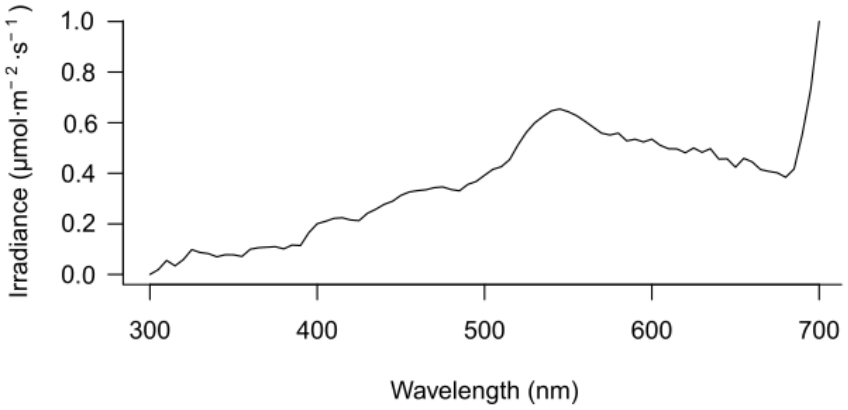

Fig. S2 Forest shade irradiance from Endler (1993) and used in the visual modelling.

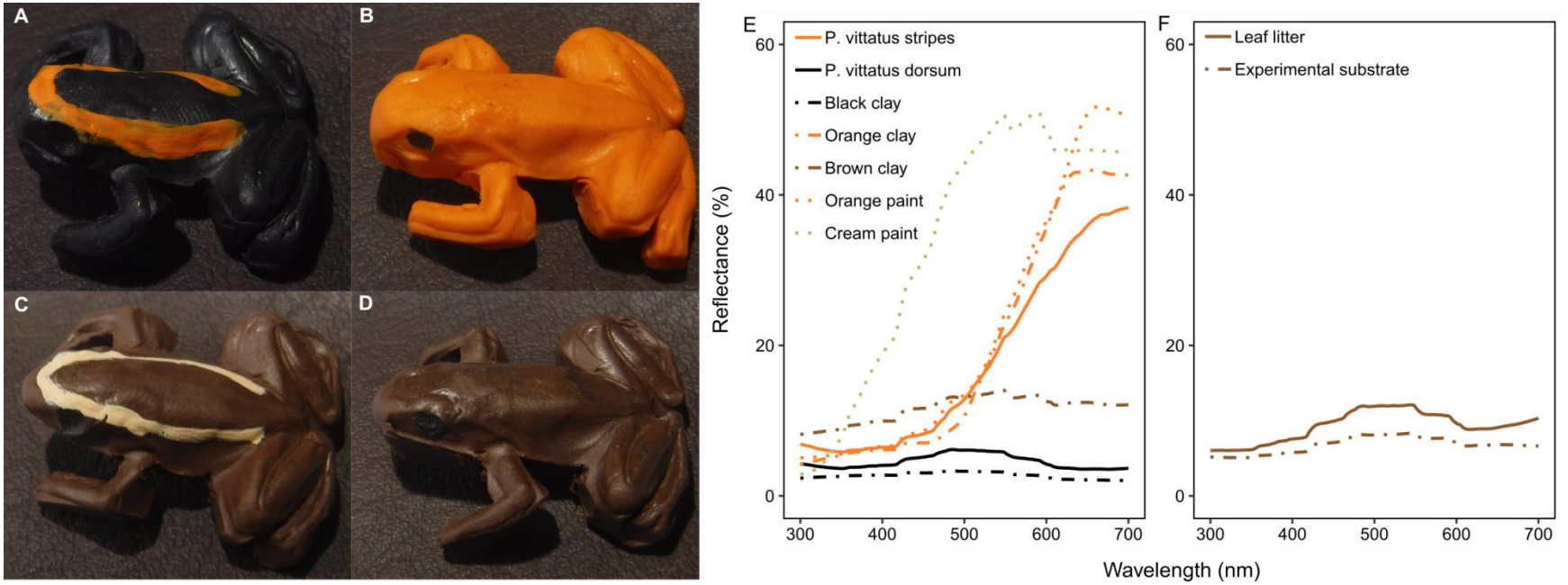

Fig. S3 Clay models used for the predation experiment on the experimental substrate (**A.** *P. vittatus*, **B.** orange, **C.** striped brown, and **D.** brown model), reflectance spectra of clay models (**E**) and experimental substrate (**F**) compared with natural colours (solid lines). For models construction, we followed Willink et al. (2014): We used BACO® non-toxic