UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

GILBERTH JOSÉ ALVARADO BARBOZA

Experimental study and anatomo-histopathological, clinico-pathological and behavioral characterization of chytridiomycosis in *Lithobates vibicarius* in the highlands of Costa Rica

São Paulo
2019

ALVARADO, G.J.

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DOUTORADO
FMVZ/USP - 2019

GILBERTH JOSÉ ALVARADO BARBOZA

Experimental study and anatomo-histopathological, clinico-pathological and behavioral characterization of chytridiomycosis in *Lithobates vibicarius* in the highlands of Costa Rica

Thesis submitted to the Postgraduate Program in Experimental and Comparative Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

Department:
Patology

Área de concentração:
Experimental and Comparative Pathology

Advisor:
Prof. Dr. José Luiz Catão-Dias, Ph.D.

São Paulo
2019

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São Paulo, 17 de junho de 2019
CEUA N 2657080715

Ilmo(a). Sr(a).
Responsável: José Luiz Catão Dias
Área: 0

Título da proposta: "Estudo experimental e caracterização anátomo-histopatológica, clínico-patológica e comportamental da quitridiomicose em Lithobates vibicarius nas terras altas da Costa Rica".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ (ID 004766)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e APROVOU a Alteração do cadastro (versão de 23/maio/2019) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Queríamos informar-lhes que por razões técnicas, de tempo e orçamento, o objetivo correspondente à metabolômica em acordo com o meu orientador, Dr. José Luiz Catão-Dias, decidimos eliminá-lo do projeto com o propósito que não seja avaliado no âmbito da tese de doutorado. Da mesma forma, por razões técnicas, na obtenção das amostras, não foi possível incluir a espécie Lithobates taylori nos resultados finais da tese. As amostras foram coletadas para Lithobates vibicarius de acordo com o que é indicado na proposta de projeto para atingir o objetivo da metabolômica, mas as mesmas serão processadas após a defesa do doutorado. Pelo exposto e buscando a concordância absoluta entre o título de defesa do doutorado (prazo final de defesa no mês de julho) e o título de aprovação pela comissão que vocês representam, queríamos respeitosamente solicitar a modificação do título do projeto acordo com a carta anexada.".

Comentário da CEUA: "Aprovada a mudança no título da tese, uma vez que não foram realizadas as análises de metabolômica, e restrito a apenas uma espécies de anfíbio, sem alteração no n. experimental inicial.".

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretária
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

UNIVERSIDAD DE COSTA RICA
VICERRECTORIA DE INVESTIGACION
COMITÉ INSTITUCIONAL PARA EL CUIDO Y USO DE ANIMALES

Lunes 29 de junio de 2015
CICUA-028-15

Doctor
Gilbert Alvarado
LEBi

Estimado Dr. Alvarado:

En la sesión número 149 del Comité Institucional para el Cuido y Uso de Animales (CICUA), del jueves 25 de junio del 2015, se evaluó y ratificó el proyecto "Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el neotrópico".

El mismo se aprueba.

Atentamente,

ORIGINAL FIRMADO

Dra. Sara González Camacho
Coordinadora
CICUA

Cc: Dr. Domingo Campos, Dirección de Gestión de Investigación
Archivo

UNIVERSIDAD DE COSTA RICA
VICERRECTORIA DE INVESTIGACION
COMITÉ INSTITUCIONAL PARA EL CUIDO Y USO DE ANIMALES

08 de julio de 2015
CICUA-032-15

Señores
Comissão da Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

Estimados señores:

Por medio de la presente hacemos constar que todos los protocolos referentes a la experimentación animal del proyecto "Estudo experimental e caracterização anatomo-histopatológica, clínico-patológica e metabolômica da doença e infecção por *Batrachochytrium dendrobatidis* em *Lithobathes vibicarius* e *Lithobathes taylori* nas terras altas da Costa Rica", correspondiente al proyecto de doctorado de Gilberth José Alvarado Barboza, estudiante del programa de doctorado de Patología Experimental y Comparada de la Facultad de Medicina Veterinaria y Zootecnia de la Universidad de São Paulo, tiene la aprobación y respectivos permisos por parte del Comité Institucional para el Cuido y Uso de Animales (CICUA) de la Universidad de Costa Rica (UCR), bajo el código CICUA 149-15 del 25 de Junio del 2015. Dicho proyecto comenzará a regir a partir del 1 de enero de 2016.

