UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS
INSTITUTO DE ECOLOGÍA

GENÓMICA DEL PAISAJE Y ECOFISIOLOGÍA DE RANAS DEL GÉNERO *Craugastor* EN UN GRADIENTE ALTITUDINAL

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

RUTH PERCINO DANIEL

TUTOR PRINCIPAL DE TESIS:	**DR. DANIEL PIÑERO DALMAU** INSTITUTO DE ECOLOGÍA, UNAM
CÓMITE TUTOR:	**DR. ALEJANDRO GONZALEZ VOYER** INSTITUTO DE ECOLOGÍA, UNAM **DR. FAUSTO ROBERTO MÉNDEZ DE LA CRUZ** INSTITUTO DE BIOLOGÍA, UNAM

CIUDAD UNIVERSITARIA, CIUDAD DE MÉXICO, JUNIO DE 2023

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M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
Presente

Me permito informar a usted que, en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada del día **13 de febrero de 2023** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **PERCINO DANIEL RUTH** con número de cuenta: **505017277** con la tesis titulada **"GENÓMICA DEL PAISAJE Y ECOFISIOLOGÍA DE RANAS DEL GÉNERO *Craugastor* EN UN GRADIENTE ALTITUDINAL".** Bajo la dirección del **DR. DANIEL IGNACIO PIÑERO DALMAU**, Tutor Principal, quedando integrado de la siguiente manera**:**

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Sin otro particular, me es grato enviarle un cordial saludo.

ATENTAMENTE
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 12 de mayo de 2023

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

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Para Sarabia
Sé que estarías feliz

Índice

Resumen

Entender cómo los factores bióticos y abióticos influyen en la variabilidad fenotípica y genotípica de los organismos es uno de los desafíos de la biología evolutiva y ecología. En ectotermos, la temperatura juega un papel fisiológico importante, determinando no sólo su temperatura corporal sino influyendo en varios procesos vitales para su desempeño. Aunado a esto, la humedad y la disponibilidad de agua determinan el balance hídrico de los anfibios que junto a la temperatura, influyen en su desempeño y reproducción. Temperatura y precipitación pueden influir en la conectividad de las poblaciones a nivel de paisaje, lo cual podría limitar el flujo génico y generar un aislamiento genético, tal como aislamiento por ambiente. Este patrón puede presentarse en ambientes heterogéneos, como lo es un gradiente de elevación. En este trabajo se evaluó el papel de factores bióticos y abióticos (temperatura, humedad y elevación) en la diferenciación genómica y rasgos fisiológicos de poblaciones de la rana del Volcán San Martín *Craugastor loki*. Esta especie se distribuye en un amplio gradiente altitudinal (120 - 2200 msnm) en el sureste de México. En los capítulos I y II se estudiaron los requerimientos térmicos e hídricos de *C. loki*, a lo largo de un gradiente altitudinal en cinco unidades ambientales, definidas por temperatura y precipitación. Se encontró que el limite térmico máximo fue variable a lo largo del gradiente altitudinal y entre los dominios bioclimáticos, disminuyendo a medida que aumenta la elevación. Las poblaciones de las tierras bajas son más vulnerables a futuros aumentos de temperatura. En el capítulo II se utilizaron modelos de agar para estimar la pérdida de agua por evaporación y la temperatura operativa a lo largo de un gradiente de elevación de *C. loki*. Se encontró que el porcentaje de pérdida de agua por evaporación fue mayor en la zona menor altitud y el resto de las localidades fue similar. Se sugiere que la humedad relativa durante la temporada de lluvias es relativamente constante, generando condiciones que permiten el balance hídrico entre las ranas y su hábitat, lo que amortigua las condiciones térmicas. El capítulo III se refiere a la genómica del paisaje de las poblaciones muestreadas a lo largo del gradiente de elevación. Se utilizó secuenciación de ADN asociada a sitios de restricción. Se encontró que las poblaciones muestreadas en elevaciones más altas (> 1500 m), probablemente corresponden a una nueva especie de *Craugastor* ya que corresponden a un clado genéticamente muy divergente y que las poblaciones de *C. loki* entre 120 m y 1500 m están estructuradas en dos grupos genéticos: vertiente del Pacífico y la Depresión Central. La estructura de la población está influenciada por la interacción entre la elevación y la precipitación, pero también por la temperatura, lo que enfatiza el papel del aislamiento por ambiente a una escala fina.

Abstract

A challenge for ecology and evolutionary biology is understanding how different biotic and abiotic environmental factors can influence the phenotype and genome expression. In ectothermic organisms, temperature plays a key role because it not only determines body temperature but also influences a variety of processes important for their performance and reproduction. Nonetheless, amphibian species must deal with water balance for their performance in addition to temperature. Temperature and precipitation can influence on population connectivity of the landscape, which can limit the gene flow and produce genetic isolation, such as isolation by environment. This pattern can be present in heterogeneous environments, such as an elevation gradient. In this work I evaluated the role of biotic and abiotic factors (temperature, humidity and elevation) on the population differentiation and physiological traits of the populations of the Volcan San Martin frog´s *Craugastor loki.* This species is widely distributed along the elevation gradient (120 – 2200 m) at the southern Mexico. In the chapters I and II I studied the thermal and hydric sensitivities of *C. loki* along an elevation gradient in five bioclimatic domains, defined as an environmental space that integrates bioclimatic variables of temperature and precipitation. I found that the critical thermal maximum is variable across elevations and among the bioclimatic domains, decreasing as elevation increases. The populations from the lowlands may be more vulnerable to future increases in temperature. For my second chapter, I used physical agar frog models to estimate evaporative water loss (EWL) and operative temperature across an elevation gradient experienced by *C. loki*. I found that the percent of EWL was similar across the elevation gradient, but it was higher in lower elevation (248m) compared with the rest localities of the gradient. The results suggest that the similarity of relative humidity during the rainy season produces moist conditions allowing the water balance between frogs and its habitat that buffers the thermal conditions to which the frogs are exposed. In chapter III, I studied the landscape genomics of populations across the elevation gradient. I used restriction-site associated DNA sequencing. I found that populations sampled at higher elevation (above 1500m) correspond to a different and undescribed new species of *Craugastor* because they represent a clade highly genetically divergent, and that populations from *Craugastor loki* between 120 m and 1500 m are clustered in two different genetic groups: a Pacific slope group and a Central Depression slope group. I found population structure to be influenced by the interaction between elevation and precipitation, but also by temperature. The results highlight the role spatial analyses can play in illuminating fine scale population structure.

Introducción

Uno de los desafíos en biología evolutiva y ecología, es entender como los factores bióticos y abióticos influyen en la variabilidad fenotípica y genotípica de los organismos. Entre los factores bióticos, están los relacionados a las características de historia de vida e interacciones ecológicas. Mientras que los factores abióticos, tal como la geografía y el ambiente pueden jugar un papel importante en la conectividad de las poblaciones a nivel de paisaje. Los factores abióticos generan gradientes ambientales lo cuales pueden ser latitudinales o altitudinales presentando marcadas diferencias ambientales. Estas diferencias pueden a su vez influir en las poblaciones que están distribuidas de una forma continua, promoviendo su divergencia o diferenciación (Savolainen et al. 2013, Muir et al. 2014). Ello se puede reflejar en los caracteres fenotípicos y en los genotípicos que están sujetos a selección divergente, produciendo por una parte aislamiento reproductivo y escaso flujo génico entre las poblaciones (Keller et al. 2013). Por lo anterior, el estudio de poblaciones a lo largo de un gradiente altitudinal provee un buen sistema para estudiar procesos de adaptación local e identificar los factores que promueven o previenen diferenciación adaptativa (Luquet et al. 2015).

En un gradiente altitudinal existe generalmente una marcada heterogeneidad ambiental dada principalmente por la temperatura y humedad. Estos parámetros, para el caso de organismos ectotermos como los anfibios, influyen en todos sus rasgos del ciclo de vida como el crecimiento, la dispersión, la sobrevivencia y la reproducción. Por ejemplo, en algunas poblaciones de anfibios, el gradiente de altitud puede restringir la dispersión de individuos y por lo tanto el flujo génico (Funk et al. 2006). Mientras que en términos fisiológicos, debido a cambios en la temperatura, para algunos individuos puede ser una limitante descender hacia altitudes menores o en el caso de individuos de tierras bajas ascender a altitudes mayores, además de que pueden o no existir barreras pre-apareamiento de flujo génico,por ejemplo, bajo éxito en reproducirse debido a diferencias en la temporada reproductiva de zonas bajas con respecto a zonas altas. Además de diferencias a lo largo del gradiente de elevación, en la selección de caracteres sexuales como pueden ser los llamados acústicos (Funk et al. 2016).

Los anfibios en ambientes terrestres deben enfrentar la pérdida de agua por evaporación, para lo cual presentan distintas adaptaciones funcionales, estructurales, fisiológicas y de comportamiento para reducir la pérdida de agua (Wells 2007). Algunas especies, sin embargo, están restringidos a ocupar microhábitats húmedos debido a la incapacidad para regular la pérdida de agua (Wells 2007). La tasa de pérdida de agua por evaporación se puede considerar como una medida para evaluar la termorregulación en anfibios (Vitt & Caldwell 2014), la cual se define como una función del gradiente de densidad de vapor entre el organismo y su ambiente. El gradiente de densidad de vapor es una propiedad física del ambiente y no puede ser modificado por el animal, a menos de que éste se mueva a un nuevo microhábitat. El gradiente puede aumentar conforme se incrementa la temperatura corporal de un anfibio o conforme la humedad del ambiente decrece (Wells 2007). Asimismo, el tamaño del organismo puede influir en la tasa de deshidratación; organismos de tallas grandes pueden tener una mayor resistencia a la pérdida de agua comparados con los de tamaño pequeño, incluso si todas las condiciones son iguales (Spotila 1972).

Además de la pérdida de agua que enfrentan los anfibios, la variación sobre las sensibilidades térmicas puede estar asociada a clinas geográficas, por ejemplo: individuos de zonas tropicales pueden exhibir estrechos límites térmicos dado que están expuestos a una menor variabilidad climática (Figura 1A), contrario a los individuos que habitan zonas templadas (Figura 1C, Janzen 1976, Ghalambor et al. 2006). Los límites críticos térmicos son los parámetros que usualmente se utilizan para describir las sensibilidades térmicas y entender cómo un organismo puede responder a la heterogeneidad ambiental (Angilletta, 2009). Estos parámetros son la temperatura critica mínima (CT_{min}) y la temperatura crítica máxima (CT_{max}), que se refieren al límite máximo y mínimo de temperatura que el organismo puede tolerar sin tener pérdida de locomoción (Navas et al. 2007). Tanto el CT_{max} como el CT_{min} definen la amplitud térmica o rango térmico y en consecuencia el desempeño de los organismos. Así, estudiar el comportamiento de estos parámetros en distintas poblaciones a lo largo de un gradiente ambiental, podría asociarse potencialmente con la adaptación de los organismos a sus ambientes (Duarte et al. 2012, Gaitán-Espitia et al. 2014) o ser resultado de una amplia plasticidad fisiológica (Figura 2).

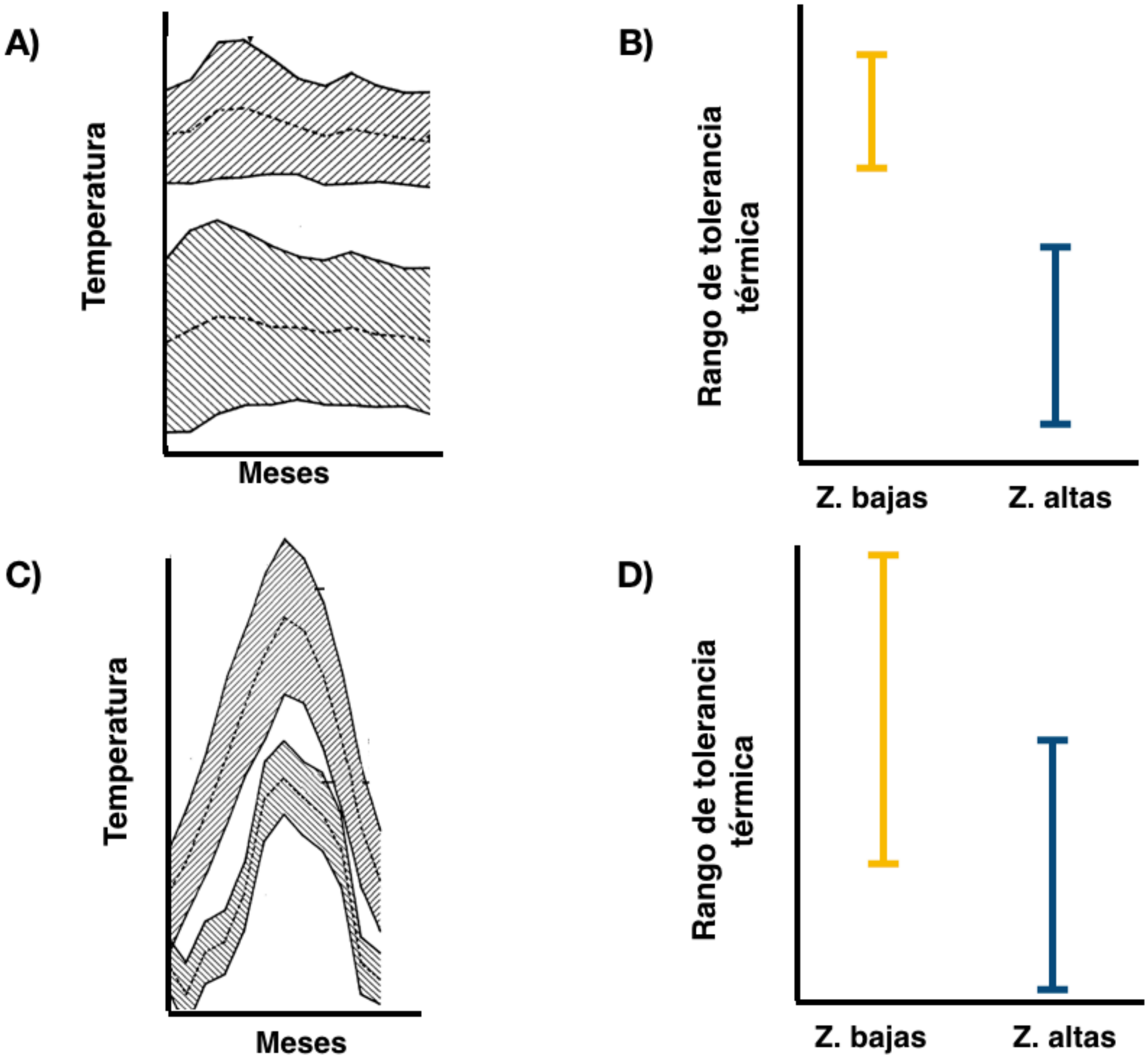

Figura 1. Representación esquemática de la hipótesis de variabilidad climática propuesta por Janzen (1967) en zonas tropicales (A-B) y en zonas templadas (C-D). Las zonas tropicales presentan una menor variabilidad climática, por lo que se se sugiere que los límites térmicos de los organismos en zonas tropicales serán estrechos (barras de color en panel B). En contraste con las zonas templadas (C) donde hay un sobrelapamiento entre las temperaturas de zonas altas y bajas, por lo cual, los límites térmicos serán más amplios y se sobrelapan (D). Esquema tomado de Ghalambor et al. (2006) y Muñoz & Bodensteiner (2019).

De acuerdo con la hipótesis de variabilidad climática (Janzen 1967), se sugiere que en especies tropicales los limites térmicos son estrechos (Figura 1B), comparados con especies de zonas templadas (Figura 1D), dado que estas especies están expuestas a una mayor variabilidad climática comparadas con las especies tropicales (Janzen, 1967). Está hipótesis ha sido ampliamente estudiada a escalas macroecológicas, mientras que a escala local como en un gradiente altitudinal, el conocimiento es aún limitado, en especial en zonas tropicales (Montejo-Kovacevich et al., 2020) donde las especies podrían ser más vulnerables a cambios climáticos. En un gradiente de elevación en zonas tropicales se esperaría que los organismos ectotérmicos, que viven en zonas bajas presenten limites térmicos estrechos, mientras que los que habitan zonas altas tengan límites térmicos más amplios (Ghalambor, 2006; Muñoz and Bodensteiner, 2019).

Paisaje y variación genética

Las características ambientales y geográficas del paisaje influyen en la estructura genética de las poblaciones; saber en qué medida ocurre esto es uno de los objetivos de la genética del paisaje (Balkenhol, et al., 2016). Por un lado, la geografía es un aspecto clave en un paisaje dado ya que ésta puede influir en la diferenciación poblacional y aislamiento reproductivo. Aún así, la geografía sólo representa uno de los componentes clave dentro del paisaje, ya que otro aspecto importante es el ambiente, el cual determina el movimiento de individuos y la conectividad poblacional, influyendo en el flujo génico (Bradburd et al., 2013).

Desde un enfoque de genómica del paisaje que permite la identificación de polimorfismos genómicos responsables de la variación, es posible detectar loci potencialmente sujetos a selección, y determinar si están asociados a adaptación local (Schwartz et al. 2010). Ambientes espacialmente heterogéneos pueden más comúnmente generar adaptación local, donde cada población local presente caracteres que permitan una mayor adecuación bajo condiciones ambientales locales. En este escenario, la selección natural puede ser suficientemente fuerte para eliminar alelos deletéreos en las poblaciones y causar que alelos ventajosos aumenten su frecuencia en un ambiente dado (Kawecki & Ebert 2004). Así, los organismos de las subpoblaciones sobrevivirán y se reproducirán mejor en su hábitat particular (Kingsolver & Pfenning 2007, 2014). Es decir, los genotipos residentes tienen una mejor adecuación en relación a los genotipos inmigrantes (Ronce 2014).

La restricción del flujo génico puede deberse, entre otros, por un aislamiento por distancia (IBD, por sus siglas en inglés) (Wright, 1943) y por aislamiento por ambiente (IBE por sus siglas en inglés, Figura 2) (Nosil, 2012). En el primer caso se refiere a que la divergencia genética incrementa con la la distancia geográfica, a mayor distancia, mayor diferenciación genética. Mientras que el segundo caso, se refiere a la relación entre la heterogeneidad ambiental y la variación espacial del flujo génico sobre un paisaje dado; es decir, en el IBE la diferenciación genética se incrementa con las diferencias ambientales, independientemente de la distancia geográfica (Wang & Bradburd 2014). El IBE es un proceso que ha tenido menos atención comparado con el IBD, dado que es difícil separar los efectos de ambos mecanismos. Sin embargo, examinando los efectos tanto de variables geográficas como ecológicas sobre el flujo génico entre las poblaciones y controlando la distancia geográfica, se puede evaluar cómo influye el paisaje en la variación genética (Orsini et al., 2013, Wang 2013).

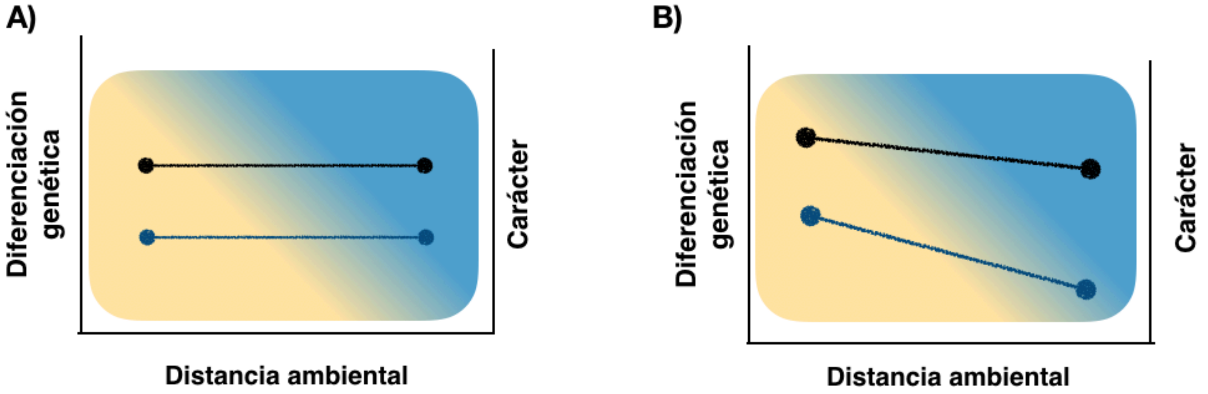

Figura 2. Representación hipótetica de la plasticidad de dos rasgos térmicos CT_{max} (línea negra) y C_{tmin} (línea azul) y del patrón de aislamiento por ambiente (IBE) representado en el eje *x* como distancia ambiental y en el eje *y* como distancia genética. Los colores representan dos poblaciones diferenciadas con ambientes diferentes. En el panel A, se observa que los rasgos fisiológicos no presentan un cambio en CT_{max} ni CT_{min} con los cambios en el ambiente (no hay plasticidad fenotípica). En el panel B, los rasgos cambian como resultado de un cambio en el ambiente (plasticidad fenotípica).

Otro patrón es el aislamiento por adaptación (IBA, por sus siglas en inglés) (Wang 2013), que se es dado por divergencia adaptativa, es decir aislamiento reproductivo y restricción al flujo génico (Nosil, 2012, Funk et al., 2016). En el IBA, el grado de divergencia fenotípica entre poblaciones está positivamente correlacionada con el grado de diferenciación genética (adaptativa), lo cual está determinado por la selección divergente que durante la especiación ecológica reduce el flujo génico entre poblaciones (Nosil 2012). Para demostrar que existe IBA se requiere controlar la distancia geográfica y potenciales barreras geograficas, tal como topografía que pueden restringir el flujo genético y por lo tanto promover divergencia fenotípica debido a deriva génica y/o selección divergente (Nosil 2012, Funk et al., 2005, 2016).

Por todo lo anterior, integrar un análisis cuantitativo de datos ecofisiológicos como son las sensibilidades térmicas y el balance de agua, en términos de tasas de deshidratación, en distintas poblaciones de ranas es un sistema de estudio que permite distinguir entre IBD e IBE. Además, con la información genética donde se evalúe la variación genética y el flujo génico podría ayudar a tener un mejor entendimiento de los procesos y/o patrones de divergencia fenotípica y genotípica que puedan estar operando y como los organismos podrían responder a un ambiente cambiante.