plasticine clay and modelled it on a putty material mould shaped by a preserved *P. vittatus* specimen. Then we cut the rests of clay with a scalpel, painted the stripes of the *P. vittatus* and brown striped models (**A, C**) with Lanco® mate paint, and drew the eyes in all models with a black Sharpie® marker.

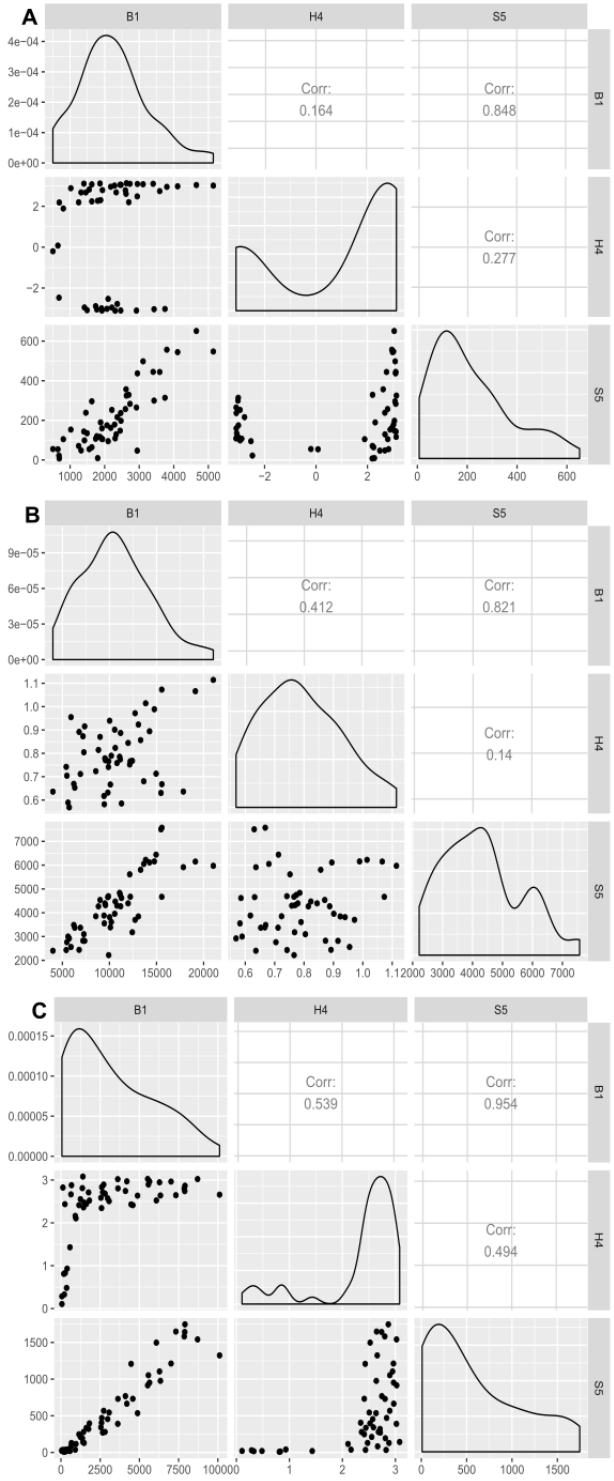

Fig. S4 Correlation matrix for colourimetric variables of Phyllobates vittatus body regions. **A.** Dorsum, **B.** Stripes, **C.** Hind limbs. B1= brightness, H4= hue, S5= chroma.