Agradeciendo la atención a la presente.

Atentamente,

Dra. Sara González Camacho
Coordinadora
CICUA-UCR

Cc: Archivo

UNIVERSIDAD DE COSTA RICA

VI Vicerrectoría de Investigación

4 de octubre de 2016
VI-6457-2016

Dr. Gilbert Alvarado Barboza
Investigador
Laboratorio de Ensayos Biológicos (LEBI)

Estimado señor:

De conformidad con lo dispuesto en el "Reglamento sobre el Acceso a la Biodiversidad en Actividades de Docencia, Acción Social y de Investigación en la Universidad de Costa Rica", la Comisión Institucional de Biodiversidad revisó el proyecto "**Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el neotrópico"**, el cual se encuentra bajo su coordinación.

Le comunico que la Comisión Institucional de Biodiversidad aprobó el permiso de acceso a los elementos de la biodiversidad que se indican en su proyecto de investigación. Por esta razón le envío la Resolución #069 de la Comisión mediante la cual se le comunican las condiciones con que se aprueba este permiso de acceso.

Le saluda con toda consideración,

UNIVERSIDAD DE COSTA RICA | VICERRECTORÍA DE INVESTIGACIÓN

M.Sc. Jorge Warner
Coordinador
Comisión Institucional de Biodiversidad

JW/lf
C. MAP. Darío Hernández Castro. Gestor, VI
Consejo Científico, LEBI
Licda. Silvia Salazar F., PROINNOVA
Archivo/Comisión de Biodiversidad

RESOLUCIÓN No. 069

La Comisión Institucional de Biodiversidad de la Universidad de Costa Rica, conoce la solicitud de permiso de acceso a los elementos y recursos genéticos y bioquímicos de la biodiversidad.

RESULTANDO

Primero: Que se presentó para su revisión por parte de la Comisión Institucional de Biodiversidad el proyecto denominado "Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el neotrópico.", del Dr. Gilbert Alvarado Barboza, Investigador del Laboratorio de Ensayos Biológicos (LEBI). El proyecto tiene como objetivo general: Determinar la respuesta inmunopatológica de Lithobates vibicarius y Lithobates taylori sometidas a infecciones experimentales con Batrachochytrium dendrobatidis mediante la caracterización anatomohistopatológica, clinicopatológica y de perfiles metabolómicos.

Segundo: Que la Comisión decidió revisar este proyecto de investigación por cuanto se encuentra contemplado en las actividades enumeradas en el Reglamento de Acceso a los Elementos de la Biodiversidad en actividades de Docencia, Acción Social y de Investigación de la Universidad de Costa Rica de conformidad con los artículos 10, 22 a 27 del citado reglamento.

Tercero: Que de la evaluación de la documentación se concluye que el proyecto tiene acceso al material genético, y que lo utilizará con fines de de investigación básica y aplicada.

Cuarto: Que para el análisis de este Proyecto y la emisión de la correspondiente Resolución se han tomado en consideración los Principios Generales, Objetivos y Criterios Aplicables contemplados en el Reglamento así como el marco legal internacional aplicable.

Quinto: Que el Reglamento de Acceso a los Elementos de la Biodiversidad en actividades de Docencia, Acción Social y de Investigación de la Universidad de Costa Rica otorga a la Comisión Institucional de Biodiversidad las competencias para hacer operativo el Reglamento y, entre otras atribuciones, la de aprobar los proyectos de investigación, docencia y acción social en los que haya acceso a los elementos de la biodiversidad.