Especie de estudio

Las ranas del género *Craugastor* se caracterizan por ser altamente diversas y fenotípicamente polimórficas (Hedges et al. 2008, Padial et al. 2014). Se conocen 126 especies que habitan principalmente en Centroamérica (Amphibiaweb 2022), que se caracterizan por tener desarrollo directo y un patrón de coloración altamente variable en muchas de las especies. A pesar de la gran diversidad de especies, son escasos los estudios genéticos sobre éstas que incluyan parámetros poblacionales (Emel & Storer 2012). Se sugiere que, en especies de ranas de desarrollo directo, por ejemplo del género *Pristimantis* (Lynch & Duellman 1997), así como del género *Eleutherodactylus* (Elmer et al. 2007, Padial et al. 2014), los taxa hermanos han sido segregados altitudinal y latitudinalmente, ocupando diferentes tipos de vegetación contigua (por ej. gradientes), lo que podría ser resultado de especiación parapátrica. Tal es el caso del grupo de ranas *Craugastor augusti,* en donde se han descrito unidades evolutivas significativas (ESUs, por sus siglas en inglés) son parapátricas y presentan flujo génico restringido (Streicher et al. 2014a), lo que sugiere que la altitud (y sus variables ambientales asociadas) probablemente sea el factor que explique esta restricción. El efecto de la altitud sobre la estructura genética se ha encontrado por ejemplo en ranas del grupo Terrana (Gonzalez-Voyer et al. 2011).

Recientemente Streicher et al. (2014b) realizaron una revisión taxonómica del grupo *Craugastor rhodopis* que contiene dos especies, *Craugastor rhodopis* y *Craugastor loki*, con base en la diversidad nucleotídica del gen mitocondrial 12S y el patrón de coloración. Encontraron cuatro linajes: 1) el clado del oeste de México lo asignaron a *Craugastor occidentalis*, 2) el clado de tierras medias del este de México a *Craugastor rhodopis,* 3) el clado del este de México, Guatemala y El Salvador de tierras bajas a *Craugastor loki* y 4) un haplotipo de clasificación confusa del Volcán San Martín en Veracruz. Estos autores delimitaron la distribución del grupo *Craugastor rhodopis* desde San Luis Potosí y Veracruz a lo largo de las tierras bajas de México hasta Belice y noroeste de Honduras, cruzando el Istmo de Tehuantepec y de ahí hacia el sureste, a lo largo de la vertiente del Pacífico de Chiapas, Guatemala hasta el Salvador.

De acuerdo al trabajo de Streicher et al. (2014b), *Craugastor loki* está presente en los estados de Veracruz, Oaxaca y Chiapas (Figura 1). Sin embargo, es interesante notar que en su delimitación la asocian principalmente a tierras bajas, aunque en Chiapas y Guatemala y posiblemente de El Salvador, se encuentran poblaciones que sobrepasan los 1800 m (Streicher et al. 2014); en las partes bajas ocurren en el bosque tropical caducifolio y en altitudes arriba de los 1200 m en bosques de pino-encino y bosque mesófilo de montaña.

Dada la distribución a lo largo de un gradiente altitudinal y el alto grado de polimorfismo de *C. loki* es un sistema ideal para evaluar el papel de la variación genética y del flujo génico. Además, la integración de aspectos ecofisiológicos de los organismos y del ambiente, podría ayudar a explicar si hay señales de adaptación local en diferentes pisos altitudinales y por lo tanto evaluar si existe un proceso como el aislamiento por ambiente (Wang 2013) y/o por adaptación (Nosil 2012).

El presente trabajo doctoral está integrado por tres capítulos, el primero se refiere a la fisiología térmica y la vulnerabilidad al cambio climático de la especie de desarrollo directo *Craugastor loki* en el sureste de México. Se estudiaron parámetros de ecología térmica que permiten evaluar la vulnerabilidad al calentamiento global. Los resultados muestran que *Craugastor loki* exhibe una amplia aclimatización, es decir, una plasticidad en los límities térmicos a lo largo del gradiente de elevación, especialmente en el límite máximo. Además, se encontró que las poblaciones que ocurren en tierras bajas presentan límites térmicos críticos muy cercanos a las temperaturas del ambiente donde éstas ocurren, por lo que sugerimos tendrán baja tolerancia al calentamiento climático. Este capítulo se publicó en la revista *Ecology and Evolution.*

En el segundo capítulo nos centramos en entender el papel de la pérdida de agua a lo largo del gradiente de elevación y de la temperatura operativa de *Craugastor loki*. Para ello se utilizaron modelos biofísicos de ranas hechos de agar. Se encontró que, en zonas bajas, la pérdida de agua es mayor comparada con las zonas intermedias y zonas altas. Sin embargo, en promedio la pérdida de agua a lo largo del gradiente de elevación es muy similar. Por el contrario, la temperatura operativa sigue un patrón clinal, es decir, la temperatura fue más alta en tierras bajas, mientras que a mayor altitud fue más baja. La humedad relativa fue muy similar a lo largo del gradiente, probablemente porque el periodo de muestreo fue en temporada de lluvias. Así, se sugiere que la humedad relativa durante esta temporada puede estar influyendo en los bajos porcentajes de pérdida de agua. Además la alta humedad relativa podría amortiguar las altas temperatura ambientales que los organismos están experimentando. Es decir, que hay una interacción entre la temperatura que experimentan los organismos con repecto a la pérdida de agua. Este capítulo será sometido a la revista *Journal of Experimental Zoology Part A*.

En el tercer capítulo se muestra el estudio sobre el papel del paisaje sobre la diferenciación genética de las poblaciones de *Craugastor loki* a lo largo del gradiente altitudinal. El objetivo fue distinguir en qué medida el ambiente, a través de la caracterización de la temperatura, la precipitación, y la elevación, influye en la diferenciación de esta especie. Se encontró que la temperatura y humedad, influyen en la estructura genética de las poblaciones, específicamente la estacionalidad en ambos parámetros ambientales. Se identificaron dos grupos genéticos, uno en la vertiente de la Depresión Central de Chiapas y el segundo en la vertiente del Pacífico. Además, se identificó que las poblaciones de tierras altas corresponden probablemente a otra especie del género *Craugastor.* Este capítulo será enviado a la revista *Heredity*.

Objetivo general

Evaluar el papel de factores bióticos y abióticos como parte del paisaje (temperatura, humedad y elevación) en la diferenciación genómica y rasgos fisiológicos de poblaciones de la rana de desarrollo directo *Craugastor loki* en un gradiente altitudinal.

Objetivos particulares

1. Identificar unidades ambientales como una medida de heterogeneidad ambiental utilizando variables ambientales de temperatura y precipitación a lo largo del gradiente altitudinal donde se distribuye *Craugastor loki*.

2. Identificar requerimientos térmicos e hídricos de individuos de *Craugastor loki* en las distintas unidades ambientales y a lo largo del gradiente altitudinal.

3. Determinar si existe diferenciación genética de *Craugastor loki* a lo largo del gradiente altitudinal, mediante el uso de marcadores nucleares polimórficos (SNPs).

Capítulo 1

Environmental heterogeneity shapes physiological traits in tropical direct-developing frogs

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Este capítulo se refiere al siguiente artículo: “Environmental heterogeneity shapes physiological traits in tropical direct-developing frogs”, en el cual se estudiaron las sensibilidades térmicas de la rana de desarrollo directo *Craugastor loki*) a lo largo de un gradiente de elevación dentro de cinco unidades ambientales (dominios bioclimáticos), definidos por la temperatura y la precipitación. Mediante la estimación de distintos parámetros como la temperatura preferida (T_{sel}), los límites críticos de tolerancia térmica (el límite máximo térmico y el mínimo térmico), se encontró que a lo largo del gradiente la temperatura corporal disminuye a medida que aumenta la temperatura ambiental. La temperatura preferida tiende a aumentar moderadamente con la elevación dentro de los dominios bioclimáticos muestreados. Además, se sugiere que el paisaje térmico ideal para esta especie se encuentra en elevaciones medias, donde la precisión térmica (d_b) y la calidad térmica del ambiente (d_e) son adecuadas. El limite máximo térmico es variable a través de las elevaciones y entre los dominios bioclimáticos, disminuyendo a medida que aumenta la elevación. Mientras que el limite mínimo térmico no es tan variable como el máximo térmico crítico. Las poblaciones de las tierras bajas pueden ser más vulnerables a futuros aumentos de temperatura.

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ORIGINAL RESEARCH

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Environmental heterogeneity shapes physiological traits in tropical direct-developing frogs

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Abstract

1. Tropical ectotherm species tend to have narrower physiological limits than species from temperate areas. As a consequence, tropical species are considered highly vulnerable to climate change since minor temperature increases can push them beyond their physiological thermal tolerance. Differences in physiological tolerances can also be seen at finer evolutionary scales, such as among populations of ectotherm species along elevation gradients, highlighting the physiological sensitivity of such organisms.
2. Here, we analyze the influence of elevation and bioclimatic domains, defined by temperature and precipitation, on thermal sensitivities of a terrestrial direct-developing frog (*Craugastor loki)* in a tropical gradient. We address the following questions: (a) Does preferred temperature vary with elevation and among bioclimatic domains? (b) Do thermal tolerance limits, that is, critical thermal maximum and critical thermal minimum vary with elevation and bioclimatic domains? and (c) Are populations from high elevations more vulnerable to climate warming?
3. We found that along an elevation gradient body temperature decreases as environmental temperature increases. The preferred temperature tends to moderately increase with elevation within the sampled bioclimatic domains. Our results indicate that the ideal thermal landscape for this species is located at midelevations, where the thermal accuracy (d_b) and thermal quality of the environment (d_e) are suitable. The critical thermal maximum is variable across elevations and among the bioclimatic domains, decreasing as elevation increases. Conversely, the critical thermal minimum is not as variable as the critical thermal maximum.
4. Populations from the lowlands may be more vulnerable to future increases in temperature. We highlight that the critical thermal maximum is related to high temperatures exhibited across the elevation gradient and within each bioclimatic domain; therefore, it is a response to high environmental temperatures.

KEYWORDS
acclimatization, amphibians, critical thermal limits, elevation gradient, thermal ecology, vulnerability, warming tolerance

1 | INTRODUCTION

A challenge for ecology and evolutionary biology is understanding how different biotic and abiotic environmental factors can influence the phenotype and genome expression (Chown et al., 2004). Temperature plays a key role affecting organisms as it influences nearly all biological processes at different levels of biological organization, from molecular kinetics to macroevolutionary rates of diversification (Dugo-Cota et al., 2015; Kingsolver, 2009). In ectothermic organisms, environmental temperature is particularly important because it not only determines body temperature but also influences a variety of processes such as periods of activity, metabolism, locomotion, foraging ability, and courtship (Angilletta et al., 2002). Therefore, temperature is not merely an abiotic factor for ectotherms, but could be considered as part of the biotic environment (Angilletta, 2009).

Body temperature in ectotherms varies with environmental conditions (Hillman et al., 2008), making thermal sensitivities also important since they relate to the way in which organism performance depends on temperature (Angilletta, 2009). Amphibians differ from other vertebrate ectotherms in the strength with which temperature and water balance affects performance (Navas et al., 2008). Additionally, the interplay between these factors is dependent on a variety of conditions such as natural history, microhabitat, latitude, and elevation (Wells, 2007).

Tropical ectotherm species have been hypothesized to have narrow physiological limits compared with temperate species (Janzen, 1967). This is known as the climate variability hypothesis (Ghalambor, 2006), which has been tested at macroecological scales in a variety of organisms (Chown et al., 2004; Gunderson & Stillman, 2015). However, at finer scales our knowledge is still limited (Montejo-Kovacevich et al., 2020). This limitation is particularly important for understanding the role of plasticity on thermal tolerance or species physiological limits. Therefore, there is some urgency to generate a better understanding of the role of thermal tolerance given that differences in physiological tolerances can also be seen at finer evolutionary scales. Such knowledge can be key to predicting species responses to climate change and species vulnerability, particularly in amphibians (Duarte et al., 2012).

In a landscape with an elevation gradient, climatic conditions vary with altitude; temperatures decrease as elevation increases, and precipitation regimes vary and are influenced by the orientation of the elevation gradient. These environmental variables grouped together in geographical space compose bioclimatic domains (Londoño-Murcia et al., 2010). Therefore, the physiological traits of organisms along the gradient are predicted to show elevated plasticity to adjust to varying environment conditions. Such physiological plasticity to environmental conditions is called thermal acclimatization, which can be defined as a reversible change in a biological trait in response to temperature variability (Gunderson & Stillman, 2015). Environmental variability includes changes in temperature or humidity due to natural fluctuations encountered along environmental gradients or different seasons (Angilletta, 2009). In other words, acclimatization refers to the plasticity in thermal physiology expressed in response to predictable or stochastic environmental fluctuations like those encountered along an elevation gradient. Moreover, ectotherms also have thermoregulation strategies to compensate for extreme environmental conditions (Muñoz & Losos, 2018) that include behavioral responses such as microhabitat selection, allowing finer modulation of body temperature in some amphibian species (Farallo et al., 2018). Nonetheless, most amphibians are considered to be thermoconformers in that their body temperature is similar to the environmental temperature. Thus, the interplay between physiology and behavior may have an important influence on how some amphibians navigate the thermal landscape (Angilletta et al., 2002).

One approach to analyze how temperature influences organism performance, in addition to field body temperature, is to study the critical thermal limits of a species to understand physiological limitations to an organism's response to environmental heterogeneity. Critical thermal limits include the upper thermal limit or critical thermal maximum (CT_{max}) and lower thermal limit or critical thermal minimum (CT_{min}). Results in the literature are mixed regarding variation in critical thermal limits, with some studies suggesting that critical thermal limits decrease with elevation (von May et al., 2017), as predicted by theory, while others do not find any association (Christian et al., 2008). The main conclusion therefore seems to be that the response depends on the studied clade (von May et al., 2017). A study on toads within the genus *Rhinella* found that two species are strong thermoconformers, being highly dependent on environmental temperature and their thermal limits are not variable with respect to seasonal variation of climatic parameters (Anderson et al., 2018). On the other hand, for two species of frogs within the genus *Craugastor* in Costa Rica, the critical thermal limits are associated with the habitat the species occupies. CT_{max} was found to be higher in the species that is more tolerant to deforestation and warming temperatures (*C. fitzingeri*), while the other (*C. crassidigitus*) is less tolerant to fragmentation and deforestation and presents lower critical thermal limits (Frishkoff et al., 2015). Studies on some ecthotherms show variation in upper and lower thermal limits associated with the species environment (Hoffmann et al., 2013). In light of global warming, the critical thermal limits are often considered as a proxy for vulnerability to climate change (Seebacher et al., 2015; Sinervo, 2011). It is important to consider life history, behavior, and the scaling of climate data to assess vulnerability to climate change (Nadeau et al., 2017). Also, the evolutionary potential to face global warming is equally important to understand the response to novel selective pressures (Muñoz et al., 2014).

Direct-developing frogs are among the most diverse group of frogs in the New World (Frost, 2020), with over 900 species the group represents ca. 1/3 of Neotropical amphibian diversity and 120 species of this group mainly occur in Middle America. Many species are also polytypic, with striking variation in color patterns and are primarily characterized by the lack of a water-dwelling larval phase and their use of substrate humidity for reproduction (Duellman & Trueb, 1994). *Craugastor loki* is a direct-developing frog species found in North and Central America from southern Mexico to northern El Salvador, inhabiting both dry and humid habitats. This species is often locally abundant and has a broad elevational distribution from sea level to 2,200 m a.s.l., particularly in southern Mexico (Lynch, 2000), but also in Guatemala and El Salvador. Consequently, this species is particularly suitable for exploring how thermal sensitivities can vary at the local scale due to plasticity or thermal adaptation. Here, we analyze whether thermal sensitivities of *C. loki* are associated with elevation and bioclimatic domains, defined as an environmental space that integrates bioclimatic variables of temperature and precipitation. We addressed the following questions: (a) does temperature preference vary according to altitude and bioclimatic domains; (b) are thermal limits, CT_{max} and CT_{min}, lower as elevation increases as predicted by the climate variability hypothesis; (c) do thermal limits change in response to local variation in average maximum and minimum temperatures; and (d) are populations from high elevations more vulnerable to a warming climate?

2 | MATERIALS AND METHODS

2.1 | Study area and sampling

The Sierra Madre de Chiapas, Mexico, is a physiographical region with elevation gradients on both slopes: Pacific versant and the interior slopes leading to the Central Depression of Chiapas, ranging from lowland humid forest to montane cloud forest. We characterized the area using bioclimatic domains as a proxy for environmental heterogeneity (Londoño-Murcia et al., 2010; Téllez-Valdés et al., 2010). We outlined rectangular polygons ca 40 × 70 km at the Sierra Madre de Chiapas that covered different environments and elevation gradients (Figure 1a). We used the 19 bioclimatic variables (Table S1) from Cuervo-Robayo et al. (2014) to determine five bioclimatic domains (Figure 1a), which are defined as *n*-dimensional space groupings, where biological processes occur (Londoño-Murcia et al., 2010). These bioclimatic domains are the combination of the 19 bioclimatic variables and differ from each other in temperature, precipitation, and elevation (Table S1, Figures S1 and S2). We used a resolution of 30 × 30 m for bioclimatic variables (Cuervo-Robayo et al., 2014). Bioclimatic domains at higher elevations have lower temperatures and lower annual precipitation than bioclimatic domains at lower elevations, which have higher temperatures and precipitation. Nonetheless, when precipitation of the driest month is low, bioclimatic domains at higher elevations are wetter compared with lower elevation ones (Table S1). In each bioclimatic domain, we identified three sampling sites and visited nineteen localities along the elevation gradient from 120 m to 2,250 m a. s. l. However, we did not find frogs in all localities (Figure 1b). In total, we studied fifteen populations of *Craugastor loki* from the Sierra Madre de Chiapas during the rainy season (June to November), when frogs are most active

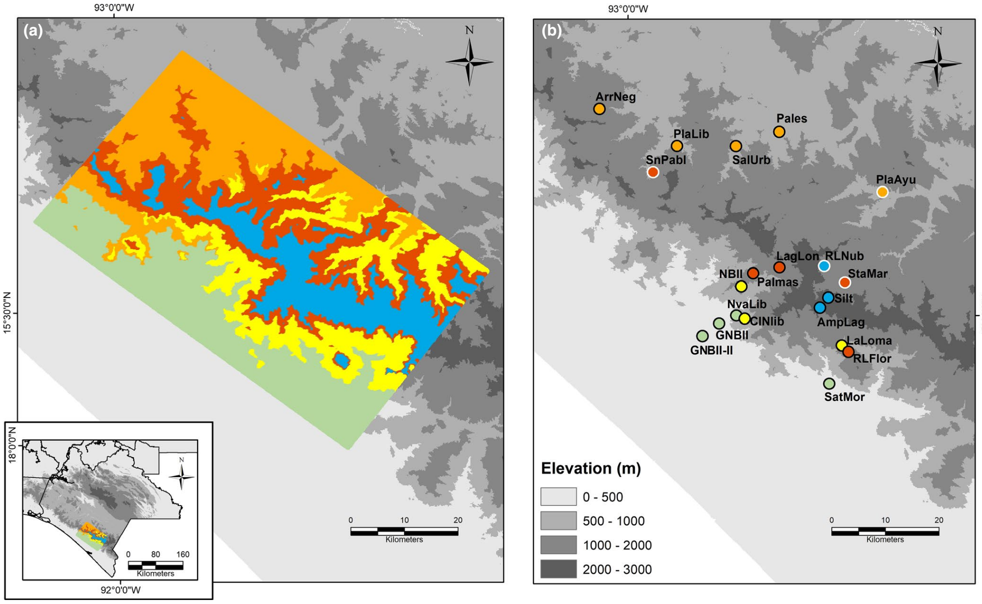


FIGURE 1 (a) Bioclimatic domains (BioDom). Green color corresponds to lowland areas and high annual temperature and precipitation. High precipitation in the warmest quarter and intermediate elevations (yellow). Intermediate elevation and temperature seasonality (orange). Higher elevations (red), and high precipitation in driest season and high elevations (blue). (b) Sampling sites of direct-developing frog *Craugastor loki* in southern Mexico in each BioDom. Circles with white outline are sampled sites where frogs were not found

(Figure 1b), in 2017 and 2018. For each population, we sampled frogs at night (20:00 hr–01:00 hr) using visual encounter surveys and locating frogs by their advertisement calls (Heyer et al., 1994). Occasionally, we sampled individuals during daytime hours. Once we found a frog, we approached the frog carefully, to avoid them moving or escaping and immediately measured body temperature (T_b) on the dorsal side using the thermocouple of the Fluke model 51-2 contact thermometer during 3–5 s. We also measured the substrate temperature and air temperature (20 cm above the substrate) where the frog was encountered, if the frogs moved, we only recorded substrate and air temperature. We also measured the local environmental temperature and humidity using data loggers (HOBO Onset) programmed to collect data every 30 min to estimate habitat temperature (see warming tolerance section below).

We brought captured frogs to our field laboratory for experimental trials. The frogs were kept for 3 days in plastic containers with the original substrate at laboratory temperature (~23°C) and environmental humidity. During the following 3 days, we recorded preferred temperature (T_{pref}) on day 1, critical thermal minimum (CT_{min}) on day 2, and critical thermal maximum (CT_{max}) on day 3. Given data collection was stressful for the animals, we divided it into 3 days to give individuals respite between trials. Due to the short amount of time we kept frogs in the laboratory, not allowing them to acclimate to a different temperature regime, we assumed that measurements reflect responses to field conditions as a response to plasticity or local adaptation (von May et al., 2017). After all experiments, we took a toe sample from each organism and in some cases for voucher specimens we extracted the liver for genomic studies.

2.2 | Operative temperatures

The operative temperature (T_e) is defined as the steady-state temperature of the organism; it is a proxy of the environmental heterogeneity as perceived by the individual (Angilletta, 2009). To estimate this parameter, we used physical models (Navas & Araujo, 2000) similar to typical *Craugastor* individuals using agar models of the same body size and similar body shape from latex molds made from rubber models using *C. loki* museum specimens. Models were made with granulated agar (Thermo Fisher Scientific), using 2.2 g into 100 ml of distilled water in which a data logger (Ibutton Thermochron, 4K) was placed. At each locality, we used 10 operative models, which were programed to record the temperature every 10 min for 24 hr. The ten frog models were placed in the microhabitats where *Craugastor* frogs were found including on top and under leaf litter, small cavities on trunks, and under fallen trunks.