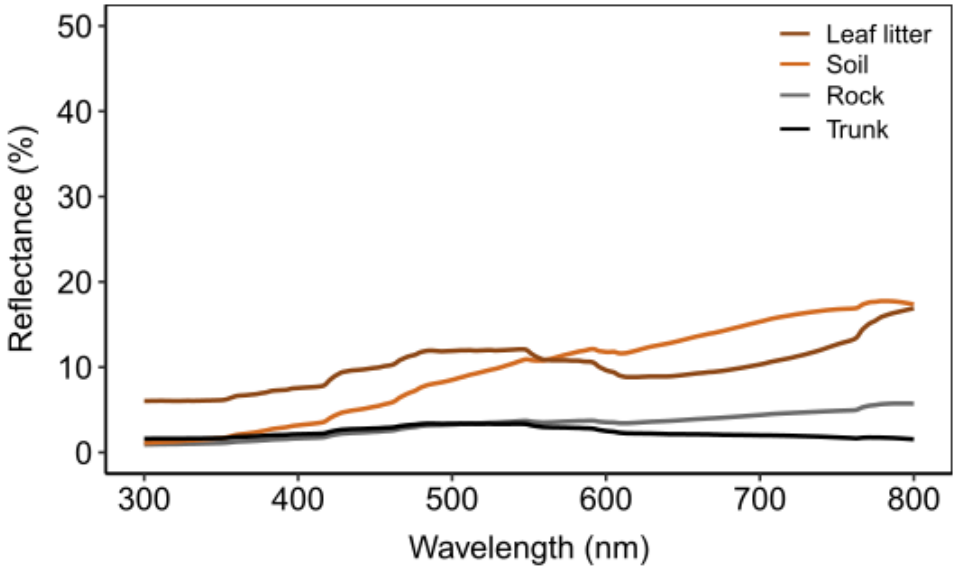

Fig. S5 Reflectance curves of the substrates where frogs were initially found. These substrates were used for calculating frogs overall conspicuousness in the visual modelling.