POR TANTO, LA COMISIÓN INSTITUCIONAL DE BIODIVERSIDAD RESUELVE:

PRIMERO: Aprobar el acceso a los elementos y recursos genéticos y bioquímicos de la biodiversidad que se indican en la propuesta para el desarrollo de la investigación básica denominada "Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el neotrópico.", del Dr. Gilbert Alvarado Barboza, Investigador, **Laboratorio de Ensayos Biológicos (LEBI).**

SEGUNDO: Si como resultado del acceso y del estudio del material biológico se pudieran generar beneficios económicos, el investigador deberá comunicarlo de inmediato y por escrito a la Comisión para que esta proceda a determinar lo que corresponda, incluyendo la necesidad de emitir una nueva Resolución.

TERCERO: Este permiso lo es únicamente para el acceso a los recursos genéticos y bioquímicos. El investigador deberá solicitar por su cuenta, cualquier otro permiso o autorización para el ingreso a los sitios donde se realicen las recolectas de los organismos o se materialice el acceso, los cuales deberán ser tramitados ante las instancias correspondientes del Sistema Nacional de Áreas de Conservación. Este permiso solo autoriza el acceso a los recursos genéticos y bioquímicos de conformidad con los términos del proyecto aprobado. La obtención de cualquier otro permiso para la realización del proyecto (CITES, certificado fitosanitario de exportación o de importación, etc.) es responsabilidad del investigador.

CUARTO: El material biológico que se requiera para referencia deberá depositarse en una colección institucional de la Universidad de Costa Rica.

QUINTO: Cualquier transferencia de material de origen biológico fuera de la Universidad de Costa Rica debe realizarse mediante un Acuerdo de Transferencia de Material (ATM) entre la institución receptora y la Universidad de Costa Rica.

Los términos de este ATM se deben negociar con la participación de PROINNOVA y de la Comisión Institucional de Biodiversidad. Estos términos deben incluir lo relativo a la distribución de posibles beneficios que surjan de la investigación y el acceso al material.

SEXTO: El investigador responsable del proyecto comunicará a la Comisión cualquier cambio en el proyecto que implique una modificación sustancial de las actividades con base en las cuales se otorgó el presente permiso.

SÉTIMO: De conformidad con el cronograma del proyecto aprobado, el presente permiso de acceso se otorga hasta el día 31 de diciembre de 2018. Al finalizar el proyecto, el investigador responsable deberá enviar a esta Comisión un informe del acceso que realizó a los recursos genéticos y bioquímicos de la biodiversidad. Este informe debe incluir lo siguiente:

-Nombre científico y cantidad de muestras recolectadas
-Nombre de recolector o recolectora y fecha en que se recolectaron las muestras
-Número del permiso de recolecta otorgado por el SINAC con el cual fueron recolectadas las muestras.
-Nombre de la colección donde se depositaron las muestras.
-Información detallada de cualquier transferencia de material biológico realizada a otra institución.
-Breve resumen de los principales resultados científicos obtenidos a partir del estudio de las muestras recolectas.

OCTAVO: La Comisión se reserva el derecho de cancelar este permiso en el caso de que se incumpla con lo establecido en esta Resolución o con la normativa legal vigente. Contra esta Resolución tienen lugar los recursos ordinarios de revocatoria ante la Comisión y la apelación ante la persona que ocupe la Rectoría, dentro de los siete días hábiles siguientes a la notificación de la presente Resolución.

Ciudad Universitaria, "Rodrigo Facio", 4 de octubre de 2016
COMUNÍQUESE

UNIVERSIDAD DE COSTA RICA | VICERRECTORÍA DE INVESTIGACIÓN

M.Sc. Jorge Warner
Coordinador
Comisión Institucional de Biodiversidad

JWP/LFC

REPUBLICA DE COSTA RICA

El Sistema Nacional de Areas de Conservación del Ministerio del Ambiente y Energía, solicita a las autoridades nacionales, funcionarios del Ministerio y a todo residente en el territorio nacional, le faciliten al portador del presente pasaporte cientffico toda la colaboración que le sea posible para la realización de la investigación autorizada.

REPUBLIC OF COSTA RICA

The National System of Conservation Areas of the Ministry of the Environment and Energy requests to the national authorities, Ministry's officials and all the residents in the national territory, to provide to the bearer of this scientific passport all the possible assistance to carry out of the authorized research.