2.3 | Preferred temperature

The preferred temperature is based on the temperature chosen by the organism in the absence of biotic and abiotic limitations (Angilletta, 2009). It can also be considered as an estimate of optimal body temperature that maximizes life-time reproductive success under unconstrained conditions (Gvoždík, 2018). We built two thermal gradients (1.0 m × 0.8 m and 0.7 m high) using wood boards. We used litter collected from each sampling locality as substrate. One end of the wood board was cooled and maintained at 8°C with ice packs, and the other was heated to 38°C with an electric hot plate. We ensured that substrate humidity was moderate along the entire gradient throughout the entire duration of the trials. We placed five individuals in each thermal gradient at a random location 1 hr before the start of the trials. We performed the trials at night, starting at 21:00 hr as it is the frogs' activity period. Each hour we recorded the preferred temperature (T_{sel}) of ten individuals in both gradients, for a total of five measures for each individual (five replicates), this process was carried out for each individual in the dark and using a red light and measuring temperature with a Fluke model 51-2 contact thermometer during 3–5 s. Each individual was weighed and measured (body and tibia length) prior to each trial.

2.4 | Thermal accuracy index and quality index

We calculated the thermal accuracy (d_b) and thermal quality index (d_e) of the environment (Hertz et al., 1993) for each population. We used these two parameters to characterize the thermal landscape that the frogs are occupying, but not to evaluate the effectiveness of thermoregulation. Amphibians depend on moisture in addition to temperature does not take into account these two parameters, but it is a proxy of how frogs are suited to the thermal landscape. To estimate the d_b and d_e, we considered the data filtered by frog activity hours, based on our observations during fieldwork. We considered that activity starts from dusk to dawn, that is, from 18:00 hr to 5:00 hr. Thermal accuracy (d_b) was calculated as the mean of the absolute value of the difference between individual body temperature (T_b) and T_{sel} ($d_b = T_b - T_{sel}$), while the thermal quality index (d_e) is the mean of the absolute value of the difference between the operative temperature (T_e) and T_{sel} ($d_e = T_e - T_{sel}$). High d_b and d_e values indicate that thermal accuracy and the thermal quality of the environment are low, while values near zero indicate high thermal accuracy and high thermal quality of environment, suggesting that under such thermal conditions the organism presents increased physiological performance (Hertz et al., 1993).

2.5 | Thermal tolerance: CT_{min} and CT_{max}

Thermal tolerance range or thermal breath is the physiological range at which the organism is active and is limited by the critical thermal minimum (CT_{min}) and the critical thermal maximum (CT_{max}). We estimated the CT_{min} using two containers. In the first one, we deposited a damp paper before the frog was placed. The container was placed within a larger container with ice to lower the temperature at a rate of ~1.5°C/min. After some minutes, the organism changes its behavior; first, frogs move and jump and then stay very still, at which point we

TABLE 1 Mean ± *SE* for the body temperature (T_b), operative temperature (T_e), and preferred temperature range (T_{sel}) of individuals from different populations by year, elevation, and bioclimatic domain (colors correspond to those in Figure 1)

Year	Bioclimatic domain	Elevation	Pop *N*	T_b	T_e	d_b (95% CI)	d_e (95% CI)	Mean of T_{sel} *n*	1st & 3rd quartile of T_{sel}
2017	– Green	124.3	GNBII-II 10	26.33 ± 0.51		4.88 (4.65–5.25)		20.57 ± 1.60	19.60–21.45
		242.24	GNBII 17	23.72 ± 0.79	23.17 ± 1.4	2.34 (1.82–2.52)	1.82 (1.2–1.6)	21.03 ± 1.06 (10)	20.3–21.38
2017		300	SatMor 24	23.81 ± 0.34	23.14 ± 0.37	0.88 (0.77–0.97)	0.27	21.77 ± 1.87 (10)	20.88–22.93
2017		768.90	NvaLib 29	21.98 ± 0.74	22.38 ± 0.71	0.09	0.52 0.0–0.5)	21.46 ± 2.17 (11)	20.30–22.50
2018		783.61	NvaLib 20	22.33 ± 1.18	22.30 ± 0.92	0.84 (0.04–1.43)	0.81 (0.4–0.9)	23.97 ± 1.66 (10)	22.98–25.0
2018	– Orange	731.08	Pales 12	20.67 ± 0.54	20.73 ± 0.70	1.13 (0.70–1.45)	0.73 (0.3–0.3)	22.70 ± 1.38 (10)	21.80–23.75
2018		836.64	SalUrb 11	21.63 ± 0.77	20.94 ± 0.95	0.57 (0.0–0.6)	0.26	20.20 ± 1.30 (10)	19.20–21.20
2017		912.27	PlanLib 11	20.07 ± 0.73	20.73 ± 0.91	0.00	0.01	20.78 ± 1.99 (12)	19.20–22.50
2017		1,072.88	ArrNeg 8	20.07 ± 0.60	19.74 ± 9.84	0.92 (0.40–1.70)	1.29 (1.0–1.5)	21.68 ± 1.47 (7)	21.0–22.70
2017	– Yellow	1,143.87	CINLib 10	20.61 ± 0.90	19.43 ± 0.98	2.79 (2.55–3.30)	3.97 (3.9–4.4)	23.86 ± 1.13 (12)	23.40–24.70
2018		1,206.18	CINLib 11	18.41 ± 0.35	17.56 ± 0.63	4.39 (4.2–4.5)	5.24	23.52 ± 1.46 (11)	22.80–24.20
2017		1,131.82	NBI 26	20.55 ± 1.23	19.49 ± 0.75	1.07 (0.3–1.7)	0.26	18.94 ± 1.20 (10)	18.35–19.60
2018		1,128.50	NBI 20	20.56 ± 1.98	19.13 ± 1.58	0.69 (0.0–1.7)	0.78 (0.7–0.7)	20.29 ± 1.81 (12)	19.20–21.42
2017		1,303.67	LaLoma 15	18.85 ± 0.78	19.25 ± 0.70	3.45 (2.9–3.8)	3.05	22.67 ± 1.23 (10)	22.3–23.30
2018		1,298.0	LaLoma 3	17.23 ± 0.15	17.57 ± 1.03	1.67 (1.5–1.8)	1.37	19.90 ± 1.74 (6)	18.90–20.20
2018	– Red	1,380.77	Palmas 5	18.68 ± 0.52	17.91 ± 0.88	1.87 (1.25–2.65)	2.64 (2.5–3.0)	21.21 ± 1.30 (11)	20.55–22.20
2018		1,471.88	LagLon 11	18.49 ± 1.17	18.21 ± 1.26	2.21 (0.8–2.9)	2.53 (2.55–3.0)	21.58 ± 1.50 (12)	20.70–22.32
2017		1,510.0	RLFlor 22	17.68 ± 1.40	17.47 ± 0.49	4.25 (3.53–5.03)	4.46	22.93 ± 2.02 (10)	21.93–24.6
2018		1,406.80	RLFlor 5	19.54 ± 0.98	17.90 ± 0.91				
2017	– Blue	2,111.17	AmpLag 22	14.76 ± 0.42	14.25 ± 0.66	9.24 (9.10–9.45)	9.75	24.66 ± 2.30 (12)	24.0–25.80
2018		2,069.33	AmpLag 3	14.90 ± 0.79	13.53 ± 1.07	5.20 (4.3–5.8)	6.57	20.80 ± 1.28 (11)	20.10–21.60

Note: N is the number of the frogs sampled for the T_b and d_b, while *n* is the number of sampled frogs for each population for T_{sel}. Thermal precision index (d_b) and thermal quality index (d_e) for each population.

Populations where the d_b index is close to zero indicate that the body temperature is close to the microenvironmental temperature. Likewise, d_e index near to zero indicates environmental thermal quality is very close to body temperature (T_b). Conversely, if d_b and d_e are different from zero, body temperature is far from microenvironmental temperature and thermal quality is not favorable to frogs. d_b, d_e, and T_{sel} are described in Materials and Methods.

measured whether there was a loss of righting response. The loss of righting response is often used in thermal physiology in ectotherms and is considered relevant in terms of thermal selection, where frogs could be vulnerable to predation (Catenazzi et al., 2014; von May et al., 2017, 2019; Navas et al., 2007). To do this, we placed the frog in a belly-up position, and if the frog could not recover its natural position, after 5 s, we measured the temperature on the ventral side. For CT_{max}, we used an electric hot plate to heat the container, with a humid substrate. Generally, the behavior displayed when the organism starts to warm up and to reach CT_{max} includes fast movements and jumping and then the organism stays still in one position. Previous to each experiment, for both CT_{min} and CT_{max} the organisms were kept at ~23°C. With the values of the CT_{max} and CT_{min}, we calculated the Thermal Tolerance Range (TTR = $CT_{max} - CT_{min}$).

2.6 | Warming and cooling tolerance

One approach to measure vulnerability to climate change is to measure the warming tolerance of organisms (Deutsch et al., 2008), which is an estimate of the difference between ambient temperatures and the organism's thermal maximum (Tuff et al., 2016). Warming tolerance is similar to the Thermal Safety Margin (TSM) from Rohr et al. (2018), but it is different according to Deutsch et al. (2008), who consider it as the difference between an organism's thermal optimum (T_{opt}) and its current climate temperature T_{hab} (TSM = $T_{opt} - T_{hab}$). Environmental temperature from the local sites was obtained from the data logger (HOBO's) in each locality. The data loggers were set up to record temperature every 30 min daily for a period of approximately 1 year and in some cases 2 years. In some localities, recording started in 2017 and in other localities in 2018. In two localities (GNBII-II and Palmas), it was not possible to obtain the recording, due to loss or damage of the datalogger. For these localities, we used the information from the datalogger closest to the locality (GNBII and LagLon, respectively). We estimated the average of the maximum and minimum daily temperatures during the recording period to estimate the temperature of the habitat at each locality (T_{hab}) (Catenazzi et al., 2014). We estimated the warming tolerance (WT) as WT = $CT_{max} - T_{hab\_max}$ and the cooling tolerance, using the daily minimum temperature to calculate the T_{hab_min} temperature.

2.7 | Data analyses

We carried out linear models, to evaluate the effects of the elevation and bioclimatic domain on T_b, substrate and air temperature, CT_{min}, CT_{max}, *TTR*, *WT*, and cooling tolerance, as well as the relationship between these variables and our microclimatic temperature measurements (T_{max_hab}, T_{min_hab}, T_{mean_hab}). In consequence, we added to the linear models the body size (snout-vent length) and mass. For T_{sel}, we used the gls function implemented in the nlme package (Pinheiro et al., 2020), to fit a linear model with heterogeneous variances using the arguments: weights and varIdent (Pinheiro & Bates, 2000), organized hierarchically by individuals and then by population from low to high elevation. For d_b and d_e indexes, we calculated the confidence intervals with bootstrap (10,000 replicates). All statistical analyses were performed using the software R v 3.6.1 (R Core Team, 2020).

3 | RESULTS

We observed 337 frogs (272 at night and 62 during the day). We measured T_b in the field for 303 frogs and obtained 337 measures for substrate and air temperature (i.e., for 34 frogs which moved prior to us being able to obtain body field temperatures, we only recorded the substrate and air temperature). Field T_b estimates for each locality varied from seven to 29 organisms (Table 1).

3.1 | Body, substrate, and air temperature

We found a linear decrease in body temperature with increasing elevation ($F_{20,177} = 144.1$, $R^2 = 0.935$, slope $= -0.006 \pm 0.03$, $p < .0005$, Table S2, Figure S3a). Substrate and air temperature also exhibited the same pattern with increasing elevation ($F_{19,266} = 125$, $R^2 = 0.892$, slope $= -0.0019 \pm 0.0018$, $p < .0005$; $F_{19,266} = 135.8$, $R^2 = 0.899$, slope $= -0.002 \pm 0.0017$, $p < .0005$, respectively; Table S2, Figure S3b,c). Both substrate and air temperatures are highly correlated with body temperature of the sampled frogs ($r = .970$, $n = 256$, $p < .005$; $r = 0.975$, $n = 256$, $p < .005$, respectively; Figure S5a,b).

3.2 | Operative temperature and preferred temperature

The operative temperature (T_e) was similar to the body temperature (T_b) in all populations along the elevation gradient (Table 1, Figure 2). Thus, T_e shows a similar pattern to body temperature (T_b); that is, T_e decreases as the elevation increases (Figure 2). The preferred temperature (T_{sel}) for each population varied with elevation ($F_{13,930} = 25.51$, $p < .0001$; Table S3, Figure 3a) and differed between the bioclimatic domains ($F_{4,930} = 9.29$, $p < .0001$; Table S3, Figure 3a). We also found that body size (snout-length SVL) is positively correlated with T_{sel}. Smaller frogs tend to select a relative lower temperature compared with larger frogs ($F_{1,930} = 7.97$, $p = .0049$, Table S3, Figure S6a). When we pooled populations, the mean of T_{sel} is 21.83 ± 2.18°C, and the minimum and maximum means of T_{sel} are 19.60 ± 1.70°C and 23.70 ± 1.30°C, respectively. The T_{sel} recorded in the lowlands and at intermediate elevations (bioclimatic domains: green, orange, and yellow) is within the range of the operative temperature (T_e) and body temperature (T_b) recorded. However, in populations above 1,100 m, T_{sel} is outside of the range recorded for T_e and T_b (bioclimatic domains: red and blue, Figures 2 and 3a).

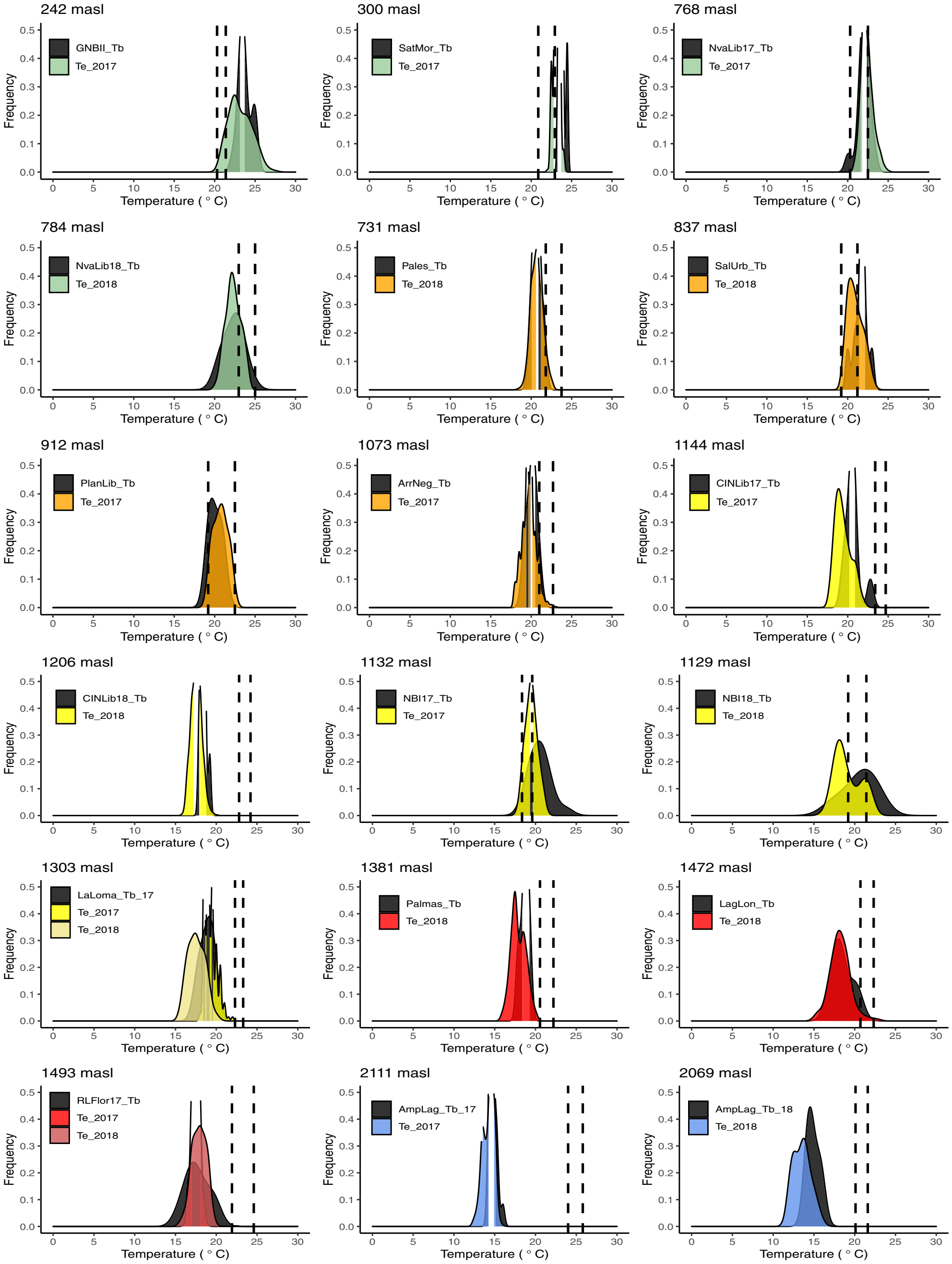


FIGURE 2 Thermal physiological traits: Body temperature (T_b in black color), selected temperature (T_{sel} dashed lines), and operative temperature (T_e) in colors according to bioclimatic domains (Figure 1). Dashed lines represent the interval between 1st and 3rd quartile of T_{sel}, *x*-axis shows the records of T_e in degrees Celsius, and *y*-axis shows the frequency of the temperature recorded

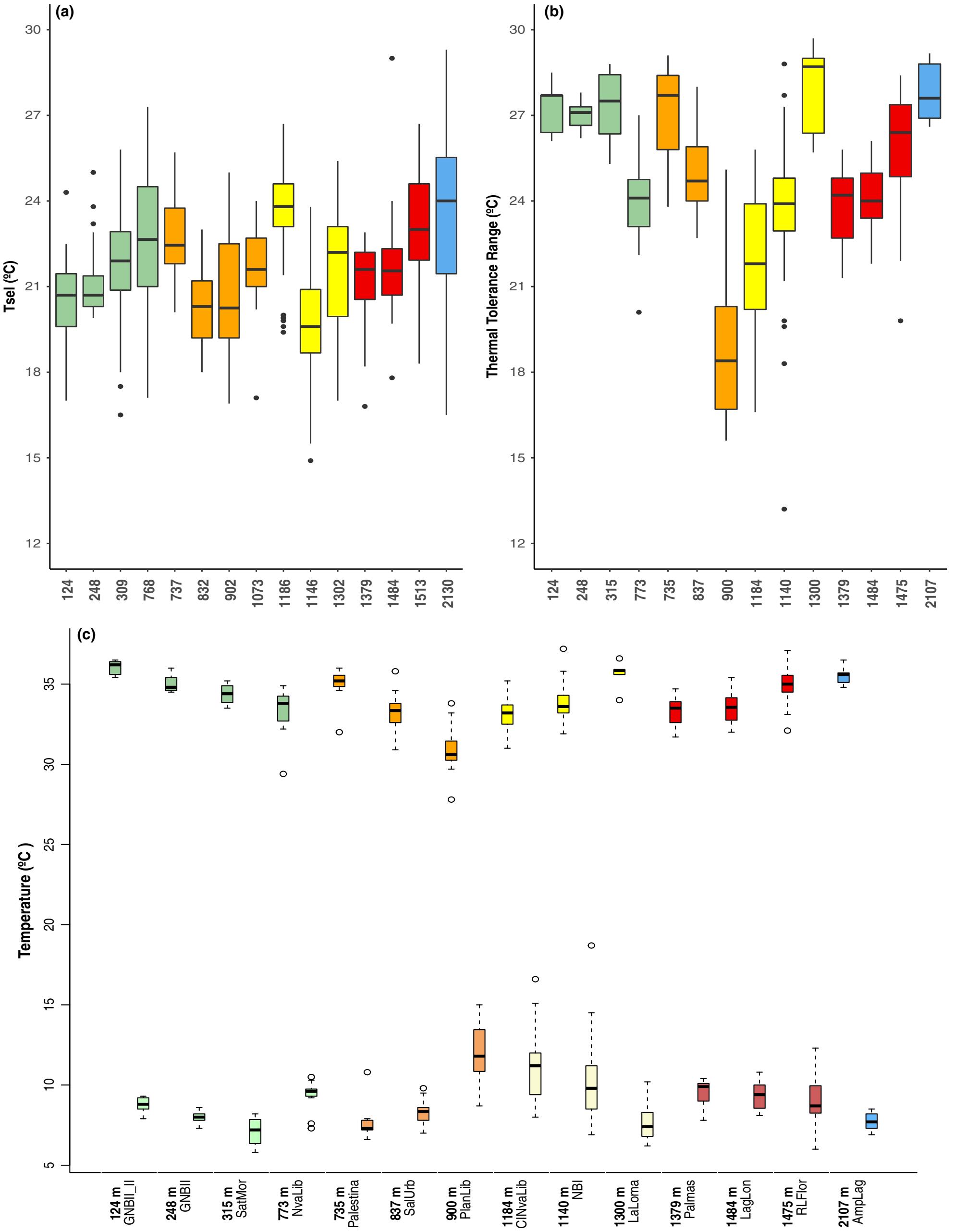
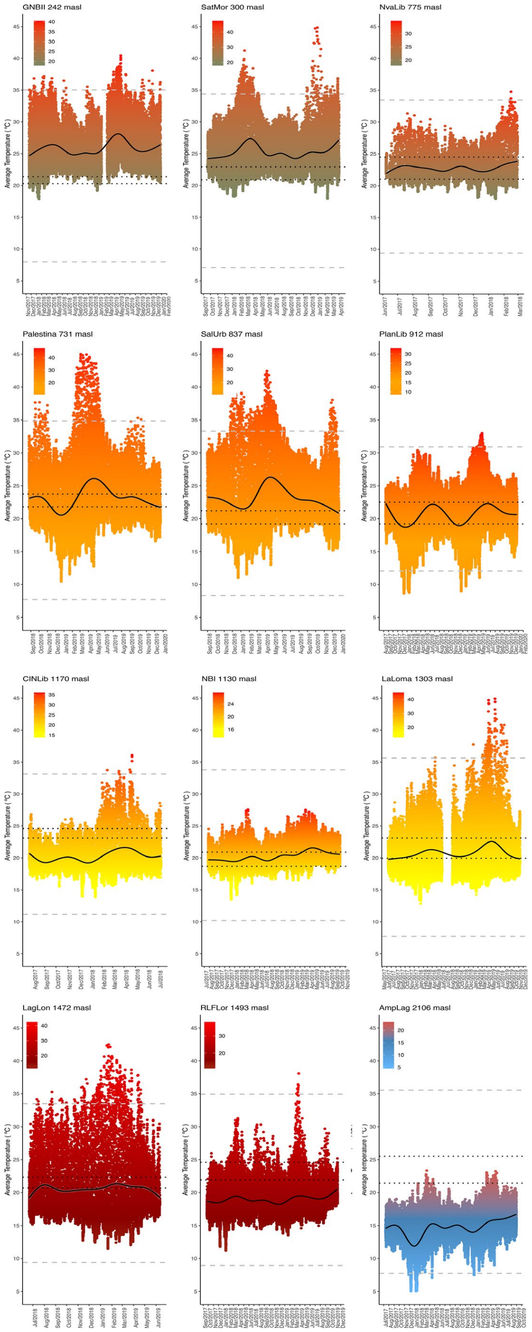


FIGURE 3 (a) Preferred temperature (T_{sel}) and (b) Thermal tolerance range (TTR) of populations sampled along the elevational gradient and bioclimatic domain (colors). The *x*-axis represents the elevation in meters above sea level. (c) Relationship between thermal tolerance limits in different elevations and bioclimatic domains. (CT_{max}: top) and (CT_{min}: bottom). The *x*-axis represents the elevation in meters and the name of population sampled

FIGURE 4 Microclimate temperatures (mean daily) and thermal limits of sampling localities between June 2017 and December 2019 at the five bioclimatic domains. Gray dashed lines represent the upper and lower thermal limits (CT_{max} and CT_{min}), respectively. Black dotted lines are the intervals of the T_{sel}, and the bold line represents mean temperature



3.3 | Thermal accuracy and thermal quality indexes

The thermal accuracy index (d_b) and thermal quality of the environment index (d_e) showed lower values in all populations from intermediate elevations (760–1,140 m, corresponding to the orange bioclimatic domain), meaning that in this bioclimatic domain the frogs are exposed to their preferred temperature (T_{sel}). Indeed, in one population (PlanLib) within the orange bioclimatic domain (Table 1), the estimates of both indexes were zero, indicating that in this site the frogs occupy more suitable temperatures. On the other hand, the populations in higher elevations (blue bioclimatic domain) show high values of d_b and d_e, indicating that these populations are exposed to environmental temperatures that differ from the preferred temperature. Also, some populations at intermediate elevations (yellow bioclimatic domain) present high values of d_b and d_e (Table 1).