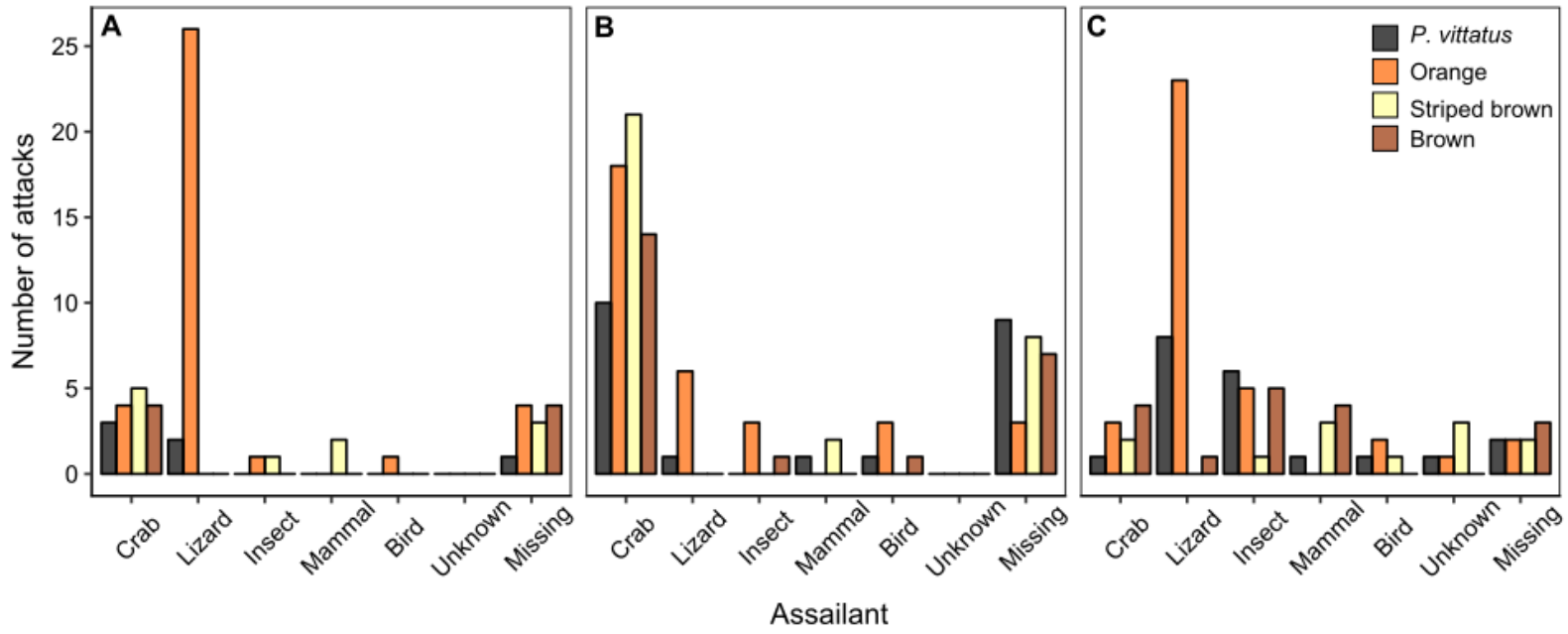

Fig. S6 Number of attacked clay models on each locality (**A.** Agua Buena, **B.** La Tarde, **C.** Piro) according to model type and the assailant to which the attack was assigned.

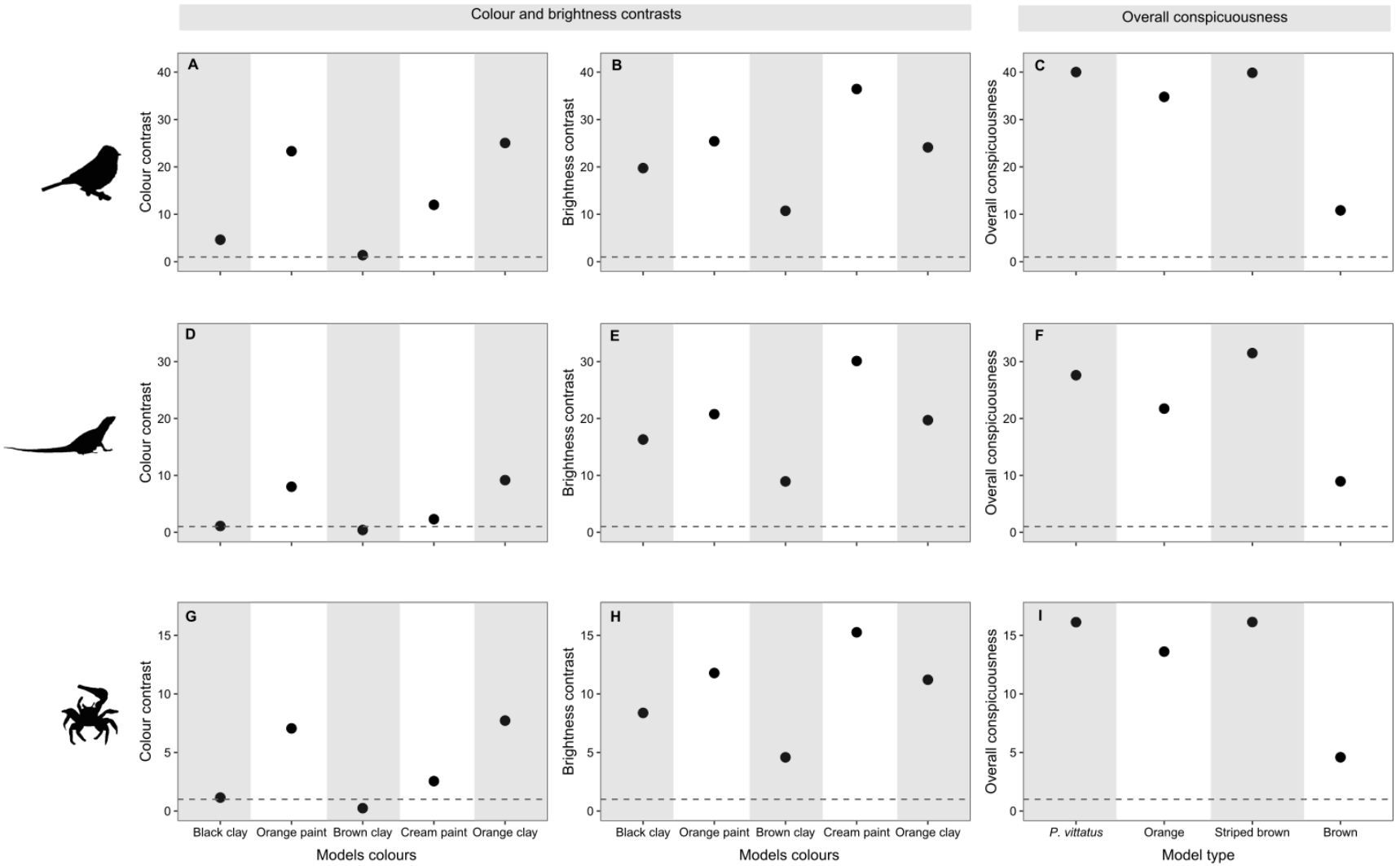
Colour and brightness contrasts
Overall conspicuousness
A
B
C
D
E
F
G
H
I
Colour contrast
Brightness contrast
Overall conspicuousness
Black clay
Orange paint
Brown clay
Cream paint
Orange clay
Models colours
P. vittatus
Orange
Striped brown
Brown
Model type