Ministerio del Ambiente y Energía
Sistema Nacional de Áreas de Conservación

PASAPORTE CIENTÍFICO
Scientific Passport

Alvarado Barboza Gilberth, José
Apellidos / *Last name* •• Nombre / *Name*

1-1162-0443
Nº Identificación / *Identification Nº*

Costarricense
Nacionalidad
Nationality

19 marzo 1983
Fecha de Nacimiento
Date of Birth

Nº Pasaporte
Passport Nº

Costa Rica
País / *Country*

Patología Vida Silvestre
Profesión / *Profession*

Escuela de Biología, Universidad de Costa Rica
Universidad o Centro de Investigación
University or Research Center

Título de la Investigación: La complejidad de las declinaciones poblacionales de anfibios: un enfoque multidisciplinario en búsqueda de una respuesta.

Área de Conservación donde se autoriza:

- ☐ Área de Conservación Cordillera Volc. Central (**ACCVC**)
- ☐ Área de Conservación Tortuguero (**ACTo**)
- ☐ Área de Conservación La Amistad Caribe (**ACLA-C**)
- ☐ Área de Conservación La Amistad Pacífico (**ACLA-P**)
- ☐ Área de Conservación Osa (**ACOSA**)
- ☐ Área de Conservación Pacífico Central (**ACOPAC**)
- ☒ Área de Conservación Arenal–Tempisque (**ACA-T**)
- ☐ Área de Conservación Arenal–Huetar Norte (**ACA-HN**)
- ☐ Área de Conservación Guanacaste (**ACG**)
- ☐ Área de Conservación Tempisque (**ACT**)
- ☐ Área de Conservación Marina Isla del Coco (**ACMIC**)

Período Autorizado:

20 / 10 / 2016 Inicio — 20 / 10 / 2017 Final

Cantidad y Material autorizado a colectar:

Para el Proyecto Investigación autorizado.
un total de: 2 puestas huevo dithobates
vibicarius y 3 parejas de la misma especie Adult
1 Pareja Atelopus senex (adultos).
1 Pareja Craugastor angelicus (adultos).
1 Pareja Craugastor escoces (adultos).
1 Pareja Isthmohyla tuvulares (adultos).

Resolución N° SINAC-ACAHN-PI-R-008-2016

Fecha de entrega informe final 20 / 01 / 2018

Nombre funcionario/a que autoriza

Sandra Díaz Alvarado
Firma

(Sello de autorización) PROGRAMA DE INVESTIGACIÓN SINAC A.C.A.H.N.

20 / 10 / 2016
Fecha

Título de la Investigación:

Infección o enfermedad a los anfibios sobrevivientes de Costa Rica: Diagnóstico de Quitridiomicosis en el Nectrópiro.

Área de Conservación donde se autoriza:

- ☐ Área de Conservación Cordillera Volc. Central (ACCVC)
- ☐ Área de Conservación Tortuguero (ACTo)
- ☐ Área de Conservación La Amistad Caribe (ACLA-C)
- ☐ Área de Conservación La Amistad Pacífico (ACLA-P)
- ☐ Área de Conservación Osa (ACOSA)
- ☐ Área de Conservación Pacífico Central (ACOPAC)
- ☐ Área de Conservación Arenal–Tempisque (ACA-T)
- ☒ Área de Conservación Arenal–Huetar Norte (ACA-HN)
- ☐ Área de Conservación Guanacaste (ACG)
- ☐ Área de Conservación Tempisque (ACT)
- ☐ Área de Conservación Marina Isla del Coco (ACMIC)

Período Autorizado:

13 / 03 / 2017 Inicio — 13 / 03 / 2018 Final

Cantidad y Material autorizado a colectar:

50 especímenes adultos de dithobates vibicareus en Parque Nacional del Agua Juan Castro Blanco, y área amortiguamiento.

Resolución N° SINAC-ACAHN-PI R-001-2017.

Fecha de entrega informe final 13 / 04 / 2018

Nombre funcionario/a que autoriza

Sandra Díaz Alvarado
Firma

(Sello de autorización) PROGRAMA DE INVESTIGACIÓN SINAC A.C.A.H.N.