3.4 | Critical thermal limits

The critical thermal maximum (CT_{max}) differs among populations at different elevations, decreasing with increasing elevation ($F_{19,137} = 10.86$, $R^2 = 0.545$, slope = −0.0118 ± 0.0042, $p < .0056$; Table S4, Figure 3c), and also differs between bioclimatic domains ($F_{4,137} = 2.6$, $p < .038$) (Figure 3c). Body size (snout-vent length SVL) was not correlated with CT_{max} ($F_{1,137} = 1.64$, $p = .20$). We also found differences in CT_{min}, among populations ($F_{19,139} = 7.29$, $R^2 = 0.43$, slope = 0.0044 ± 0.0062, $p < .0001$; Table S4, Figure 3c), decreasing with increasing elevation, but no differences among bioclimatic domains ($F_{1,137} = 1.64$, $p = .20$). Body size (SVL) is negatively correlated with CT_{min}, with small frogs having higher CT_{min} ($F_{1,139} = 11.79$, $p < .001$; Table S4, Figure S7). The thermal tolerance range (*TTR*) slight increases with elevation ($F_{19,136} = 11.98$, $R^2 = 0.573$, slope = −0.0162 ± 0.0081, $p < .049$; Table S4, Figure 3b), and body size has an effect on thermal breath ($F_{1,136} = 10.62$, $p < .0014$), but not bioclimatic domains ($F_{4,136} = 0.38$, $p = .821$; Figure 3b).

With respect to the local environmental data gathered by the data loggers, we found a 10°C difference between the extreme low and high populations (mean = 25°C ± 3.45 and 14.70°C ± 2.35, respectively; Table S5). On the other hand, we observed a wide range of recorded temperatures for some sites (Figure 4), where the daily temperature can exceed the range of critical thermal limits (Figure 4). This happens in more than 60% of the sampled localities (Figure 4).

There was not a significant relationship between the physiological thermal limits (CT_{max}) with respect to local environmental temperature (T_{max_hab}) ($F_{1,137} = 0.22$, $p = .63$), but we found differences between in elevation by bioclimatic domain ($F_{19,137} = 10.86$, $R^2 = 0.5415$, slope = −0.012 ± 0.004, $p < .05$, Table S4, Figure S8a). Furthermore, we did not find a relationship between CT_{min} with T_{min_hab} ($F_{1,139} = 0.74$, $p = .39$, Table S4, Figure S8b). When we plotted the CT_{max}, CT_{min}, and the elevation with bioclimatic domain, we found decreasing CT_{max} as CT_{min} increases ($F_{20,135} = 10.73$, $R^2 = 0.556$, slope = −0.123 ± 0.057, $p < .0001$, Table S4, Figure S9). This could be a consequence of the intrapopulation variability in both thermal limits, but it could also be driven by body size (see discussion).

3.5 | Warming and cooling tolerance

Populations from low elevations (green bioclimatic domain) have lower warming tolerance ($F_{19,137} = 143.1$, $R^2 = 0.944$, slope = −0.0114 ± 0.0041, $p < .0001$, Table S4, Figure 5a) than those from other bioclimatic domains. Conversely, populations from high elevations have lower cooling tolerance, in particular the populations at elevations ~1,370 m–1,500 m ($F_{19,139} = 28.66$, $R^2 = 0.769$, slope = −0.0044 ± 0.0061, $p < .0001$, Table S4, Figure 5b), which are within the red bioclimatic domain.

4 | DISCUSSION

We have shown that thermal sensitivities of the direct-developing *Craugastor loki* are influenced by the particular environmental conditions where the populations are found, as defined by elevation and the bioclimatic domains. Our results show there is greater plasticity in critical thermal maxima (CT_{max}), compared with critical thermal minimum (CT_{min}), as CT_{max} shows greater variability in response to changes in elevation or bioclimatic domain. The CT_{max} is higher in the lowlands and tends to decrease as elevation increases. Also, it is noteworthy that the T_{sel} exhibits a narrow range along the elevation gradient and even tends to increase with elevation within each bioclimatic domain.

4.1 | Body temperature

Body temperature (T_b) decreases significantly with increased elevation, which is a pattern found in most ectotherms that do not exhibit thermoregulation behavior (Angilletta, 2009). Tropical amphibians, especially terrestrial frogs, are highly dependent on the substrate and local environment for their body temperature (Navas, 1996). This pattern has also been found in *C. loki*, where T_b is highly correlated with substrate and air temperature, confirming that *C. loki is* a thermoconforming species. The T_b influence on individual performance (Navas et al., 2008) impacts activities such as foraging, courtship, and locomotion. As a consequence, T_b is important as a trait for resilience to climate variability. On the other hand, if T_b is associated with environmental variables (Navas et al., 2013), it implies differences in the effects of microclimate on individual performance and traits (Clusella-Trullas & Chown, 2014) such as development, growth and performance, all of which impact fitness (Kingsolver & Huey, 2008). Also, T_b is linked to other parameters such as preferred temperature (T_{sel}) and

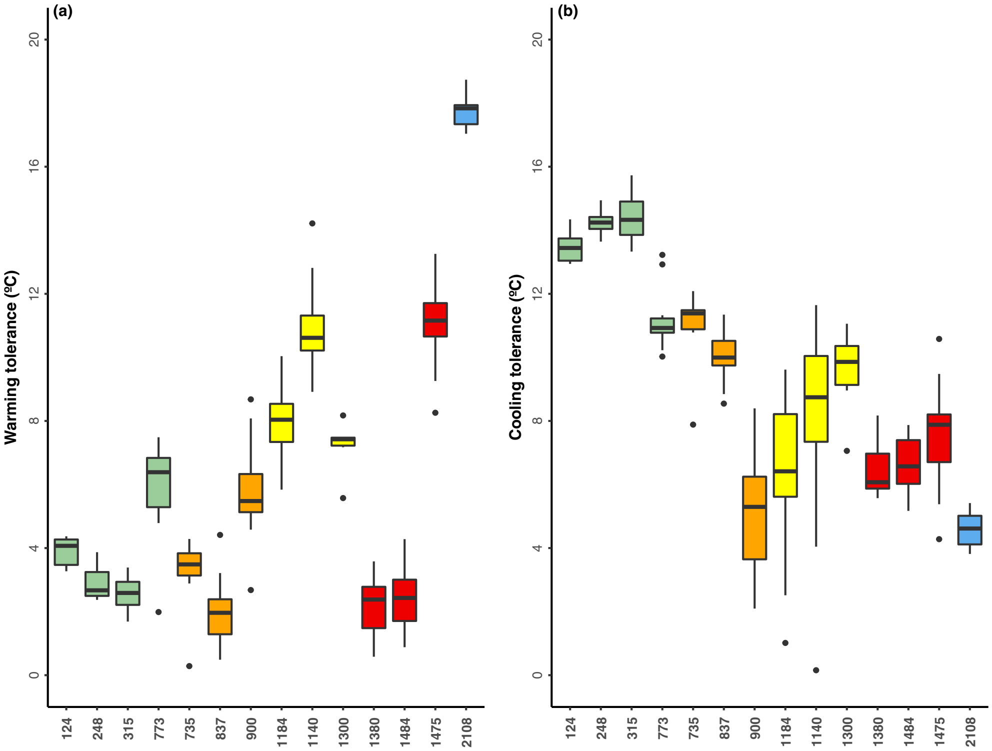


FIGURE 5 Warming and cooling tolerance of *Craugastor loki* along the elevational gradient. Colors correspond to each bioclimatic domain (Figure 1)

critical thermal limits, which are considered as traits integrated in the species thermal niche (Gvoždík, 2018).

In our study, the relationship between T_b, and T_{sel} varies across the elevation gradient and bioclimatic domain. In lowland populations, T_{sel} is relatively lower than the range of T_b (GNBII and SatMor, Figure 2). At intermediate elevations (orange environmental domain, Figure 2), T_{sel} is within the range of T_b, and in one population (PlanLib) T_{sel} matches T_b exactly. As elevation increases, part of the narrow range of T_b falls within the range of T_{sel}. Since these thermal parameters partly define the thermal niche, population growth and development are expected to be affected (Gvoždík, 2018). Our field observations of highland populations indicate that the reproductive season starts later compared with the remaining populations (at lower altitudes) and has a shorter duration, which indirectly affects population growth. Disentangling how the thermal niche could impose costs directly to population growth and development is an important question to be addressed in future work, particularly with respect to how these populations can face forthcoming climate change challenges.

4.2 | Preferred temperature and thermal indices

The preferred temperature (T_{sel}) in *Craugastor loki* shows a narrow range across elevations, but it tends to slightly increase as the elevation increases within each bioclimatic landscape (Table 1). This increase is important given that some studies show that T_{sel} is close to T_b or even near the upper thermal limit (Angilletta, 2009). Our findings show that T_{sel} tends to slightly increase across the elevational gradient; however, in the highest populations T_{sel} is closer to the T_{sel} recorded in lowland sites. These results are similar to studies of nocturnal geckos, some of which are thermoconforming species (Pianka & Vitt, 2003) and tend to have a constant T_{sel}. In our study, the T_{sel} mean for each sampled population was between 18.94 ± 1.20 and 24.66 ± 2.30, with the latter value recorded in the high-elevation population (Table 1, Figure 3a). T_{sel} is generally considered a conserved trait among species (Anderson et al., 2018). Here at the population level, differences in T_{sel} show a narrow range along all elevation localities. These observed variations could be the results of interpopulation variation in T_{sel}.

However, in highland populations where the mean average temperature is less than 20 degrees Celsius, the T_{sel} of frogs was ~5 degrees higher than the air temperature, which could be related to a pattern known as counter gradient variation. This pattern is observed when individuals from colder environments select higher temperatures in comparison with individuals from hot environments (Freidenburg & Skelly, 2004). Also, counter gradient has been related to the organisms having higher CT_{max} (Llewelyn et al., 2017). Further work is necessary to evaluate whether this hypothesis can be applied to frogs and *Craugastor* in particular. In addition, highland populations prefer higher temperatures than they experience, suggesting that this imposes an important challenge for individuals. These data suggest impacts on their "performance" and on fitness. During sampling in the highland population (AmpLag), we saw some frogs active during the day, not calling, but moving among the sunny patches. These frogs were possibly looking for warmth and staying in suitable microhabitats, which suggests that at high elevations frogs have to deal with thermal niche by exhibiting behaviors not shown in the lowlands.

On the other hand, it is important to point out that our preliminary studies on landscape genomics for these populations suggest that one highland population (AmpLag; blue environmental domain) might correspond to a divergent evolutionary lineage. Therefore, the maximum elevation range of *C. loki* might only extend to ~1,500 m a. s. l. and populations above that elevation could correspond to another species (Fig. S10). In the highlands of the Sierra Madre de Chiapas, at least four species of *Craugastor* that are very similar in coloration, size, and habits are believed to occur. Therefore, the sampled highland populations (blue environmental domain) might be similar to *C. loki* from higher elevations (red domain) in terms of thermal landscape. Effectively, the populations in each elevational extreme and bioclimatic domain confer the same thermal pressures on these physiological traits.

The results from the thermal accuracy (d_b) and the thermal quality of the environment (d_e) show that the populations from the orange environmental bioclimatic domain (characterized by the temperature seasonality at intermediate elevations) have high thermal accuracy and thermal quality. These conditions seem optimal for the frogs, given their preferred temperature is available in that bioclimatic landscape. It is noteworthy that one population (PlanLib) shows estimates close to zero for both indices, indicating remarkable thermal quality. Those frogs do not have to deal with extreme temperatures as they do in the lower or higher elevations, experiencing close to an ideal thermal niche. These thermal parameters used mainly in lizards studies have proven useful in understanding the thermal landscape in frogs as well. Our data at the population level permit the splitting of thermal niche and how it can vary with spatial and temporal scale (Gilbert & Miles, 2019). While we did not take into account humidity, our sampling was performed in rainy season where the humidity is mainly similar and oscillated between 90% to 100% humidity (Table S6). As a result, understanding the thermal niche in these frogs is a step toward understanding the causes that produce phenotypic variation in thermal traits in buffering high temperatures and coping with rising temperatures.

4.3 | Critical thermal limits

The critical thermal limits measured in our study showed thermal acclimatization on the upper thermal limit associated mainly with altitude and the environment, though patterns were less clear in lower thermal limits. That is, within each of the sampled bioclimatic domains as elevation increases, the upper thermal limit decreases, especially in populations from 200 m to 1,000 m a. s. l. In most interspecific studies of ecthotherms, species show that upper thermal limit is a conserved trait among species or within a particular species (Muñoz et al., 2014). Likewise, in frogs of the genus *Pristimantis* along an elevation gradient in the Andes, CT_{min} is more variable than CT_{max} by elevation. This finding is explained by microclimatic thermal variation, mainly in minimum temperatures, suggesting that this group of frogs has increased cold tolerance to buffer against lower temperatures (Pintanel et al., 2019). However, another study focused on terrestrial breeding frogs distributed along a tropical elevation gradient has shown that CT_{max} and CT_{min} exhibit substantial variation across closely related species (von May et al., 2017), where CT_{max} and CT_{min} decrease with increasing elevation. Other studies found interspecific variation in critical thermal limits related to body size, where CT_{max} tends to increase with increasing body size (González-del-Pliego et al., 2020; von May et al., 2019), and CT_{min} tends to decrease with increasing body size (von May et al., 2019). In our study, we only found that CT_{min} tends to decrease as body size increases. This contrasts with interspecific studies in terrestrial breeding frogs, which suggested that larger body size is common at high-elevation habitats (Gonzalez-Voyer et al., 2011; Hedges, 1999; Santa-Cruz et al., 2019). Here, it is clear that lowland populations of *C. loki* tend to have a larger body size (Figure S4). Also, we found a correlation between CT_{max} and CT_{min} (Figure S9), which could be the consequence of body size. That is, intrapopulation variability in both thermal limits could be driving by body size, and results in larger individuals tend to have higher CT_{max} and lower CT_{min} (larger individuals better tolerate heat and cold, because they are less susceptible to relatively rapid temperature change). While smaller individuals tend to have lower CT_{max} and higher CT_{min}, because they are more susceptible to relatively rapid temperature change.

The role of critical thermal limits in ectotherms has an ecological importance (von May et al., 2019). This was a consequence of the organisms experiencing temperatures closer or exceeding CT_{max}, which could result in death. But in the case of CT_{min}, the organism does not necessarily face death as becoming more inactive. However, even when temperatures are low *C. loki* is active nocturnally. During the day spend, their time apparently inactive and are exposed to elevated air temperatures in leaf litter, where the $T_{hab\text{-}max}$ can be very high in every environmental domain independent of elevation. Microclimatic temperatures at intermediate and high elevations can reach high temperatures similar to those recorded in lowland sites and at night lowlands are usually warmer but show greater variability during the day (Ghalambor, 2006).

According to the climatic variability hypothesis (Janzen, 1967), climatic variation and physiological tolerance in tropical and temperate regions are considered equivalent to conditions in elevation gradients (Ghalambor, 2006). We therefore expected lowland populations to present higher thermal limits than populations from the highlands. At an interspecific level, we expect that environmental variation could result in an adaptive change, while at the intraspecific level, variation could be due to thermal plasticity instead of an adaptive response. However, the role of plasticity could be more important. Thermal tolerance range (*TTR*) in *C. loki* exhibits a different pattern than expected across the elevation gradient. Populations at low and high elevations have narrower *TTR*, while at intermediate elevations, TTR is wider. Microclimatic temperatures could explain this pattern, as the average maximum temperature ($T_{max\text{-}hab}$) in lowlands and highlands are relatively similar. That is, $T_{max\text{-}hab}$ do not show a pattern with respect to elevation and neither does the bioclimatic domain, while T_{mean} and T_{min_hab} both show a slight decrease with elevational increase. This trend is not shown in $T_{max\text{-}hab}$, where in lowlands (i.e., GNBII, 300 m elevation), $T_{max\text{-}hab}$ can reach around 30°C, as well as in highlands (i.e., LagLon, 1,500 m elevation).

Studies with other terrestrial breeding frogs suggest that thermal limits are related to microclimate temperatures (González-del-Pliego et al., 2020; Pintanel et al., 2019). That is, thermal limits exhibit variation according to the type of microhabitat they occupy including open forests (González-del-Pliego et al., 2020; Nowakowski et al., 2017, 2018). In our study, *C. loki* is mainly found in leaf litter (more than 70% of the sampled individuals); therefore, microhabitat temperatures, especially at night, do reflect temperatures to which frogs are usually exposed. This is probably not the case at daytime when they are inactive in the leaf litter and temperatures are higher. As a consequence, further work is needed to test how habitat modifications affect thermal traits. This is particularly critical since our study area has changed drastically over the course of the study (Percino-Daniel, pers. obs.), especially at intermediate elevations where coffee plantations and open areas are increasing.

4.4 | Vulnerability to climate change

Our study provides insights regarding the sensitivity of these terrestrial frogs to climate change. First, our results show that lowland populations can be more vulnerable to high daily temperatures due to low warming tolerance (Figure 5a) while highland populations could benefit or be less vulnerable to temperature increases. Macroecological studies suggest that tropical organisms are more vulnerable to climate change for two reasons: (a) They experience environmental temperatures near their critical tolerance temperatures (Deutsch et al., 2008; Kingsolver, 2009); and (b) they have a narrow thermal tolerance (Janzen, 1967) and are therefore more sensitive to changes in climate. Here, at a fine scale we found that lowland frogs are more vulnerable to warming due to their limited thermal tolerance. Also, populations at high elevations are less vulnerable (bioclimatic blue domain). However, these findings can vary within each bioclimatic domain. For example, populations from elevations sampled ca ~1,300–1,400 m also exhibited a low warming tolerance (Figure 5a) and these sites experience high temperatures (Table S2). Previous research on other terrestrial breeding frogs includes species distributed from mid- to high elevations (Catenazzi et al., 2014), as well as species distributed from low to high elevations (González-del-Pliego et al., 2020; von May et al., 2019). However, the maximum daily temperature recorded at some sites in our study reached above 40°C (Table S2), which are dangerously high temperatures for thermoconformers. Indeed, IPCC reports (2019) predict the global temperature will increase to or exceed ~1.5°C, making it critical to understand how the frogs will respond to this warming increase. Hence, integrating an approach from a species' niche rather than a traits-based approach could make for a better understanding of general patterns of tolerance (Frishkoff et al., 2015).

In conclusion, our findings indicate that the future increase in global temperatures is likely to negatively affect performance and population growth of thermoconforming species such as *C. loki*. Predictive models can be used to test distinct scenarios for distribution shifts of species that integrate microclimate variables. As in this study, local temperatures can reveal if daily temperatures approach or exceed thermal limits for a species. This approach could better characterize long-term persistence of amphibian populations.

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CONFLICT OF INTEREST

The authors declare no conflicts of interests exist.