Fig. S7 Conspicuousness for the visual systems of potential predators: birds (**A, B, C**), lizards (**D, E, F**), and crabs (**G, H, I**), of clay models and paint, against the experimental substrate used for the predation experiment. Colour (**A, D, G**) and brightness (**B, E, H**) contrasts are presented for the clay and paint used for model fabrication. Overall conspicuousness (**C, F, I**) refers to the Euclidean distance between colour and brightness contrasts of the colours used in the clay model (see Fig. S3). The units of the model are just noticeable differences (jnd), where values above 1 (dashed horizontal line) indicate that the two colours are distinguishable for the viewer; the higher the value, a more rapid discrimination is achieved (Siddiqi et al., 2004).

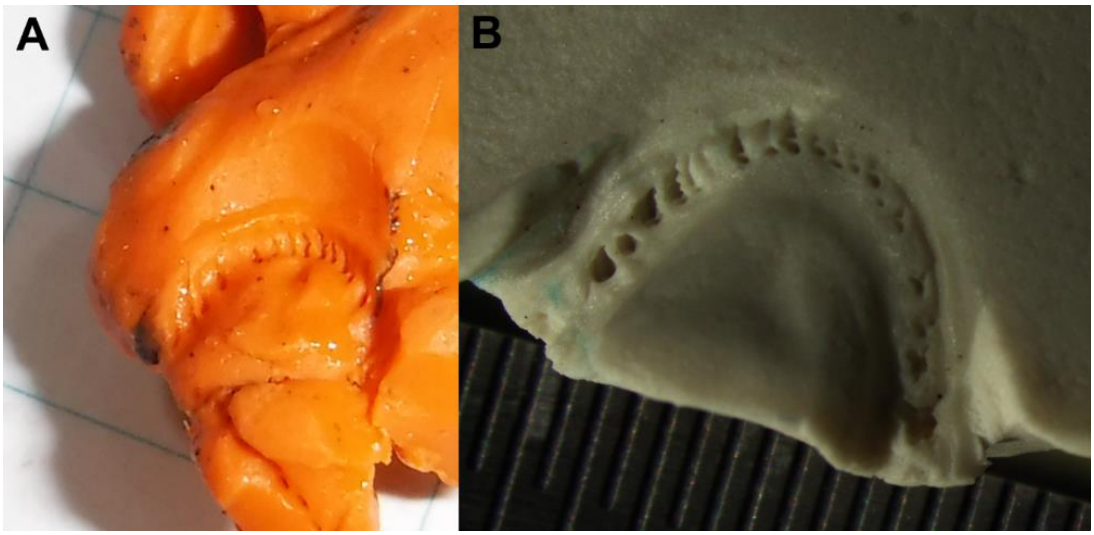

Fig. S8 Example of a lizard mark on a clay model in the field (**A**), and the mark imprinted on clay from a preserved specimen of *Basiliscus basiliscus* from the Zoology Museum at the University of Costa Rica (**B**). Pictures are not in the same scale. Comparing marks on clay models in the field with marks from preserved specimens could be an important tool to objectively identify the assailants. However, this method must be properly standardized in order to be reproducible.

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