23 / 03 / 2017
Fecha

Título de la Investigación:

Dinámica poblacional de Lithobates vibicarius en el Parque Nacional del Agua Juan Castro Blanco, Alajuela, Costa Rica

Área de Conservación donde se autoriza:

- ☐ Área de Conservación Cordillera Volc. Central (ACCVC)
- ☐ Área de Conservación Tortuguero (ACTo)
- ☐ Área de Conservación La Amistad Caribe (ACLA-C)
- ☐ Área de Conservación La Amistad Pacífico (ACLA-P)
- ☐ Área de Conservación Osa (ACOSA)
- ☐ Área de Conservación Pacífico Central (ACOPAC)
- ☐ Área de Conservación Arenal–Tempisque (ACA-T)
- ☒ Área de Conservación Arenal–Huetar Norte (ACA-HN)
- ☐ Área de Conservación Guanacaste (ACG)
- ☐ Área de Conservación Tempisque (ACT)
- ☐ Área de Conservación Marina Isla del Coco (ACMIC)

RESOLUCIÓN
SINAC-ACAHN-PI-R-001-2017

El Sistema Nacional de Áreas de Conservación y el Área de Conservación Arenal Huetar Norte a través de las facultades y competencias del Director Regional, al ser las dos horas con treinta minutos del día trece de marzo del dos mil diecisiete.

RESULTANDO:

PRIMERO: Que el día dos de febrero del año dos mil diecisiete, el señor **GILBERTH JOSÉ ALVARADO BARBOZA,** costarricense, cédula de identidad número UNO MIL CIENTO SESENTA Y SIETE CERO CUATROCIENTOS CUARENTA Y TRES (1-1167-0443), quien es M.Sc. en Biología y licenciado en Medicina Veterinaria (DMV), labora en la Escuela de Biología de la Universidad de Costa Rica. Solicitó permiso de investigación y colecta científica según el proyecto titulado: **"INFECCIÓN O ENFERMEDAD EN LOS ANFIBIOS SOBREVIVIENTES DE COSTA RICA: DIAGNOSTICANDO QUITRIDIOMICOSIS EN EL NEOTRÓPICO"**, que se desarrollará en el Parque Nacional del Agua Juan Castro Blanco.

SEGUNDO: Que el día dos de febrero del año dos mil diecisiete, entre los documentos que presentó el investigador referido se adjuntó: solicitud, proyecto, copia de la cédula de identidad, curriculum vitae, resolución de la Universidad de Costa Rica.

TERCERO: Que la Resolución N° 069 del cuatro de octubre del año dos mil dieseis de la Comisión Institucional de Biodiversidad de la Universidad de Costa Rica, mediante la cual se le otorgó un permiso para el acceso a los elementos y recursos genéticos y bioquímicos de la biodiversidad, según los términos que contempla la propuesta para el desarrollo del proyecto de investigación básica mencionado en el resuelve primero, con un periodo de vigencia que comprende hasta el treinta y uno de diciembre del año dos mil dieciocho; así como el oficio número V1-6457-2016 de fecha cuatro de octubre del dos mil dieciséis, firmado por el M.Sc. Jorge Warner, Coordinador de la Comisión Interinstitucional de Biodiversidad de la Universidad de Costa Rica.

CUARTO: Que el proyecto de investigación contempla dentro de los aspectos metodológicos, el análisis en laboratorios del Departamento de Patología y Microbiología del Colegio Atlántico de Veterinaria de la Universidad del Príncipe Eduardo, Charlottetown, Prince Edward Island en Canadá.

PROGRAMA DE INVESTIGACIÓN SINAC ACAHN

QUINTO: Que el día trece de marzo del dos mil diecisiete, la funcionaria, Sandra Díaz Alvarado, encargada del Programa de Investigación del Área de Conservación Arenal Huetar Norte, presentó oficio SINAC-ACAHN-PI-07-2017, Informe Técnico Número 04-2017; donde emite recomendación y análisis del expediente administrativo número AH04-001-2017, en el cual se consigna la solicitud del investigador Gilberth José Alvarado Barboza.