AUTHOR CONTRIBUTIONS

Ruth Percino-Daniel: Conceptualization (lead); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing-original draft (lead); writing-review & editing (lead). **José M. Contreras López:** Data curation (equal); methodology (equal); validation (supporting). **Oswaldo Téllez-Valdés:** Data curation (equal); methodology (equal); writing-review & editing (supporting). **Fausto R. Méndez de la Cruz:** Conceptualization (equal); methodology (equal); validation (equal); writing-review & editing (equal). **Alejandro Gonzalez-Voyer:** Conceptualization (equal); methodology (equal); validation (equal); writing-review & editing (equal). **Daniel Piñero:** Conceptualization (equal); funding acquisition (lead); project administration (lead); resources (lead); supervision (equal); validation (equal); writing-review & editing (equal).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Capítulo 2

Interaction of water loss and temperature in tropical direct-developing frogs along an elevation gradient

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Para ser enviado a la revista *Journal of Experimental Zoology Part A*

Este capítulo se refiere al siguiente artículo: “Interaction of water loss and temperature in tropical direct-developing frogs along an elevation gradient”, el cual será enviado próximamente a la revista *Journal of Experimental Zoology Part A.* Se utilizaron modelos biofísicos de ranas de agar para estimar la pérdida de agua por evaporación (EWL) y la temperatura operativa a lo largo de un gradiente de elevación de la especie *Craugastor loki*, que se distribuye entre los 240 m y 2250 m en Chiapas, México. El objetivo principal fue analizar cómo la pérdida de agua por evaporación y la temperatura operativa varían a lo largo del gradiente de elevación en cinco paisajes ambientales contrastantes durante la temporada de lluvias. Se encontró que el porcentaje de EWL fue similar a lo largo del gradiente de elevación, pero fue mayor en la elevación más baja (248 m), comparado con el resto de las localidades a lo largo del gradiente. Además, la EWL fue mayor durante el día en comparación con la noche. La temperatura operativa presenta un patrón de variación clinal, donde en tierras bajas fue más alta y a mayor altitud fue más baja. Además, durante el día, la temperatura operativa es más alta en comparación con la noche. Los resultados sugieren que debido a que la humedad relativa durante la temporada de lluvias es relativamente constante, genera condiciones que permiten el balance hídrico entre las ranas y su hábitat, lo que amortigua las condiciones térmicas a las cuales los individuos están expuestos.

Interaction of water loss and temperature in tropical direct-developing frogs along an elevation gradient

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Abstract

Ectothermic organisms are temperature dependent for all physiological processes; however, amphibian species also have to deal with water balance for their survival, growth and reproduction. As a consequence, thermal and hydration physiology are key approaches to understand how amphibians respond to climatic changes in temperature and humidity. Here, we used physical agar frog models to estimate evaporative water loss (EWL) and operative temperature across an elevation gradient (240 m to 2250 m) experienced by the direct-developing frog species *Craugastor loki* in Chiapas, Mexico. Our goal was to analyze how evaporative water loss and operative temperature varies across the elevation gradient in five contrasting environmental landscapes during the rainy season, when frogs are active. We found that the percent of EWL was similar across the elevation gradient, but it was higher at the lowest elevation (248m) compared with the remaining localities. Also, EWL was higher in daytime when compared to nighttime. The operative temperature presents clinal variation, with higher temperatures in the lowlands that decreased with increasing elevation. Also, in the daytime, the operative temperature is higher compared with the night. Our results suggest that the similarity of relative humidity during the rainy season produces moist conditions allowing the water balance between frogs and their habitat that buffers the thermal conditions to which the frogs are exposed.

Keywords: dehydration, hydrothermal physiology, physical agar models, amphibians, thermoregulation.

1. INTRODUCTION

Understanding the effect of temperature on biology, ecology and life history of ectothermic organisms has been keystone in evolutionary biology (Angilletta et al., 2002; Angilletta, 2009). A variety of studies have shown that temperature influences all physiological process such as growth, development, performance and ecological interactions (Hillman et al., 2008). Nonetheless, amphibian species must deal with water balance for their performance in addition to temperature (Navas, 1996; Navas et al., 2008).

Amphibians require moist skin for respiration (Duellman and Trueb, 1994). They exhibit thermoregulatory behavior and body posturing in order to maintain optimal thermal performance (Kearney et al., 2009), as well to prevent water loss (Heatwole et al., 1969). Evaporative water loss rate is defined by the difference between water vapor density between an organism and air, which in warmer temperatures can represent a deficit of vapor pressure and water loss (Katz and Graham, 1980). Water availability for amphibians, such as water vapor, soil moisture or surface water is mainly influenced by precipitation patterns (Schewe et al., 2014), and has been altered by the effects of climate change (Hagemann et al., 2013). Furthermore, empirical studies on dehydration physiology in amphibian habitats and along environmental gradients are still scarce (Greenberg and Palen, 2021) as vulnerability and extinction risk studies usually only consider the thermal biology approach (Deutsch et al., 2008; Sunday et al., 2019). In those studies hydration physiology should be integrated, especially for amphibian species, so that vulnerability to distinct scenarios of environmental change are not underestimated (Lertzman-Lepofsky et al., 2020; Greenberg and Palen, 2021; Hoffmann et al., 2021). Therefore, temperature and precipitation are key abiotic niche axes for amphibian species that will help to understand the interaction between them and to illuminate how amphibians respond to climate warming (Lertzman-Lepofsky et al., 2020; Greenberg and Palen, 2021) or emerging diseases (Catenazzi et al., 2014).

Like many other landscape features, montane habitats are predicted to be affected by climate change (Pounds et al., 1999). As a result, the risk of extinction for montane organisms increases under unfavorable climate change scenarios. Among a suite of climatic variables, temperature and humidity parameters show significant changes along elevation gradients (MacArthur, 1972; Barry, 2008). Temperature often has a linear pattern with elevation whereas precipitation trends with elevation are generally nonlinear (McCain and Colwell, 2011). The interaction between these two abiotic variables plays a role on a variety of patterns for amphibians, such as determining global and local species range limits (Duellman and Sweet, 1999; Hedges, 1999), and generally on all ecophysiological activities. Understanding how species that experience a variety of conditions across montane environments respond to natural environmental change could be critical to predicting species responses under various climate change scenarios (Duarte et al., 2012).

Some studies have used physical agar models for thermal biology studies in amphibians to simulate the thermal characteristics of the organisms (e.g., Navas and Araujo, 2000; Lertzman-Lepofsky et al., 2020), as physical models are useful to estimate evaporative water loss (EWL) (Spotila and Berman, 1976; Lemenager et al., 2022). Physical agar models are a well-established tool that can accurately estimate water loss when designed properly (Navas and Araujo, 2000; Tracy et al., 2008; Lertzman-Lepofsky et al., 2020). Both operative temperature and EWL can be estimated through the use of agar models, which are important parameters for understanding thermoregulatory behavior and habitat selection. This knowledge can be critical in predicting amphibian responses to climate change and extinction risk (Lertzman-Lepofsky et al., 2020). Direct developing frogs of the genus *Craugastor* are characterized by the lack of a water-dwelling larval phase and are highly dependent on humidity for their fitness, growth and reproduction (Duellman and Trueb, 1994). This group of frogs is therefore a useful model to understand the interaction between these abiotic niche axes at local scales along an elevation gradient.

Here, we analyze how variation in evaporative water loss and temperature along an elevation gradient affect frogs in the genus *Craugastor*. To do so, we used physical agar models as a proxy to simulate the physiological properties of a frog by measuring the rate of EWL and the operative temperature (defined as the temperature of an animal in steady-state; Angilletta, 2009). We expect EWL to increase at higher temperatures, such as in lowlands, given that in warmer conditions EWL tends to increase (rates of EWL can act antagonistically with temperature; Lertzman-Lepofsky et al 2020). In addition, we expect that the operative temperature presents a negative linear pattern across the elevation gradient, given the operative temperature is highly correlated with the environmental temperature.

2. METHODS

2.1 Agar frog model

To estimate EWL, we used physical agar models of frogs (Navas & Araujo, 2000) in the field (see below). These physical models were designed from *Craugastor loki* body size and shapes from latex molds made from rubber models using *C. loki* museum specimens. Models were made with granulated agar (ThermoFisher Scientific), using 2.2 g into 100 mL of distilled water in which a data logger (Ibutton Thermochron, 4K) was placed to record the operative temperature (see below). Agar frog models and live frogs dehydrated at a similar rate when we measured their mass at regular intervals (30 min) in the lab ($F_{2,39} = 0.32$, $P = 0.73$). Thus, the agar frog models are valid proxies for studying EWL of frogs in the field environments.

At each locality, we deployed 10 physical agar models. We estimate hourly rates of EWL, recording the mass of each fully hydrated agar model at the start of each trial (*Mi*) and again (*Mt*) every four hours during the day (8:00-12:00, 12:00-16:00, 16:00-20:00), and every 12 hours at night (20:00-8:00) (Lertzman-Lepofsky et al., 2020; Percino-Daniel et al., 2021). We recorded weight with the use of an electronic balance at 0.01g (Ohaus Scout). In the case of agar frog model damage, either by animals (e.g. opossum, mice, ants or slugs) or hard rain, we replaced them with another new full hydrated model at the start of each trial. For each model we calculated the hourly rates of EWL by dividing the relative change in mass (*Mi* - *Mt*) by the time between measurements (*t,* hours, Lertzman-Lepofsky et al., 2020).

2.2 Operative temperature model

Operative temperature was recorded using a data logger inserted in each model, programmed to record temperature every 10 min for 24 h (Percino-Daniel et al., 2021). For each locality, the agar frog models were new and fully hydrated. Each model was placed in the microhabitats where *Craugastor* frogs were found including on top and under leaf litter, small trunks cavities and under fallen logs.

Additionally, we measured the local environmental temperature and humidity using data loggers (HOBO Onset) programmed to collect data every 30 min to estimate temperature and humidity for each locality.

2.3 Agar frog models deployment in the field

We deployed the 140 physical agar models at 14 different localities in the Sierra Madre del Sur in Chiapas, Mexico, which is a physiographical region with an elevation gradient from 250 m to 2250 m.a.s.l., with lowland humid forest to montane cloud forest. These localities correspond to five different environmental landscapes as a proxy for environmental heterogeneity (for more details see Percino et. al., 2021). For each landscape, we visited three localities during the rainy season (June to November) when frogs are most active, in 2017 and 2018. In Figure 1, we show the five different environmental landscapes.

2.3 Data analyses

We explored our data and used non parametric tests because the data were non-normally distributed. To test for differences between EWL and operative temperature among the localities along the elevation gradient, we used a Kruskal-Wallis test and the *pos hoc* Wilcoxon pairwise test. We assessed EWL during the daytime using Mann-Whitney-Wilcoxon tests. We also fitted generalized linear models (GLM) to evaluate the effect of elevation, operative temperature, locality, environmental landscape, environmental temperature and humidity on EWL using gaussian distributed errors and identity link function. We used analyses of variance to compare the Akaike Information Criterion (AIC) scores and to select the most parsimonious GLM with the lowest AIC score. All statistical analyses were performed using the software R v 3.6.1 (R Core Team, 2020).

3. RESULTS

3.1 Evaporative water loss

Evaporative water loss (% of body mass loss/h) differed across localities (Kruskal-Wallis test, $X^2_{13} = 169.67$, $P<0.0001$, Fig.2). The EWL average across all localities was 0.68% and the lowest elevation locality (248 m.a.s.l.) had a significantly higher EWL (2.16%), while the locality with highest elevation (2137 m.a.s.l.) was 0.62%. There are some localities with no significant differences among them, especially at the intermediate elevations (orange and yellow environmental landscapes, Supplementary material Table S1, Fig. 2).

The EWL varied between day and night, with a higher EWL during the day (0.73%) than night (0.57%) (Mann-Whitney-Wilcoxon Test, $W = 77827$, $p < 0.001$, Fig 3). There was variation in both daytime and nighttime EWL across localities along the elevation gradient (Kruskal–Wallis test, $X^2_{13} = 129.07$, $P < 0.0001$; $X^2_{13} = 75.232$, $P < 0.0001$; Fig 3a). The locality at the lowest elevation had higher EWL during the daytime (2.56%) and nighttime (1.59%) than all other localities. However, some localities at intermediate elevations belong to orange and yellow environmental landscapes and EWL were not statistically different (Supplementary material Table S3, Fig. 2).

3.2 Operative temperature

The operative temperature shows a clinal pattern along the elevational gradient and environmental landscape (Kruskal-Wallis test, $X^2_{13} = 656.63$, $P<0.0001$, Fig. 3b). The higher operative temperature was recorded at lower elevations and lowest operative temperature at higher elevations. Higher operative temperatures occurred at daytime than night (Mann-Whitney-Wilcoxon Test, $W = 130872$, $P<0.0001$, Fig. 3b). Further, operative temperatures at daytime and nighttime varied among localities across the elevation gradient (Kruskal-Wallis test, $X^2_{13} = 437.26$, $P<0.0001$, $X^2_{13} = 296.77$, $P<0.0001$, respectively. Fig. 3b, Table S4, Table S5). However, at intermediate elevations (i.e. yellow and red environmental landscapes), operative temperatures at nighttime did not show any statistical difference (Table S5).

The relative humidity was higher during the rainy season in comparison with the dry season (Mann-Whitney-Wilcoxon Test, $W = 220172$, $p < 0.001$, Table 1). For each locality, the relatively humidity varied across the elevation gradient, both in the rainy season (Kruskal-Wallis test, $X^2_{11} = 29666$, $P<0.0001$, Table 1, Table S6) and in the dry season (Kruskal-Wallis test, $X^2_{11} = 19420$, $P<0.0001$, Table 1, Table S7). The environmental temperature was higher during the dry season than rainy season ($W = 1.5062e+10$, $P < 0.0415$, Table S8). For each locality across the elevation gradient, in both rainy season ($X^2_{11} = 104118$, $P<0.0001$) and dry season ($X^2_{11} = 69684$, $P<0.0001$), the environmental temperature showed a clinal pattern (Supplementary material Table S8).

The GLM model that best explains which variables have an effect on EWL includes the locality ($P < 0.0001$), elevation ($P=0.0035$) and operative temperature ($P<0.0001$) (Fig. 4. Supplementary material, Table S9, S10).

4. DISCUSSION

Rates of EWL of ectotherms vary depending on multiple of factors, including, organism size, sex, characteristics of habitat such as windspeed, humidity, temperature (Claussen, 1969; Heatwole et al., 1969; Spotila and Berman, 1976), among others. In our study, we found that EWL across an elevation gradient is notably different at lower elevation localities than higher elevations. However, EWL at intermediate and higher elevations did not differ. In contrast, the operative temperature presents a clinal pattern, where lower elevation localities show higher temperature and higher elevations show lower temperatures. Relative humidity results showed a similar elevational gradient as operative temperature.

Evaporative water loss represents the differences between the concentration of water vapor within the animal (below the skin) and its environment (free air beyond the adhering boundary layer of air next to animal surface). In rainy conditions, the relative concentration of humidity is expected to be high. In our study, we found that during the rainy season, relative humidity across the elevation gradient was maintained around 90 % (Table 1). In contrast, during the dry season, relative humidity decreased ~15 - 20% in comparison to the rainy season (Table 1). This change in humidity can have a negative effect on the availability of the microrefugia for these frogs (Hoffmann et al., 2021), particularly when they are not active and need to spend time in microrefugia. Additionally, during the dry season, the environmental temperature and soil temperature are higher and interact to intensify the soil dryness and stress the water balance in the frogs. If the frogs are inactive it could be dangerous because they could exceed the physiological thresholds.

Moreover, in the rainy and dry seasons in our study area, there were days with environmental temperatures that exceeded the thermal maximum limits of the frogs (Percino-Daniel et al., 2021). In warmer conditions, EWL can increase with higher vapor pressure deficit and potentially have a fitness cost on dehydration condition (McCain and Colwell, 2011). Various frog studies have documented the effects of dehydration in amphibians, which produce deficits in some metabolic processes that support locomotion (Beuchat et al., 1984; Moore and Gatten, 2022) and ultimately influence performance (McCain and Colwell, 2011). Unfortunately, we did not evaluate performance traits in this study, but in previous work Percino-Daniel, et al., (2021) reported that frog populations at lower elevations present high vulnerability due to low warming tolerance. This study suggests that the high relative humidity in the rainy season in lowlands could be ameliorating thermal conditions and therefore does not affect the performance of the organisms. The humidity conditions and thermal variation across the elevation gradient shows a potential trade-off between thermal and hydration physiology traits in *Craugastor* frogs.

This study contributes to understanding how a terrestrial frog confronts humidity and thermal landscapes, fundamental variables for the persistence of individuals. Such information is critical for assessing conservation needs and concerns as the climate changes. There is a lack of thermal biology and hydration physiology studies that have evaluated EWL of amphibians inhabiting Middle America. Our contribution provides critical empirical data for a terrestrial breeding frog species with broad elevational distribution and it is a member of a group of frogs that has suffered major losses at the species and population level in the recent past. In addition, the vulnerability to climate change and prediction of extinction risk studies usually only take into account thermal traits, but for amphibians, hydration physiology is fundamental for generating an integrated approach. At least these two abiotic axes will provide more reliable predictions (Bovo et al., 2018; Greenberg and Palen, 2021) and are less likely to underestimate vulnerability in climate change scenarios (Lertzman-Lepofsky et al., 2020; Hoffmann et al., 2021).

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CONFLICT OF INTEREST

The authors declare no conflicts of interests exist.

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DATA AVAILIBILITY STATEMENT

Figures

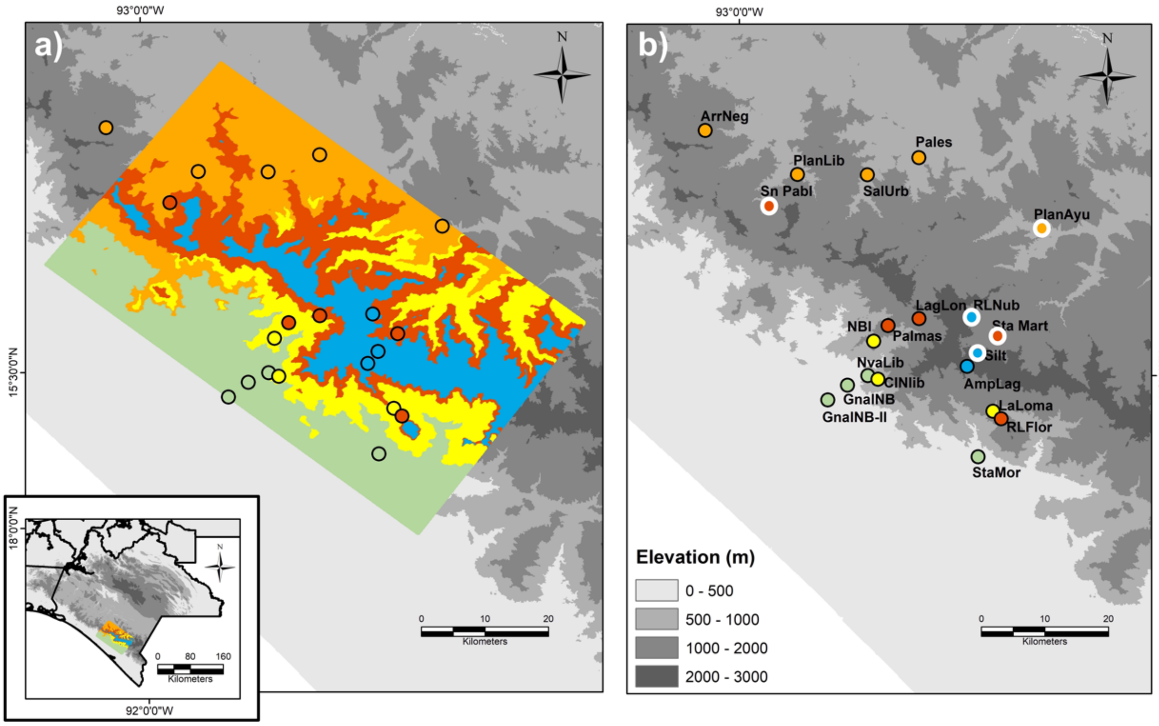

Figure 1. Map of study area in the southern Mexico a) indicating the study localities. Colors represent the five different environmental landscapes. Green corresponds to lowland areas and high annual temperature and precipitation. High precipitation in the warmest quarter and intermediate elevations (yellow). Intermediate elevation and temperature seasonality (orange). Higher elevations (red), and high precipitation in driest season and high elevations (blue). b) Sampling sites were the agar frog models were deployed.

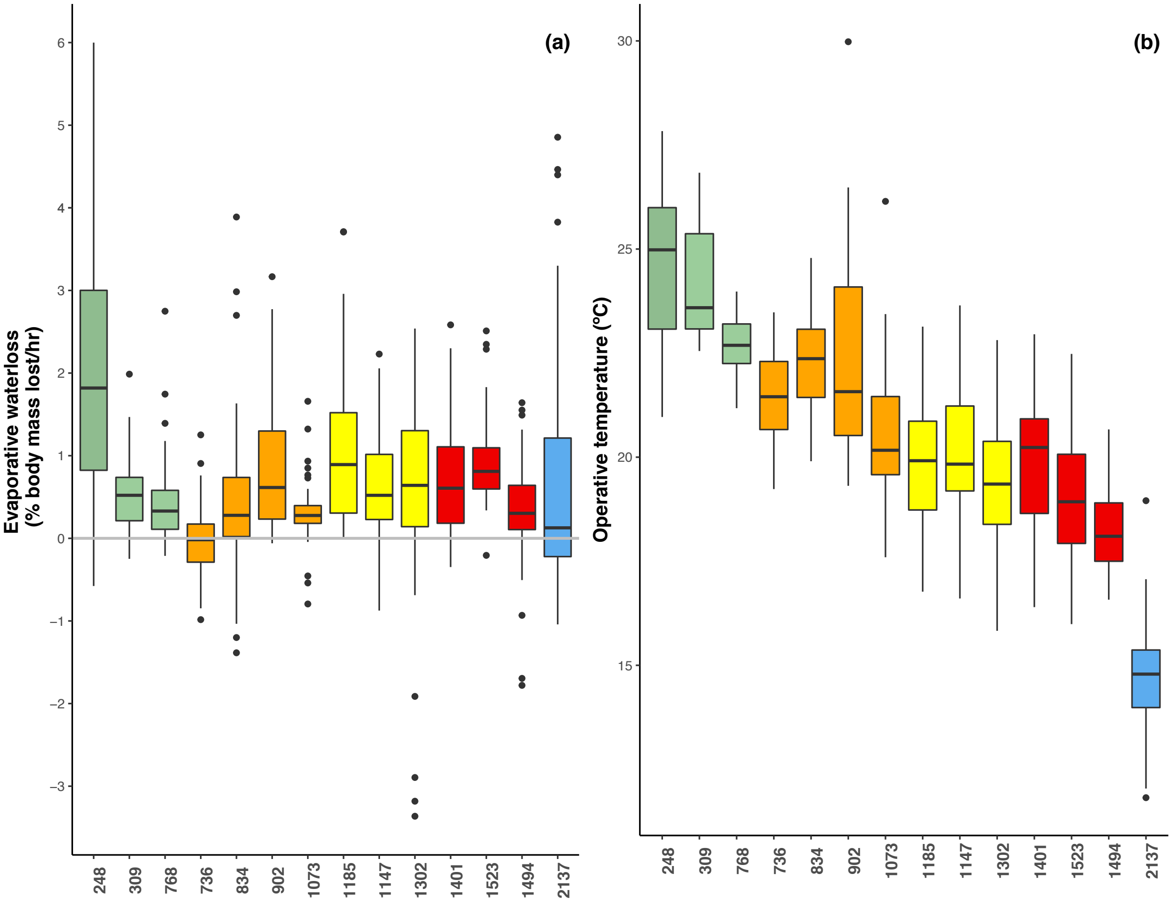

Figure 2. Evaporative water loss (EWL) (a) measured as percent of the body mass lost /h of the agar frog models and operative temperature frog models (b) along the elevation gradient. Colors correspond to each environmental landscape of Fig. 1. The x- axis represents the elevation in meters above sea level.