CONSIDERANDO:

PRIMERO: Que la Ley de Conservación de la Vida Silvestre N° 7317, del treinta de octubre de mil novecientos noventa y dos y sus reformas, en los artículos 1 y 3, establece que: "*La vida silvestre está conformada por el conjunto de organismos que viven en condiciones naturales, temporales o permanentes en el territorio nacional,...*" que "*Se declara de dominio público la fauna silvestre que constituye un recurso natural renovable.... De interés público la flora silvestre...*" y que únicamente pueden ser objeto de apropiación particular y de comercio mediante las disposiciones contenidas en los tratados públicos, en los convenios internacionales y en la Ley Nº 7317 y su reglamento, artículos 3 y del 36 al 50 y otras normativas vinculantes.

SEGUNDO: Que según la Ley Nº 7317, Ley de Conservación de la Vida Silvestre y sus reformas, en el artículo 6, establece que el Sistema Nacional de Áreas de Conservación del Ministerio de Ambiente y Energía es el órgano competente en materia de planificación, desarrollo y control de la vida silvestre, en igual sentido dicha competencia se encuentra establecida en el artículo 22 de la Ley de Biodiversidad Nº 7788.

TERCERO: Que según la Ley Nº 7317, Ley de Conservación de la Vida Silvestre y sus reformas, en el artículo 7, inciso e) establece que el Sistema Nacional de Áreas de Conservación del Ministerio de Ambiente y Energía tiene entre sus competencias el promover y ejecutar investigaciones en el campo de la vida silvestre.

CUARTO: Que según la Ley Nº 7317, Ley de Conservación de la Vida Silvestre y sus reformas, en los artículos 40 al 49, faculta al SINAC para dar permisos de investigación así como rechazar cualquier solicitud contraria al interés público y fiscalizar la ejecución de estas actividades.

QUINTO: Que la Ley N° 4594, Depósito de duplicados de Material Zoológico, Botánico o Mineral en la UCR y el Museo Nacional, del primero de julio del año mil novecientos setenta, en su artículo 1 indica que "*todo científico o institución que personalmente o en representación, recoja material botánico, zoológico o mineral con fines taxonómicos, en cualquier zona o lugar del territorio nacional, tiene la obligación de dejar duplicados de sus colecciones a la Universidad de Costa Rica y al Museo Nacional. La Universidad de Costa Rica y el Museo Nacional, conjuntamente, pueden exonerar al científico o institución de que se trata, de la obligación a que se refiere el párrafo anterior, tomando en consideración motivos calificados que le impidan dejar esos duplicados.*"

Dirección: Alajuela, San Carlos, Quesada 150 metros Norte y 250 metros Este del Hospital San Carlos.
Teléfono: (506)2460.06.20 • Fax: (506)2460.06.44 • Apdo.: 11384-1000 San José, Costa Rica
www.sinac.go.cr

SEXTO: Que el Decreto Ejecutivo N° 12329-A, Reglamento de Investigaciones del Servicio de Parques Nacionales, del 6 de marzo de 1981, establece lo referente a las investigaciones en los parques nacionales y áreas afines administradas por el Servicio de Parques Nacionales.

SETIMO: Que de conformidad con el criterio del ente técnico competente sea la Secretaria Técnica Nacional Ambiental (SETENA), según acuerdo ACP-70-2015 adoptado en la Sesión Ordinaria N° 088-2015-SETENA de la Comisión Plenaria de la Secretaría Técnica Nacional Ambiental del 23 de junio de 2015, la evaluación de impacto ambiental no resulta de aplicación para los permisos de funcionamiento para centros de rescate, mariposarios, acuarios, herbarios, museos naturales, bancos de germoplasma y exhibiciones que impliquen construcciones menores a 300 metros cuadrados o que no impliquen nuevas construcciones, así como los viveros artesanales comerciales, los permisos de investigación, las licencias de caza de subsistencia y las licencias de colecta científica o académica, los permisos de importación y de exportación presuponiendo que los establecimientos respectivos de procedencia o de destino de los especímenes sí cuentan con una EIA, los permisos para desarrollar actividades relacionadas con costumbres comunitarias y por ende los mismos quedaron exentos del trámite referente a la evaluación de impacto ambiental establecido en el numeral 26 de la Ley de Conservación de la Vida Silvestre N° 7317, según el acuerdo previamente citado.

OCTAVO: Que de conformidad con lo dispuesto en el transitorio del artículo 4 de la ley N° 7788, Ley de Biodiversidad, se emitió el Reglamento sobre el acceso a la biodiversidad en actividades de docencia, acción social y de investigación de la Universidad de Costa Rica, en adelante denominado **El Reglamento**, aprobado por la rectora de dicha universidad mediante resolución N° 5861-2005 y publicado en La Gaceta Universitaria N° 9-2005 del 29 de setiembre de dos mil cinco.

NOVENO: Que conforme a lo dispuesto en el artículo 10 de EL Reglamento, la Universidad de Costa Rica reconoce cuatro modalidades esenciales de acceso a los recursos genéticos, a saber, (1) Taxonomía, colección y catalogación, (2) Investigación básica y aplicada, (3) Bioprospección y (4) Comercialización.

DECIMO: Que en el artículo 22 de El Reglamento se determina que los proyectos universitarios correspondientes a la categoría de Taxonomía, catalogación y colección quedan exentos de todo trámite de permiso, debido al interés científico que estos revisten.

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DECIMO PRIMERO: Que el artículo 23 de El Reglamento se indica que los proyectos universitarios correspondientes a la categoría de Investigación básica y aplicada deben ser aprobados directamente por el Consejo Científico o la Comisión de Investigación de cada Unidad Académica o de Investigación, respectivamente, pudiendo dichas entidades, facultativamente y cuando así lo determinen, someter algunos de tales proyectos ante la Comisión Institucional de Biodiversidad de la Universidad de Costa Rica, para su correspondiente análisis y eventual aprobación.

DECIMO SEGUNDO: Que según lo que establecen los artículos 24 y 26 de El Reglamento, los proyectos universitarios correspondientes a las categorías de bioprospección y Comercialización deben ser aprobados por la Comisión Institucional de Biodiversidad de la Universidad de Costa Rica, todo ello sujeto a la obtención del consentimiento informado previo por parte de los directores de las áreas de conservación, los dueños de los predios y fincas particulares o las autoridades indígenas, según donde se realice la colecta de los materiales que se utilizaran para el acceso a los elementos y recursos genéticos o bioquímicos.

DECIMO TERCERO: Que en el artículo 29 de El Reglamento se establece que, adicionalmente al permiso de acceso otorgado por la Comisión Institucional de Biodiversidad de la Universidad de Costa Rica, los permisos de ingreso a los terrenos privados o a los terrenos del Estado para colecta de muestras deberán ser tramitados en las respectivas instancias por parte de los investigadores a menos que la Universidad logre la firma de acuerdos institucionales con ese fin.

DÉCIMO CUARTO: Que para los efectos de la solicitud presentada por el interesado, se tiene como aprobado el proyecto de investigación por la Comisión Institucional de Biodiversidad de la Universidad de Costa Rica; y que para los efectos de la licencia de exportación de aquellas muestras autorizadas, se requiere un permiso de investigación emitido por el Sistema Nacional de Áreas de Conservación.

DÉCIMO QUINTO: Que el solicitante GILBERTH JOSÉ ALVARADO BARBOZA, cumplió con los requisitos de inscripción de investigaciones y colecta científica establecidos en la Ley Nº 7317, Ley de Conservación de la Vida Silvestre, sus reformas y reglamento.

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POR TANTO
EL DIRECTOR REGIONAL DEL AREA DE CONSERVACIÓN ARENAL HUETAR NORTE

RESUELVE:

PRIMERO: Aprobar la solicitud al investigador principal **GILBERTH JOSÉ ALVARADO BARBOZA**, e inscribir la investigación básica titulada: **"INFECCIÓN O ENFERMEDAD EN LOS ANFIBIOS SOBREVIVIENTES DE COSTA RICA: DIAGNOSTICANDO QUITRIDIOMICOSIS EN EL NEOTRÓPICO"**, a desarrollar en el **PARQUE NACIONAL DEL AGUA JUAN CASTRO BLANCO**, Cuyos objetivos son:

A) Objetivo General: Determinar la respuesta inmunopatologica de Lithobates vibicarius y Lithobates taylori sometidas a infecciones experimentales con Batrachochytrium dendrobatidis mediante la caracterización anatomohistopalógica, clínico patológica y de perfil metabolómicos.