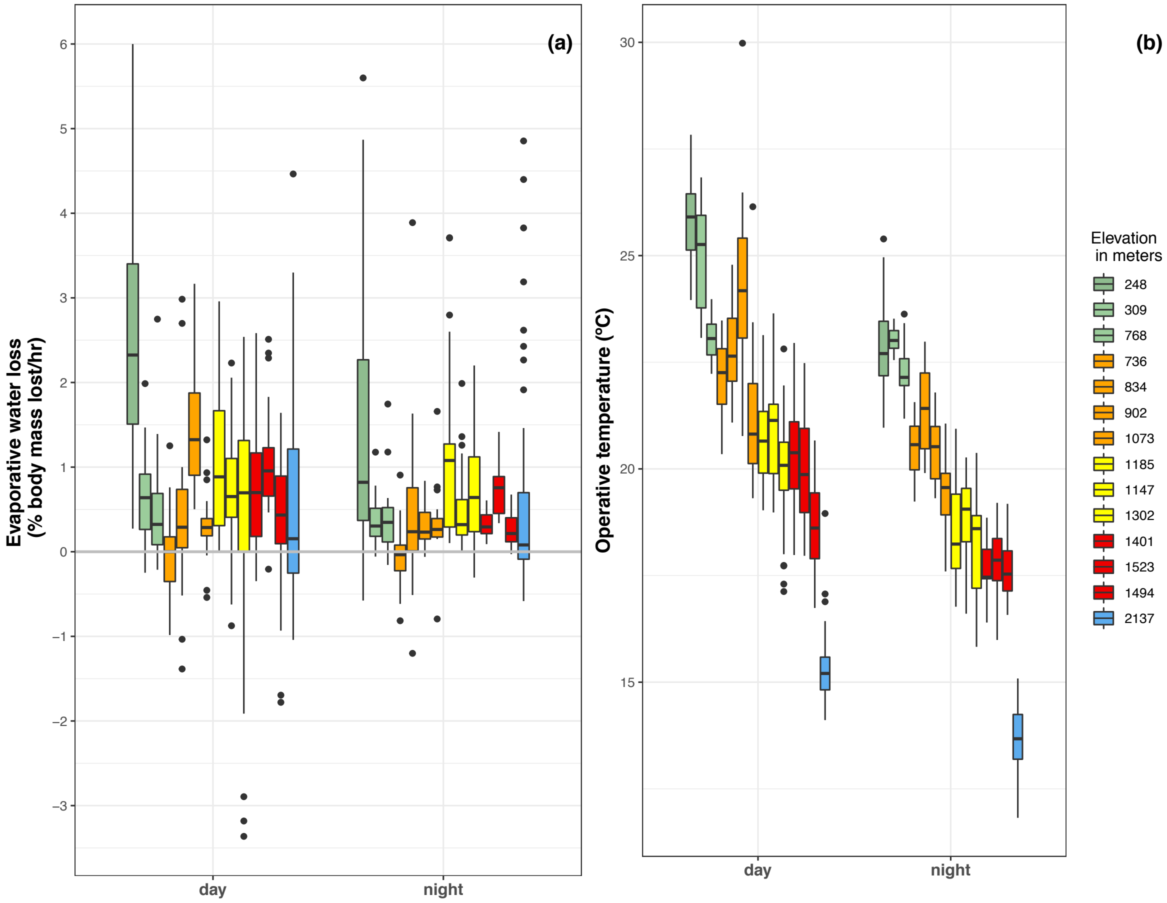

Figure 3. Evaporative water loss of the agar frog models by daytime and nighttime. The colors represent the environmental landscapes and it is ordered by elevation, from lowlands to highlands.

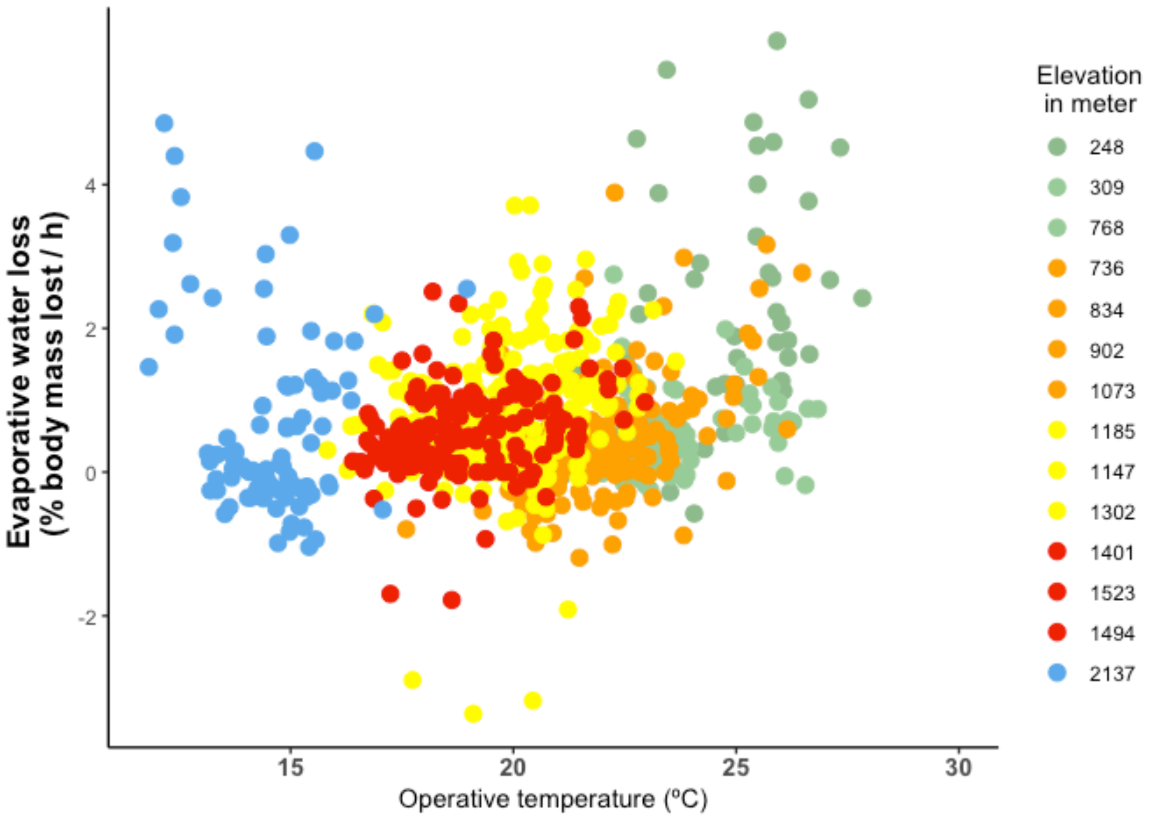

Figure 4. Evaporative water loss and operative temperature of the agar frog models. The colors represent the environmental landscapes.

Tables

Table 1. Mean local humidity in percent per locality sampled during the rainy and dry seasons, as recorded by dataloggers. The number in parentheses represents the minimum and maximum recorded.

		Rainy season	**Dry season**
Environmental landscape / Locality	Elevation	Mean % Humidity	Mean % Humidity
Green			
GNBII	248	94.19 (43.56 – 100)	80.05 (28.27 – 100)
SatMor	309	93.84 (45.99 – 100)	80.87 (27.5 – 100)
NvaLib	768	93.20 (54.12 – 100)	70.79 (37.27 – 99.43)
Orange			
Pales	736	92.41 (43.57 – 100)	75.02 (11.09 – 100)
SalUrb	834	91.70 (34.42 – 100)	75.42 (17.51 – 100)
PlanLib	902	97.75 (48.42 – 100)	85.33 (22.04) – 100)
Yellow			
CINvaLib	1185	94.85 (52.99 – 100)	80.51 (36.50 – 100)
NBI	1148	98.40 (50.17 – 100)	87.04 (44.01 – 100)
LaLoma	1302	93.73 (31.71 – 100)	78.84 (16.75 – 100)
Red			
LagLon	1523	87.97 (39.06 – 100)	77.24 (20.48 – 100)
RLFlor	1494	95.56 (41.43 – 100)	83.09 (27.79 – 100)
Blue			
AmpLag	2137	99.31 (67.72 – 100)	93.74 (39.16 – 100)

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Capítulo 3

Landscape genomics of direct-developing frogs: spatial and environmental divergence along a tropical gradient

Ruth Percino-Daniel, Kara Jones, Tom Maigret, David Weisrock & Daniel Piñero

Para ser enviado a la revista *Heredity*

Este capítulo se refiere al siguiente artículo: “Landscape genomics of direct-developing frogs: spatial and environmental divergence along a tropical gradient” que será próximamente sometido a *Heredity.* El objetivo fue cuantificar si factores abióticos como la temperatura, la precipitación y la elevación tienen un efecto sobre la conectividad y el flujo génico en poblaciones de *Craugastor loki*. Se utilizó secuenciación de ADN asociada a sitios de restricción (*RADseq*) para generar una variedad de loci (SNPs). El muestreo ser realizó en diferentes poblaciones de *Craugastor loki*, que se distribuyen desde los 100 m a los 2250 m de elevación en 13 localidades de la Sierra Madre de Chiapas en el sur de México. Se encontró que las poblaciones muestreadas en elevaciones altas (> 1500 m) probablemente corresponden a una especie previamente no descrita de *Craugastor* ya que se agrupan en un clado altamente divergente y que las poblaciones de *C. loki* entre 120 m y 1500 m están diferenciadas en dos grupos genéticos diferentes, uno en la vertiente del Pacífico con elevaciones de 100 a 1500 m y el otro en la vertiente de la Depresión Central entre los 700 a 1100 m. Además se encontró que la estructura de la población está influenciada por la interacción entre la elevación y la precipitación, pero también por la temperatura. Los resultados resaltan el efecto de los factores abióticos sobre la diversidad genética y enfatizan el papel que pueden desempeñar los análisis espaciales para entender la estructura genética de la población a una escala fina.

Landscape genomics of direct-developing frogs: spatial and environmental divergence along a tropical gradient

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Keywords: elevation gradient, *Craugastor,* environmental heterogeneity, genomics, RADseq

Running title: Landscape genomics of frogs in a tropical gradient

Abstract

Abiotic factors such as geography and environment influence population genetic structure and diversity. However, to identify which abiotic factors are most important to define population structure, both spatiotemporal scales and species-specific life history attributes and ecology must be accounted for. Our study aims to quantify the extent to which abiotic factors such as temperature, precipitation, and elevation are important drivers of gene flow. To do so, we used a direct-developing frog species of the genus *Craugastor* that occurs along a steep elevation gradient. We used restriction-site associated DNA sequencing (RADseq) to generate large numbers of independent SNP loci from individual frogs collected from 100 m to 2250 m asl at 13 localities in the Sierra Madre de Chiapas in Southern Mexico. We quantified population structure using a variety of clustering model-based and landscape genomics approaches. We found that populations sampled at higher elevation (above 1500m) correspond to a different and undescribed species of *Craugastor*, and that populations from *Craugastor loki* between 120 m and 1500 m are clustered in two different genetic groups: a Pacific slope group with elevations of 100 to 1500 m and a Central Depression slope group with elevations from 700 to 1100m. We found population structure to be influenced by the interaction between elevation and precipitation, but also by temperature; a null model of isolation-by-distance was not supported. Our results underscore the

effect of abiotic factors in driving genetic diversity, and highlight the role spatial analyses can play in illuminating fine scale population structure.

Introduction

Biotic and abiotic factors modulate the balance between geneflow and population differentiation (Coyne & Orr, 2004) and can yield a wide range of patterns of genetic and phenotypic variation across the landscape (Cushman et al., 2015). Biotic factors such as life history and ecological interactions can determine population structure (Steele et al., 2009; Travis, 1994). Abiotic factors, such geography and environment also play a key role in population connectivity across a given landscape (Cushman et al., 2015).

Restriction of gene flow across a landscape can be explained by the ubiquitous pattern of isolation by distance (IBD; Wright, 1943), where genetic differentiation increases with the increase in geographic differences. However, IBD assumes a uniform landscape and does not account for landscape heterogeneity. In contrast, a model of isolation by resistance (IBR) considers how characteristics of the landscape inhibit gene flow and result in spatial genetic structure (McRae, 2006). An IBR model predicts a positive relationship between genetic differentiation and resistance to gene flow. Gene flow can also be limited in environmentally heterogeneous landscapes due to divergent selection driving genetic differentiation, leading to isolation by environment (IBE). IBE can be identified by assessing the contribution of environmental factors on genetic variation while controlling for geographic distance (Wang, 2013; Wang & Bradburd, 2014). IBD, IBR and IBE can easily be confused and disentangling which of these processes is more important is key to understand how the landscape influences genetic variation.

Recent studies in amphibians have shown that different climatic variables (e.g., annual mean temperature, isothermally, temperature range and precipitation) function as drivers of population divergence, thus producing a pattern of IBE (Medina et al., 2021; Páez-Vacas et al., 2022). Thermal differences can act as an environmental pressure on phenotypic traits through divergent selection and increase population differentiation (Páez-Vacas et al., 2022). Likewise, seasonal variation in temperature influences the movement and behavior of frogs (Seebacher et al., 2012) resulting in limited gene flow. Indeed, candidate SNPs and structural variants associated with temperature in frogs have been identified and suggest a potential functional role in life history strategies (Cayuela et al., 2021; Medina et al., 2021). Precipitation can limit amphibian distribution at both the regional and local scales (Ochoa-Ochoa et al., 2019). In dry seasons, frogs tend to move less because their ability to absorb water from the ground decreases when soil water potential decreases (Vitt & Caldwell, 2014; Wells, 2007). Despite an increasing number of studies that explore the contribution of environment on population divergence in ectotherm species, much remains to be explored in the way in which environmental variables shape gene flow and genetic diversity at a fine scale. This is particularly true in tropical forests, where the potential for biodiversity loss is higher.

At small scales, tropical mountains are highly heterogeneous in temperature, precipitation and humidity (Stevens, 1992). Thus, they are ideal to study the potential for IBR and IBE to limit gene flow (Funk et al., 2016). Elevation gradients can also be an ideal model to study which factors can drive the adaptive differentiation and identify local adaptation (Luquet et al., 2015).

The Sierra Madre de Chiapas is a physiographic region with an ample variety of habitats, where lowlands and intermediate habitats are influenced by the orientation of both slopes: the Pacific and the Central Depression. The Pacific slope is more humid in comparison to the Central Depression, due to the continental influence. Furthermore, the region offers a natural elevation gradient in both slopes where amphibian species inhabit. Thus, given the natural differences of temperature and precipitation, we can expect that isolation by environment could contribute more than isolation by distance to population structuring, especially for ectothermic organisms, with low vagility and high site fidelity (Zamudio et al., 2016).

Here, we use the amphibian species *Craugastor loki*, a direct-developing frog species found in North and Central America, inhabiting a variety of humid habitats in Chiapas, Mexico. This group of frogs has a wide elevational distribution range spanning from sea level up to 2200 meters above sea-level (masl), particularly in southern Mexico (Frost, 2020; Streicher et al., 2014), and is often locally abundant. Consequently, this species is particularly suitable for investigating fine scale genetic differentiation patterns across landscape-level gradients. Our study aims to (1) quantify both fine-scale genetic differentiation and more distant evolutionary relationships between populations of *C. loki* in the Sierra Madre de Chiapas, (2) test whether patterns of genetic variation in *C. loki* are best explained by geographic distance or environmental conditions, and (3) identify which specific environmental variables might be associated with patterns of genetic differentiation in *C. loki*.

Materials and Methods

Study area and sampling

We collected 90 tissue samples of the direct-developing frog *C. loki* from 13 different localities in the Sierra Madre de Chiapas of southern Mexico (Figure 1, Table S1). Our sampling regime was designed to capture environmental variation along an elevation gradient, with sampling sites ranging from 120 to 2200 masl (Percino-Daniel et al., 2021). We collected mostly toe and, in some cases, liver tissues. Sampling was performed in the rainy season (June to November in 2017). The number of samples varied from five to eight individuals per site. We included *Craugastor pygmaeus* as an outgroup, as it is closely related to the members of the *Craugastor rhodopis* group, which includes *C. loki*. We also included *Craugastor rupinius*, which inhabits the Sierra Madre de Chiapas, is distantly related to *C. loki,* and is nested within the *C. punctariolus* species group (Hedges et al., 2008).

DNA extraction and library preparation

Genomic DNA extractions were obtained using a QIAamp DNA Mini kit. Quality of extractions (20ng/µl) were checked using a Qubit 3 Fluorometric Quantitation Kit (Invitrogen). The library and the sequencing protocol were performed in Floragenex Inc (Eugene, Oregon, USA). We sequenced 94 *C. loki* samples using a restriction-site associated DNA sequencing (RAD-seq) method, employing the restriction enzyme *SbfI.* Sequencing libraries were run on two lanes of an Ilumina HiSeq 2000.

Preprocessing and data assembly

We trimmed adaptors, filtered out low-quality data and demultiplexed raw data of the 94 samples using process_radtags in Stacks v.2.5. (Catchen et al., 2013). We subsequently assembled the data using Ipyrad v.0.7.30 (Eaton & Overcast, 2020), exploring parameter optimization with a focus on the maximum allowed divergence among allelic variants and the number of samples required per locus.

We made two datasets, the first (dataset 1) considered all samples from lowlands and intermediate elevation (120 to 1500 masl), the highlands (samples above to 2000 masl), plus the two outgroup samples of *C. pygmaeus* and *C. rupinius.* The second dataset (dataset 2) only included samples from lowlands to intermediate elevation (100 to 1500 masl). Due that samples from higher elevations likely represent a cryptic species and the dataset 2 was used for intraspecific landscape genomic analysis. For dataset 1, assembly and variant calling was performed using a clustering threshold of 0.85 and a minimum number of samples per locus of

35. For dataset 2, we used 0.85 as clustering threshold and 30 as the minimum number of samples per locus. We filtered one SNP for each RAD locus using VCFTOOLS v.4.2 (Danecek et al., 2011) and PLINK v.1.0.7 (Chang et al., 2015), and converted to VCF format for landscape genomic analysis. Finally, we filtered dataset 2 allowing for at least 50% missing data, a minimum mean read depth of 26, and a minor allele frequency (MAF) of 0.01.

Phylogenetic analyses

We reconstructed phylogenetic relationships among individuals of *Craugastor loki* along the elevation gradient and outgroup species using dataset 1 and two tree-building methods. First, we used FastTree v.2.1.11 (Price et al., 2009), using nearest-neighbor interchange for the topology and subtree-prune and regraft moves (NNI + SPR) for branch length optimization. We used a general time-reversible nucleotide substitution model with a single rate per site (GTR + CAT). Second, we performed a maximum likelihood analysis in RAxML-HPC v.8.2.12 (Stamatakis, 2014) using a GTR + GAMMA nucleotide substitution model and saving the best-scoring tree. For node support we ran 500 bootstrap samples for both analyses. Analyses were performed on the CIPRES Science Gateway v.3.3 (Miller et al., 2010)

Non-spatial population structure analyses

We used two non-spatially aware methods to evaluate population structure. First, we used the non-parametric Discriminant Analysis of Principal Components (DAPC) with the R package Adegenet v.2.1.1. (Jombart & Ahmed, 2011) to analyze both dataset 1 and dataset 2. To determine the number of the groups, we used the find.clusters function and selected the optimal *K* using the lowest BIC value. The optimal number of PCs to retain was selected using a cross-validation procedure (Jombart et al., 2010). Second, we used the model-based method Admixture v.13 (Alexander et al., 2009) to analyze dataset 2. We ran *K* from 1 to 5, with 10 iterations per *K* and selected the *K* with the lowest cross-validation error.

Spatially-informed population structure analyses

We used multiple approaches to investigate the relationship between geographic and genetic information. First, we inferred the strength of an IBD pattern and compared a spatial model with a non-spatial model using the R package conStruct v.1.3 (Bradburd et al., 2018) implemented in R v.3.6.1. conStruct uses an explicit spatial component and models genetic variation in genotyped individuals as partitioned within or admixed specific number of discrete layers, within each layer the relatedness decays as a parameter function of the distance between samples (Bradburd et al., 2018). We analyzed *K* from 1 to 5, each with 10 replicates with 1000 MCMC iterations. We also used a cross-validation function to determine the statistical support for models with and without a spatial component. Because conStruct is sensitive to missing data, we collapsed some closely related individuals from the same population into a single, multi-individual samples (https://github.com/gbradburd/conStruct.git) and ran the cross-validation test using 35 multi-individual samples from 10 localities.

Next, we studied spatial and genomic variation across our sampled elevation gradient using a spatial Principal Component Analysis (sPCA; Jombart, 2008) of dataset 2 implemented in Adegenet v.2.1.1. sPCA relies on an ordination approach for analyzing spatial and genetic patterns; using Moran's *I* index, it identifies eigenvectors which maximize genetic variation and spatial autocorrelation and maps selected eigenvectors on to geographic space.

Finally, we used the ResistanceGA v.4.0.14 software (Peterman, 2018) to associate specific landscape attributes with levels of gene flow. The landscape attributes we used were elevation, temperature, and precipitation (during the rainy season from July to November).

ResistanceGA uses pairwise genetic dissimilarity and a genetic algorithm to optimize resistance surfaces. We employed least cost paths to estimate the effective distances across the landscape (Peterman, 2018) and used a linear mixed effects model with maximum likelihood to test for landscape effects, where pairwise genetic distance is the response variable and the optimized effective distance is the predictor variable. We used both approaches for each individual variable and for all combinations of the individual surface and null model.

Isolation by environment (IBE)

We tested for a signature of isolation by environment using both temperature and precipitation variables, factors highly relevant to amphibian activity. Analyses were performed in the BEDASSLE v1.5 R package (Bradburd et al., 2013), which models the covariance of allelic frequencies as a Gaussian process and uses a Bayesian model to estimate the contribution of environmental and geographic variables. For environmental variables, we obtained bioclimatic variables available for Mexico from Cuervo-Robayo et al. (2014) interpolated to ~90m resolution. We used annual temperature, annual precipitation, temperature seasonality, and precipitation seasonality, variables that have been associated with genetic differentiation in studies of amphibians (Cayuela et al., 2021; Medina et al., 2021; Páez-Vacas et al., 2022). Pairwise Euclidean distances were generated between sampling sites for environmental variables. Pairwise great-circle geographic distances were generated between sampling sites using the function rdist.earth with the package field in R. We standardized both distance matrices by dividing values by their standard deviation constants. We performed two replicate Markov chain Monte Carlo runs to ensure convergence of the parameters using the beta-binomial model, running one analysis for 5 million generations, and the second for 8 million generations, sampling every 1000 generations in both runs. Performance of the model was assessed by visualizing plot acceptance rates and parameter trace plots. We discarded the first 50% of samples as the burn-in and estimated the contribution of the environmental distance versus geographic distance to genetic differentiation using the ratio (αE/αD).

Results

Sequence data and bioinformatics

We obtained ~661 million reads for the 94 samples, with 6.9 million mean reads per sample. The ensembled loci number produced with Ipyrad was 431059 variants. After filtering, we obtained a total variant calling of 848 for dataset 1 and 1801 for dataset 2 (Table 2S). The assembled RADseq data set are available on Dryad repository as VCF files.

Phylogenetics and population structure

Phylogenetic trees generated with both FastTree and RAxML placed samples from the elevations above 2000 m (highlands group) in a separate clade with 100% bootstrap support in both analyses, suggesting that they may belong to a different species of *Craugastor* (Figure 2a). Some samples from intermediate elevations (1200 -1300 m.a.s.l) clustered with the outgroup samples: ArrNeg8 clustered with *C. pygmaeus* and the samples NBra1_1 and NBra1_4 grouped with *C. rupinius*. This is likely explained by the difficulty in differentiating adult members of *C. pygmaeus* from *C. loki*, and the difficulty in differentiating juveniles of *C. rupinius* from *C. loki* (Hedges et al. 2008). Samples from the low-elevation localities and the majority of samples from intermediate-elevation localities were recovered as a clade of *Craugastor loki* in both analyses.

DAPC results for dataset 1 identified a $K = 3$ as the best-fitting model (Figure S1a). The first axis accounted for 39.1% of the variation and identified the greatest separation between a cluster containing samples from the highlands, the outgroup samples (*C. pygmaeus* and *C. rupinius*), and the intermediate-elevation samples associated with those clades in the phylogenetic trees, and two clusters containing samples from the lowland and intermediate elevations (Figure 2b). Interestingly, four additional samples (GnNB2_4 LaFlor2, LaLoma1, LaLoma7) from intermediate elevations were placed closer to the "highland" cluster in ordination space. To verify the identity of these four samples, we reviewed fieldnotes and confirmed uncertainty in our taxonomic identification; thus, we omitted these individuals from the dataset 2.

For dataset 2, DAPC shows three groups, one of which is represented by the orange cluster that corresponds to the Central Depression slope (Figure S1b). The other two groups (light green and dark green) identified in the discriminant space are samples from the lowlands and intermediate elevations of the Pacific slope (Figure S1b), accounting for 16.26% of the conserved variance, BIC score is stabilized at values of K=3 (Figure S1c). The Admixture analysis for dataset 2 reinforces the patterns we found for dataset 1, showing two groups with the cross-validation support for K=2 (Figure 2c). One genetic cluster recovered samples from the Central Depression slope and the other clustered the lowland and intermediate samples from the Pacific slope (Figure 2c).

Our conStruct analyses recovered two genetic clusters (Figure 2c): Central Depression slope (orange color) and Pacific slope (green color) including samples from low and intermediate elevations. However, there is no difference between non-spatial model and spatial model of conStruct (Figure S4).

Landscape genomics

The sPCA detected a significant pattern both globally (P = 0.003) and locally (P = 0.002). The first axis shows that there is a break between the two clusters (Figure S5), Central Depression slope and Pacific slope, both identified with Admixture and conStruct. (Figure 3). Despite this, some sampled localities fall at the boundaries of the genetic break, corresponding to a lower elevation area.

The resistance surface analysis with ResistanceGA found the highest support for a model containing rainy season precipitation and elevation, although a competing model containing only temperature was nearly as well-supported (Table 1). Little to no support was found for a null model of no geographic structuring or for isolation-by-distance.

Isolation by environment

Bayesian analysis of the relative contribution of isolation by environment (IBE) and isolation by distance (IBD) to genetic differentiation in BEDASSLE showed evidence of IBE when elevation was considered. Both temperature seasonality and precipitation seasonality show an important contribution on genetic differentiation across elevation more than the geographic distance (Table 2, Figure S6). The annual mean temperature and annual precipitation never reached convergence despite running for 8 million of MCMC iterations. The mean of the ratio of α_E/α_D can be interpreted as the relative effect of changing one degree Celsius or one millimeter precipitation with one meter elevation distance between populations when the IBD have the same effect size of IBE. That is, for elevation, we would expect that 1°C change of the variation of temperature have a similar effect on genetic divergence as a shift of 857.98 m.

Discussion

Disentangling which abiotic factors have an important role in population connectivity and limiting gene flow can be challenging, particularly given that abiotic environmental variables such as temperature and precipitation are usually autocorrelated with geography (Bradburd et al., 2013), and frequently also with elevation (Barry, 2008). Here, we found significant genetic differentiation between populations of the direct-developing frog *Craugastor loki,* and further found that patterns of gene flow were best explained by the interaction between temperature and precipitation, with elevation suggesting a pattern of isolation by environment.

In terms of *Craugastor loki* specifically, we found that it inhabits areas from sea level to about 1500 masl. Previous work on the evolutionary relationship of the *Craugastor rhodopis* group (including *C. rhodopis, C. occidentalis* and *C. loki*) using mitochondrial DNA suggested three clades of *C. loki* occurring from Mexico to Central America, with the southern clade inhabiting mainly lowland areas (Streicher et al., 2014). Here we confirm that *C. loki* occupies mainly lowland and intermediate elevations and that samples from above the 2000 m of elevation possibly correspond to another species. This group of frogs are highly polytypic and field identification is challenging due to a lack of diagnostic characters. Here we show that the members of *Craugastor* found above 1500 masl cluster separately from *C. loki*, indicating that they either belong to another species that we did not include in the analysis (e.g., *C. matudai*, *C. montanus, C. greggi*) or possibly an unnamed species. Our sampling covers different localities that occur near the type locality of these species, like Cerro Ovando (Figure 1), the type locality where *C. matudai* (Taylor, 1941) and *C. montanus* occur (Taylor, 1942). Both species were described by museum specimens as *C. loki*. *Craugastor greggi* (Lynch, 1965) is also supposed to be present in this area, inhabiting cloud forest like the highland ecosystem where we sampled. Even if all those species represent different groups in the taxonomical classification of

Craugastor, in the field these frogs could not to be distinguished from each other and the original descriptions did not describe the vivid coloration of live individuals. Further work and a geographic more extensive sampling are needed to clarify the evolutionary relationships among these taxa.

Within *Craugastor loki*, we identified two main groups; one cluster from the Central Depression slope and the other from the Pacific slope. Our spatial analyses (sPCA) show a discontinuity in the area sampled that matches a valley that breaks the range of the Sierra Madre de Chiapas (Figure 3). It may be that the dispersal of frogs along the Sierra Madre de Chiapas could be restricted by this break in elevation. Frogs often have low dispersal capacity and limited movement up and down elevation gradients (Duellman & Trueb, 1994). In addition, it seems that the break identified by our spatial analysis is reflected in local forest communities: at the point where the range breaks, to the southeast local conditions become more humid, and to the northwest of the Sierra Madre de Chiapas conditions are usually drier and more influenced by the Central Depression, resulting in a continuous dry forest habitat.

Our landscape resistance and IBE analysis both suggest an effect of the climatic variables on spatial genetic differentiation. Precipitation and temperature are important factors influencing the natural history and ecology of these direct-developing frogs, characterized by the lack of a water-dwelling larval phase and their use of substrate humidity for reproduction (Duellman & Trueb, 1994). The Central Depression slope cluster presents a thermal landscape different from the Pacific slope, where the former is characterized mainly by temperature seasonality (Percino-Daniel et al., 2021). Hence, our results suggest that the abiotic variables considered in our study, temperature and precipitation, have an important role in the pattern of isolation by environment.

Several studies on amphibians have documented patterns of genetic differentiation across elevation gradients (Funk et al., 2005, 2016) and have found evidence of phenotypic divergence associated with temperature and elevation, explained by a pattern of isolation by environment (Medina et al., 2021). In addition, Medina et al. (2021) found a clinal pattern of genomic differentiation associated with temperature and identified some candidate SNPs associated with temperature and body size across the clinal gradient. Our results suggest a pattern of isolation by resistance and isolation by environment. When we used specific layers of temperature and precipitation, we found that the seasonality of both parameters show signatures of isolation for environment when elevation is taken into account. Both environmental variables are intrinsically associated with elevation and the orientation of the slope. In a previous study, Percino et al (2021), studied some physiological traits from the same localities of this study. The frogs that inhabit at the Central Depression slope showed a different thermal sensitivity compared with the populations from Pacific slope. First, they exhibit high thermal accuracy, meaning that the body temperature is close to the microenvironmental temperature, and high thermal quality, that is, the thermal landscape offers suitable microhabitats with temperature close to body temperature. Thus, it is suggested that the landscape plays an important role on frog populations and eventually could drive to phenotypic and probably genotypic divergence, as a result of potential local adaptation. Further work is necessary to specifically study the role of local adaptation or thermal plasticity coupled with the evapotranspiration rates across the elevation in the Sierra Madre de Chiapas that could be associated with genetic differentiation using common garden experiments and studies of genetic expression using RNAseq.

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Competing interests

The authors declare no conflicts of interests exist.

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Figures

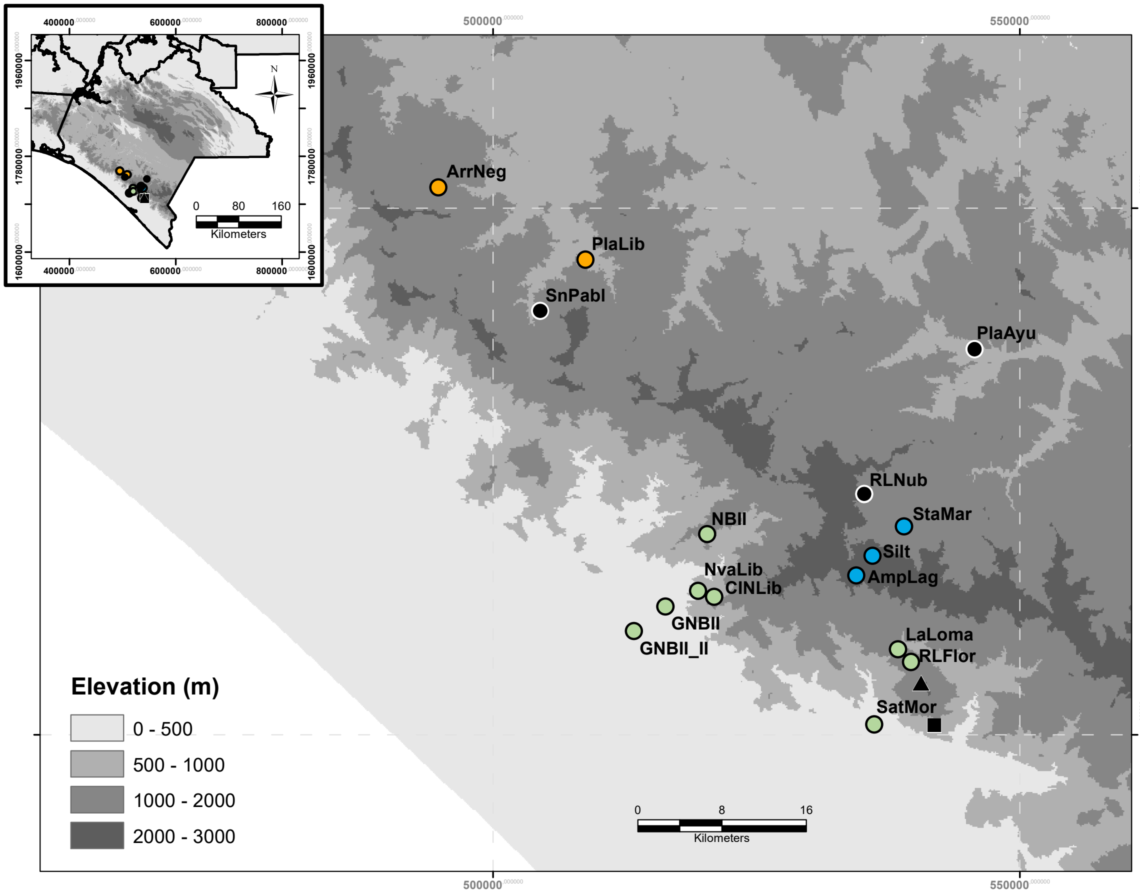

Figure 1. Sampled localities of *Craugastor* in Chiapas, Mexico. The orange and green circles correspond to the two genetic clusters of *C. loki* identified by Admixture and conStruct analysis, as seen in Figure 2. Blue circles correspond to the localities of *Craugastor* sp. from the highlands (see Supplementary material). Black circles with white outline are visited localities where we did not find frogs. Triangle and square symbols correspond to the type localities of the *C. montanus* and *C. matudai,* respectively.

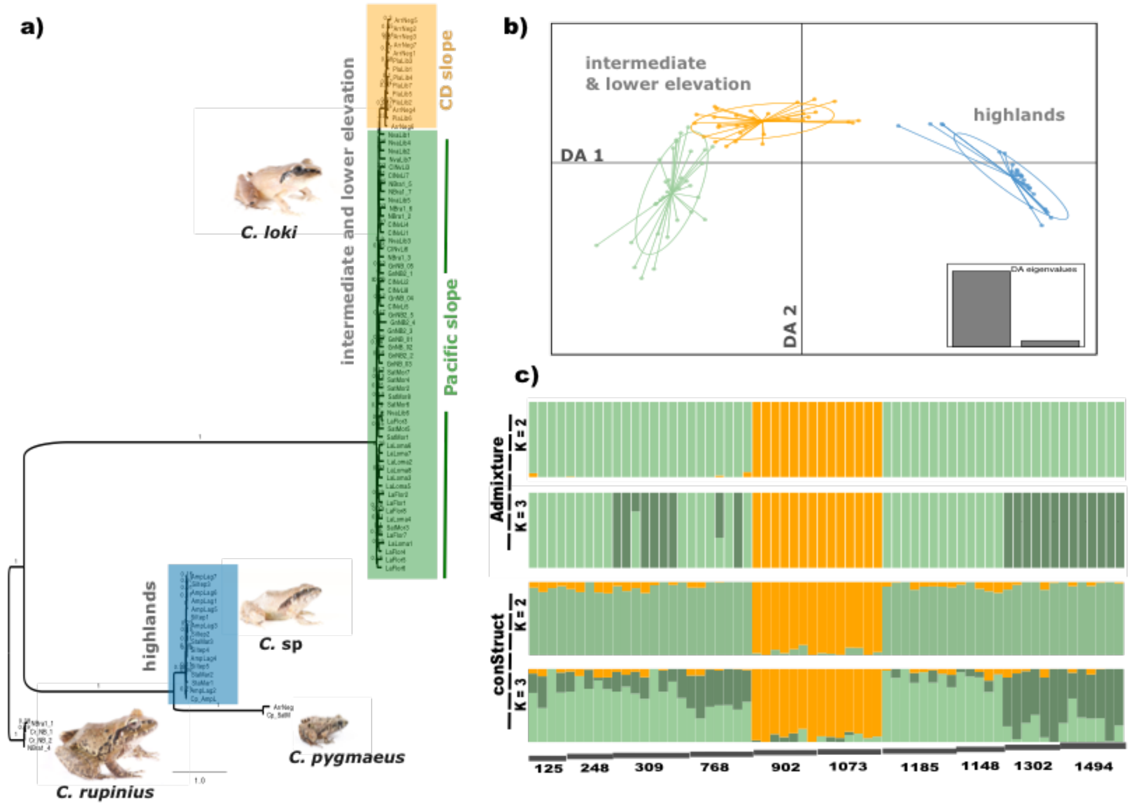

Figure 2. Maximum likelihood phylogenetic tree obtaining from RAXML and (b) discriminant analysis of principle components (DAPC), both applied to dataset 1. Individuals from populations of higher elevation may correspond to a different species of *Craugastor*, and not to *C. loki*. Colors correspond to sampling locations shown in Figure 1. (c) Genetic assignment of individuals using Admixture and non-spatial model conStruct analyses, both applied to dataset 2. Results from $K = 2$ & 3 are shown for both analyses with individuals ordered from lower to higher elevations. Elevation (in meters) is represented on the horizontal axis. A Central Depression cluster and Pacific slope cluster are shown in orange and green, respectively. Frog photos by R. Percino-Daniel (*C. rupinius* & *C.* sp) and J. E. Pérez Sanchez (*C. pygmaeus*).

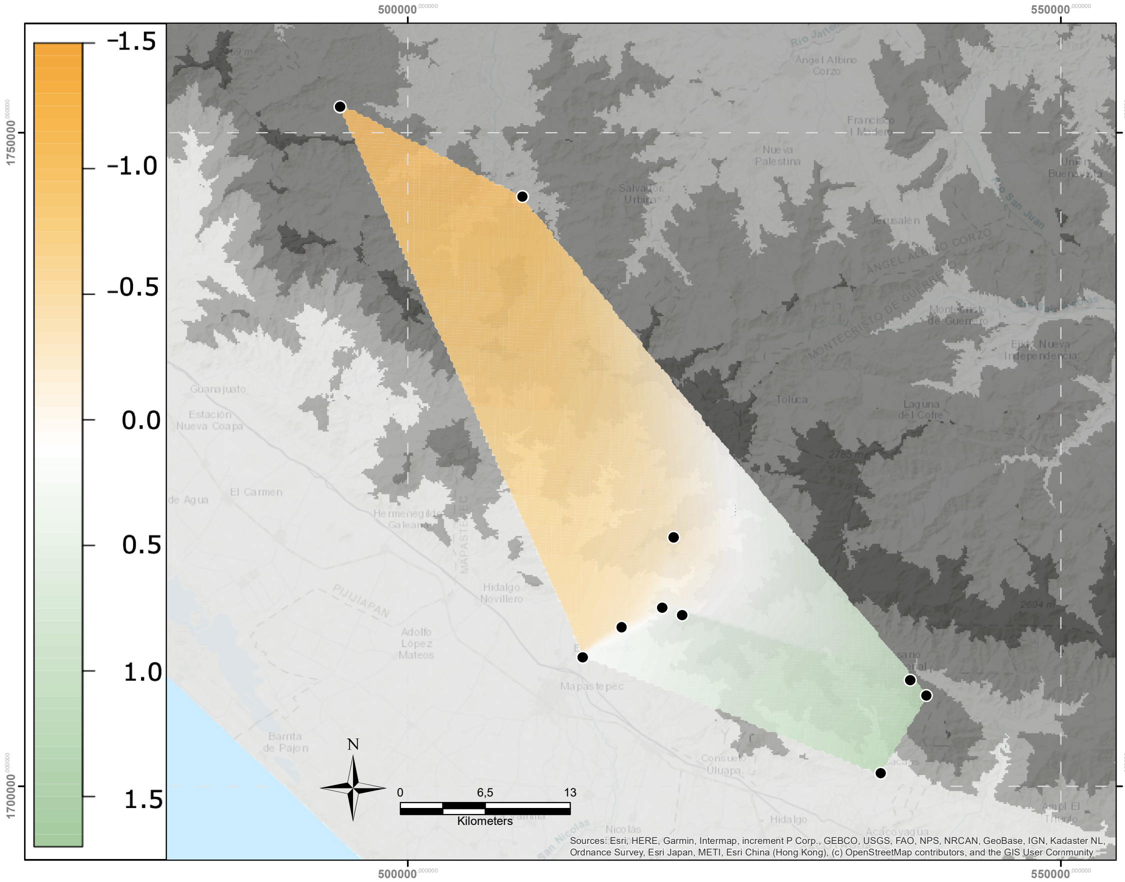

Figure 3. Interpolated spatial genetic structure based on sPCA superimposed over elevation map. The bar at left represents the interpolated vector scores, which exemplified the degree of differentiation between individuals. The white color represents the break of the mountains edge of the highlands at Sierra Madre de Chiapas (lower elevation area). Black dots represent sampled sites.

Tables

Table 1. Summary of model selection for the generalized linear mixed -effects models carried out in ResistanceGA. The null model assumes no geographic structure and the alternative models performed with environmental surfaces using temperature, precipitation and elevation, where the interaction between elevation and precipitation is well fitted to the model.

Layer	k	AIC	AICc	R2m	R2c	LL	delta.AICc	weight
Elevation and Precipitation	7	-7893.07	-7891.07	0.0516	0.1276	3953.53	0	0.3965
Temperature	4	-7891.19	-7890.51	0.0425	0.1129	3949.60	0.5546	0.3005
Precipitation and temperature	7	-7891.14	-7889.14	0.0487	0.1247	3952.57	1.9287	0.1512
Elevation and temperature	7	-7890.51	-7888.51	0.0486	0.1213	3952.25	2.5596	0.1103
Precipitation	4	-7885.98	-7885.30	0.0513	0.1389	3946.99	5.7669	0.0222
Distance	2	-7884.71	-7884.51	0.0260	0.0950	3944.35	6.5607	0.0150
Elevation, Precipitation and temperature	10	-7885.42	-7881.27	0.0494	0.1256	3952.71	9.7999	0.0030
Elevation	4	-7880.71	-7880.02	0.0260	0.0949	3944.35	11.0420	0.0016
Null	1	-7836.83	-7836.77	0	0.0630	3919.41	54.3006	6.41E-13

Table 2. Results of the BEDASSLE analysis. We ran two replicates for each analysis. The first runs compared the relative effect of the precipitation seasonality (αE) and geographical distance (αD) on genetic differentiation. The third and fourth runs compared the relative effect of the temperature seasonality (αE) and geographical distance (αD). The fifth and sixth runs compared the relative effect of the precipitation seasonality (αE) and elevation distance (αD) on genetic differentiation, while the last two runs compared the relative effect of the precipitation seasonality (αE) and elevational distance (αD).

	Ratio (αE/αD)	**95% Confidence Intervals**
Precipitation seasonality and geographical distance	0.428	(0.212 – 0.695)
Precipitation seasonality (replicate)	0.390	(0.192 – 0.621)
Temperature seasonality and geographical distance	0.483	(0.052 – 1.196)
Temperature seasonality (replicate)	0.436	(0.214 – 0.710)
Precipitation seasonality and elevation distance	12.674	(1.192 – 29.929)
Precipitation seasonality (replicate)	10.323	(1.119 – 25.548)
Temperature seasonality and elevation distance	857.980	(446.588 – 1629.37)
Temperature seasonality (replicate)	932.536	(453.102 – 1826.62)

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Data Accessibility

The data are accessible to the repository in Dryad (XXXXXXX). The repository contains the data in vcf format.

Author Contributions

RPD and DP designed the research. RPD performed the fieldwork and lab work. RPD, KJ, TM analyzed the data. DP, DW contributed with analytical tools. RPD lead the manuscript writing with input of all authors.

Discusión general

Con la integración de los enfoques ecofisiológico y genómico se quiso entender cómo diferentes factores bióticos y abióticos pueden influir en aspectos de variabilidad fenotípica, específicamente las sensibilidades térmicas y el balance de agua, de organismos ectotermos. Así también, entender si estos factores abióticos dentro del paisaje influyen en la variabilidad y estructura genética a lo largo de un gradiente altitudinal.

Sensibilidades térmicas, tolerancia a la pérdida de agua y vulnerabilidad al calentamiento

Las sensibilidades térmicas estudiadas en la rana *Craugastor loki* varían de acuerdo con el paisaje ambiental y a lo largo del gradiente de elevación. La temperatura corporal decrece conforme incrementa la elevación, lo cual es esperado dado que los anfibios son organismos ectotermos. Los límites térmicos estudiados exhiben una aclimatización, específicamente del límite térmico superior CT_{max} a lo largo del gradiente de elevación, que es más alto en elevaciones bajas y tiende a disminuir conforme la elevación incrementa.

Por otro lado, la integración de los parámetros de temperatura corporal y la temperatura preferida (T_{sel}), así como los límites críticos térmicos aportan información del nicho térmico de una especie, e influyen en el desempeño y crecimiento de los organismos (Gvoždík, 2018). No obstante, aunque no realizamos un estudio sobre el desempeño de los organismos a distintas temperaturas, es probable que las diferencias encontradas de la T_{sel} con respecto a la temperatura corporal (T_{sel} fue baja comparada con la temperatura corporal zonas bajas, mientras que T_{sel} fue relativamente alta con respecto a la temperatura corporal a lo largo del gradiente de elevación afecten el desempeño y reproducción de los organismos. Esto es, dado que se observó que en las poblaciones de zonas más altas la temporada reproductiva inicia más tarde y es muy corta a diferencia del resto del gradiente de elevación, donde los individuos podrían estar activos a lo largo de la temporada lluviosa.

La temperatura preferida (T_{sel}) de *Craugastor loki* en la zona de estudio tiende a aumentar conforme aumenta la elevación en cada unidad ambiental, aun cuando es un rango estrecho a lo largo del gradiente, es decir la T_{sel} en zonas bajas es similar a la que de zonas altas. Algunos autores sugieren que la T_{sel} puede ser cercana al límite superior (Angilletta, 2009), por ejemplo en especies que son termoconformadores nocturnos (organismos que su temperatura es igual a la temperatura ambiental), como las lagartijas nocturnas (geckos; Pianka and Vitt, 2003), donde se sugiere que la T_{sel} es un rasgo conservado entre especies (Anderson et al., 2018). Sin embargo, en zonas de mayor elevación se observó que la T_{sel} es más alta de lo que el paisaje térmico está ofreciendo, lo cual puede estar relacionado con un patrón conocido como contra gradiente de variación. En este escenario los organismos que habitan en ambientes fríos tienen a seleccionar temperaturas altas, en comparación de organismos que viven en zonas calientes; ello también está relacionado con que presentan CT_{max} más altos (Llewelyn et al. 2017). Estudios futuros podrían evaluar si esta hipótesis aplica en las ranas del género *Craugastor*, por ejemplo mediante un estudio de jardín común.

Por otro lado, la pérdida de agua por evaporación representa las diferencias entre la concentración de vapor de agua dentro del animal (debajo de la piel) y su entorno (aire libre más allá de la capa límite adherida a la superficie del animal). En condiciones de lluvia, se espera que la concentración relativa de humedad sea alta. En nuestro estudio encontramos que durante la temporada de lluvias la humedad relativa a lo largo del gradiente de elevación se mantuvo alrededor del 90%. En la estación seca, la humedad relativa puede bajar a un 15-20% en comparación con la estación lluviosa, lo que puede tener un efecto negativo en la disponibilidad de los microrefugios para estas ranas (Hoffmann et al., 2021). Durante la estación seca, la temperatura ambiental y la temperatura del suelo son más altas, lo que intensifica la sequía del suelo, generando estrés sobre el balance hídrico de las ranas, y si éstas están inactivas podría ser peligroso si exceden sus umbrales fisiológicos.

Con respecto a los dos parámetros sobre precisión térmica (d_b) y la calidad térmica del ambiente (d_e), que utilizan los datos de la T_{sel} y de temperatura corporal, se observó que las poblaciones en el paisaje caracterizado por la estacionalidad de la temperatura en elevaciones intermedias, tienen alta precisión y calidad térmicas. Lo que sugiere que en ese paisaje ambiental las condiciones podrían considerarse óptimas para *C. loki*, es decir las ranas no tienen que lidiar con temperaturas extremas como lo hacen en las elevaciones más bajas o más altas, son temperaturas de un nicho térmico ideal.

Aunque el muestreo se realizó en la temporada de lluvias y la humedad prácticamente no varió (osciló entre 90 y 100%), se registraron días con temperaturas ambientales altas, incluso superando los límites térmicos máximos (Percino-Daniel et al., 2021). En condiciones más cálidas, la pérdida de agua puede aumentar debido a que hay un mayor déficit de presión de vapor y potencialmente tener un costo ya que las ranas pueden experimentar mayor deshidratación (McCain y Colwell, 2011). Diversos estudios han documentado los efectos de la deshidratación en anfibios, señalando que se pueden producir déficits en algunos procesos metabólicos que están relacionados con la locomoción (Beuchat et al., 1984; Moore y Gatten, 2022) y, en últimas consecuencias, podrían influir en el desempeño de los organismos para todas sus actividades (McCain y Colwell, 2011). Desafortunadamente, no evaluamos rasgos de desempeño, sin embargo con los datos obtenidos se observó que las poblaciones en elevaciones más bajas presentan una alta vulnerabilidad debido a la baja tolerancia al calentamiento. En este sentido, se sugiere que la alta humedad relativa en la temporada de lluvias podría estar amortiguando las condiciones térmicas en las tierras bajas y por lo tanto, no se vería afectado el desempeño de los organismos. Es decir, las condiciones de humedad y la variación térmica a lo largo del gradiente de elevación permiten un balance en los rasgos fisiológicos térmicos y de hidratación en *C. loki* en la zona de estudio.

Limites térmicos críticos

Se observó que el límite térmico superior (CT_{max}) presentó aclimatización asociada a la altitud y a la unidad ambiental, siendo menos claro para el límite inferior (CT_{min}). El proceso de aclimatización se refiere a la plasticidad térmica fisiológica que pueden exhibir los organismos en un ambiente heterogéneo, es decir se define como los cambios que puede presentar un rasgo biológico, el cual es reversible, como respuesta a la variabilidad del ambiente (Gunderson and Stillman, 2015). Encontramos que dentro de cada unidad ambiental a mayor elevación el CT_{max} disminuye. Esto se observa claramente en las poblaciones que van desde los 200 hasta los 1000 m. Notablemente, se ha señalado que el CT_{max} en organismos ectotermos usualmente es un rasgo conservado dentro de una especie en particular (Muñoz et al., 2014), mientras que el CT_{min} puede ser más plástico. Por ejemplo, en ranas del género *Pristimantis* que se distribuyen a lo largo de un gradiente de elevación en la zona de los Andes, el CT_{min} es más variable que CT_{max}. Ello se ha relacionado con la variación térmica microclimática que se presenta en la zona, la cual está dada principalmente en las temperaturas mínimas, lo que sugiere que este grupo de ranas ha aumentado su tolerancia al frío para amortiguar las temperaturas más bajas (Pintanel et al., 2019). Sin embargo, otro estudio interespecífico de ranas terrestres distribuidas a lo largo de un gradiente de elevación tropical ha demostrado que CT_{max} y CT_{min} muestran una variación sustancial entre especies estrechamente relacionadas (von May et al., 2017), donde ambos limites térmicos disminuyen con el aumento de la elevación. Aún así, otros estudios encontraron una variación interespecífica en los límites térmicos críticos relacionados con el tamaño corporal, donde CT_{max} tiende a aumentar con el aumento del tamaño corporal (González-del-Pliego et al., 2020; von May et al., 2019), y CT_{min} tiende a disminuir conforme el tamaño corporal (von May et al., 2019). En este estudio, como ya se mencionó, el CT_{max} de *C. loki* tiende a disminuir conforme aumenta la elevación, pero ello no está relacionado con el tamaño corporal, a diferencia del CT_{min} que tiende a disminuir a medida que aumenta el tamaño corporal. Esto contrasta con los estudios interespecíficos en ranas terrestres, que sugieren que un tamaño corporal más grande es común en hábitats de gran altitud (Gonzalez-Voyer et al., 2011; Hedges, 1999; Santa-Cruz et al., 2019). Además, observamos que las poblaciones de tierras bajas tienden a tener un tamaño corporal más grande. Por otro lado, se observó una correlación entre CT_{max} y CT_{min} que podría ser consecuencia del tamaño corporal. Es decir, la variabilidad dentro de la población en ambos límites térmicos podría estar asociada con el tamaño corporal, lo que da como resultado que los individuos más grandes tiendan a tener un CT_{max} más alto y un CT_{min} más bajo; así, los individuos más grandes toleran mejor el calor y el frío, porque son menos susceptibles a cambios de temperatura relativamente rápidos. Mientras que los individuos más pequeños tienden a tener un CT_{max} más bajo y un CT_{min} más alto, porque son más susceptibles a cambios de temperatura relativamente rápidos.

Los límites térmicos críticos en ectotermos son importantes en términos de su vulnerabilidad al cambio climático (Deutsch et al., 2008). Por ejemplo, el incremento de la temperatura a futuro podría ocasionar la muerte de los organismos que experimentan temperaturas más cercanas o superiores a su CT_{max}. Mientras que en el caso de CT_{min} éstos se vuelven más inactivos (von May et al., 2019). Aún cuando en la zona y temporada de nuestro estudio las temperaturas son bajas, los individuos de *C. loki* están activos durante la noche. Durante el día, están aparentemente inactivos y expuestos a temperaturas elevadas del aire y en la hojarasca, como lo registramos en los diferentes paisajes ambientales, independientemente de la elevación. Es decir, las temperaturas microclimáticas registradas diariamente en elevaciones intermedias y altas pueden alcanzar temperaturas altas, similares a las registradas en sitios de tierras bajas. Algunos estudios con ranas terrestres de desarrollo directo sugieren que los límites térmicos están relacionados con las temperaturas del microclima (González-del-Pliego et al., 2020; Pintanel et al., 2019). Es decir, los límites térmicos presentan variación según el tipo de microhábitat, ya sea bosques abiertos o bosques conservados (González-del-Pliego et al., 2020; Nowakowski et al., 2017, 2018). Por lo que estudios futuros en la zona deberían incluir la variable del tipo de hábitat; por ejemplo evaluar si está modificado y/o alterado y compararlos con bosques conservados, para tener un mejor análisis sobre si los límites críticos térmicos cambian dependiendo del hábitat.

En la hipótesis de la variabilidad climática (Janzen, 1967) se propone que la tolerancia fisiológica y la variabilidad climática en regiones tropicales y templadas podrían ser equivalentes a las condiciones en un gradiente de elevación (Ghalambor, 2006). Por ello, se podría asumir que a nivel interespecífico, la variación ambiental pueda resultar en un cambio adaptativo, mientras que a nivel intraespecífico, la variación podría deberse a la plasticidad térmica, en lugar de una respuesta adaptativa. Esto último es lo que sugieren los resultados obtenidos en este estudio. El rango de tolerancia térmica (*TTR*) en *C. loki* exhibe un patrón diferente al esperado a lo largo del gradiente de elevación. Las poblaciones en elevaciones bajas y altas tienen una *TTR* más estrecha, mientras que en elevaciones intermedias el *TTR* es más amplio. Las temperaturas microclimáticas podrían explicar este patrón, ya que la temperatura máxima promedio ($T_{max\text{-}hab}$) en las tierras bajas y altas es relativamente similar. Es decir, $T_{max\text{-}hab}$ no muestra un patrón con respecto a la elevación, ni tampoco a la unidad ambiental, mientras que la temperatura media anual (T_{mean}) y la temperatura mínima por cada unidad ambiental muestran una ligera disminución con el aumento de la altitud. Esta tendencia no se muestra en $T_{max\text{-}hab}$, donde en tierras bajas (por ejemplo, en la localidad GNBII, 300 m de elevación) puede alcanzar temperaturas de alrededor de 30°C, así como en tierras altas (por ejemplo, en la localidad de LagLon, 1500 m de elevación).

Vulnerabilidad al cambio climático

Los resultados de este estudio proporcionan información sobre la sensibilidad térmica de *C. loki* al cambio climático. Primero, se muestra que las poblaciones de las tierras bajas pueden ser más vulnerables a las altas temperaturas diarias debido a la baja tolerancia al calentamiento (Capítulo I), mientras que las poblaciones de las tierras altas podrían beneficiarse o ser menos vulnerables a los aumentos de temperatura. Algunos estudios macroecológicos sugieren que los organismos tropicales son más vulnerables al cambio climático por dos razones: 1) experimentan temperaturas ambientales cercanas a sus limites críticos térmicos (Deutsch et al., 2008; Kingsolver, 2009) y 2) tienen una estrecha tolerancia térmica (Janzen, 1967) y por lo tanto son más sensibles a los cambios de clima. Aquí, a pequeña escala, se encontró que los individuos de *C. loki* de las zonas bajas son más vulnerables al calentamiento debido a su tolerancia térmica limitada, mientras que aunque potencialmente las de elevaciones altas pudieran ser menos vulnerables, ello varía dentro de cada unidad bioclimático. Por ejemplo, las poblaciones muestreadas entre ~1300-1400 m también exhibieron una baja tolerancia al calentamiento y estos sitios experimentan altas temperaturas. Además la temperatura diaria máxima registrada en algunos sitios superó los 40 grados centígrados, siendo temperaturas extremadamente peligrosas para organismos termoconformadores. De hecho, los informes del IPCC (2019) predicen que la temperatura global aumentará o superará los ~1.5ºC, por lo que es fundamental comprender cómo responderán las ranas a este aumento del calentamiento. Por ello, un enfoque integrativo del nicho térmico de una especie junto con los rasgos fisiológicos podría ayudar a comprender mejor los patrones generales de tolerancia térmica (Frishkoff et al., 2015).

Asimismo, el papel de la humedad aunado al paisaje térmico puede ayudar a entender como estos organismos podrían enfrentar distintos escenarios de calentamiento climático. Cabe señalar que son escasos los estudios sobre biología térmica y fisiología de la hidratación que hayan evaluado el papel de la pérdida de agua y de la vulnerabilidad en especies distribuidas en Mesoamérica. De ahí también la contribución de este trabajo, donde a partir de datos empíricos de una especie de rana terrestre de desarrollo directo que presenta una amplia distribución altitudinal nos ayuda a entender cómo podrían organismos ectotermos termoconformadores hacer frente a distintos escenarios de cambio climático. Generalmente, los estudios de vulnerabilidad al cambio climático y predicción del riesgo de extinción solo toman en cuenta las características térmicas; sin embargo, en anfibios, el balance de agua es fundamental para generar un enfoque integrado con al menos estos dos ejes abióticos que proporcionará predicciones confiables (Bovo et al., 2018; Greenberg y Palen, 2021), para así no subestimar la vulnerabilidad ante el cambio climático (Lertzman-Lepofsky et al., 2020; Hoffmann et al., 2021).

Genómica del paisaje

Los factores abióticos tales como temperatura y precipitación tienen un papel importante en la conectividad de las poblaciones, ya que influyen positiva o negativamente en el flujo de genes. En qué medida es uno de los desafíos a estudiar, dado que estas variables generalmente están autocorrelacionadas con la geografía (Bradburd et al., 2013) y frecuentemente con la elevación (Barry, 2008). Es este estudio se encontró que las poblaciones de *C. loki* estudiadas presentan diferenciación genética explicada por resistencia y por un patrón de aislamiento por ambiente.

Se identificaron dos grupos genéticos, uno en la vertiente de la Depresión Central y el otro de la vertiente del Pacífico. Se identificó una discontinuidad en el zona de estudio que coincide con el parteaguas de la Sierra Madre de Chiapas. Está discontinuidad refleja diferencias en condiciones ambientales, tales como mayor húmedad al sureste , mientras que al noroeste de la Sierra Madre de Chiapas las condiciones suelen ser menos húmedas, además que una marcada estacionalidad (lluvias y secas). Las especies de anfibios en general son especies poco vágiles, es decir que tienen una capacidad limitada para la dispersión. Sin duda el parteaguas de la Sierra Madre representa una barrera que resulta en que las poblaciones de ambas vertientes estén diferenciadas genéticamente. Aunado a esto, otra barrera es la ambiental; de acuerdo a los resultados de resistencia y de aislamiento por ambiente, se observó un efecto de las variables climáticas sobre la diferenciación genética. El grupo genético de la Depresión Central presenta un paisaje térmico diferente al de la vertiente del Pacífico, donde el primero se caracteriza principalmente por la estacionalidad de la temperatura. Además, la temperatura y la humedad son importantes en la biología y ecología de anfibios en general. *Craugastor loki* se caracteriza por no presentar una fase larvaria, es decir son de desarrollo directo, por lo que necesitan de hábitats húmedos, sobretodo del sustrato húmedo para la reproducción (Duellman & Trueb, 1994).

Varios estudios de anfibios han documentado patrones de diferenciación genética a lo largo de un gradiente de elevación (Funk et al., 2005, 2016) y han encontrado evidencia de divergencia fenotípica asociada con la temperatura y la elevación, reflejando un patrón de aislamiento por ambiente (Medina et al., 2021). Los resultados del capítulo tres muestran que este patrón de aislamiento por ambiente probablemente explica la diferenciación genética encontrada en las poblaciones estudiadas en la Sierra Madre de Chiapas para *C. loki,* donde la estacionalidad en temperatura y precipitación, variables que convergieron en el análisis de aislamiento por ambiente, sustentan este patrón. No obstante, son necesarios estudios futuros para poner a prueba si tanto divergencias fenotípicas y genotípicas, son resultado de una posible adaptación local o si es debido a la plasticidad térmica. Entre los estudios que son ampliamente usados para identificar variación adaptativa y potencial adaptación local son los experimentos de jardín común, así como estudios de expresión genética usando RNAseq. Así como estudios de asociación genotipo ambiente, identificando polimorfismos en ambientes contrastantes.

Conclusiones

- La temperatura corporal en individuos de *Craugastor loki* disminuye a medida que aumenta la temperatura ambiental. La temperatura preferida tiende a aumentar moderadamente con la elevación dentro de las unidades ambientales muestreadas.
- Se sugiere que el paisaje térmico adecuado para esta especie se encuentra en elevaciones intermedias, donde la precisión térmica (d_b) y la calidad térmica del ambiente ($\mathrm{d_e}$) son óptimas.
- El límite térmico máximo (CT_{max}) varía a lo largo del gradiente de elevación y entre los dominios bioclimáticos, disminuyendo a medida que aumenta la elevación. Mientras que el limite térmico mínimo (CT_{min}) no es tan variable como el CT_{max}.
- Las poblaciones de tierras bajas podrían ser más vulnerables a futuros aumentos de temperatura.
- El porcentaje de pérdida de agua (EWL) fue similar a lo largo del gradiente de elevación, aunque mayor en la localidad de menor elevación (248 m) en comparación con el resto de las localidades del gradiente. Asimismo, la EWL fue mayor durante el día en comparación con la noche.
- La temperatura operativa presenta una variación clinal. En las tierras bajas la temperatura fue más alta, mientras que más baja a mayor altitud. Durante el día, la temperatura operativa es más alta en comparación con la noche.
- Se sugiere que la humedad relativa, la cual fue similar a lo largo del gradiente durante la temporada de lluvias, produce condiciones de humedad que permiten el equilibrio hídrico entre las ranas y su hábitat, que amortigua las condiciones térmicas a las cuales están expuestas.
- Las poblaciones muestreadas en elevaciones más altas (por encima de los 2000 m) corresponden probablemente a una nueva especie de *Craugastor*.
- Las poblaciones de *Craugastor loki* entre 120 m y 1500 m conforman dos grupos genéticos diferentes: un grupo de la vertiente del Pacífico con elevaciones de 100 a 1500 m y un grupo de la vertiente de la Depresión Central en elevaciones de 700 a 1100 m.

- La estructura de la población está influenciada por la interacción entre la elevación y la precipitación, pero también por la temperatura.
- Los resultados sustentan que los factores abióticos influyen sobre la diversidad genética y se sugiere que un aislamiento por resistencia y ambiente está explicando la diferenciación genética que se encontró en el gradiente de elevación.

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