B) Objetivos específicos:

1. Evaluar la presencia o ausencia de cambios anatomohistopatológicos a través de los diferentes estadios de infección por *Batrachochytrium dendrobatidis* que puedan presentar los animales de ambas especies.
2. Determinar alteraciones en parámetros hematológicos y de química sanguínea a través de los diferentes estadios de infección por *Batrachochytrium dendrobatidis* que puedan presentar los individuos de ambas especies.
3. Explorar las diferencias en los perfiles metabolómicos que puedan presentar durante los diferentes estadios de infección por *Batrachochytrium dendrobatidis* los individuos de ambas especies utilizando perfiles de 1H-RMN.

SEGUNDO: Se anota en el Pasaporte Científico Número 2539 a nombre de Gilberth José Alvarado Barboza; quien está autorizado según el permiso de investigación y colecta científica del proyecto titulado "Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el Neotrópico".

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TERCERO: Se autoriza la colecta de cincuenta (50) especímenes adultos de *Lithobates vibicarius en el Parque Nacional del Agua Juan Castro Blanco y su área de amortiguamiento, que corresponde al Área de Conservación Arenal Huetar Norte (ACAHN), según se detalla a continuación:*

Nombre de la especie	***Lithobates vibicarius***	
Sectores propuestos:	**Cantidad I Trimestre 2017**	**Cantidad II Trimestre 2017**
Laguna El Congo	5	5
Potreros Pozo Verde	5	5
Laguna Tamara	5	5
La Legua	5	5
Las Brisas	5	5
Total	**25**	**25**

Este permiso no faculta para colectar en fincas privadas, sin el respectivo permiso del propietario registral o de quien está legalmente autorizado para otorgarlo.

CUARTO: Autorizar el uso de los siguientes métodos para el desarrollo de la investigación:

1. Se colectarán animales adultos clínicamente saludables de *Lithobates vibicarius* en los alrededores del Parque Nacional del Agua Juan Castro Blanco, en San José de la Montaña, Ciudad Quesada; San Carlos, Alajuela.

2. Cada rana será colocada en un contenedor de plástico individual (70X95X150 mm3) para su transporte. Las ranas serán colocadas en contenedores plásticos individuales (230X230X350 mm3) en temperaturas (18-20°C) y luz (12 horas luz/12 horas oscuridad).

3. El agua será cambiada diáriamente y las ranas serán alimentadas con grillos domésticos grandes (*Acheta domestica*) empolvado con carbonato de calcio superfino (Cattlekare, Dandenong, VIC) y polvo multivitamínico (Reptivite, Zoo Med Laboratories Inc., San Luis Obispo, CA), ad libitum cada día.

4. *Las ranas de ambas especie: Lithobates vibicarius* de ACAHN y *Lithobates taylori* con el permiso de investigación del Área de Conservación Cordillera Volcánica Central, serán asignadas aleatoriamente a cada uno de los dos ensayos experimentales. Antes de que cada ensayo comience, cada rana será clínicamente examinada por un veterinario especialista (investigador principal), será medida, pesada y se le realizará un hisopado de su cuerpo para la determinación de *Batrachochytrium dendrobatidis* mediante el análisis de la reacción en cadena de la polimerasa en tiempo real.

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Todas las muestras serán realizadas en triplicado y comparadas con los estándares de la Sociedad Zoológica de Londres. Todas las ranas serán negativas antes de iniciar con los ensayos experimentales.

El diseño experimental consiste en los siguientes pasos:

5. **Diseño experimental 1:** Ranas sin infectar: Este será diseñado como un proyecto piloto para validar las metodologías de parámetros hematológicos y químico clínicos, así como los patrones metabolómicos en ranas saludables de ambas especies.
En el inicio del experimento 1 (Día 0), *L.* vibicarius (n=25) y *L. Taylori* (n=25) serán anestesiadas para obtener las muestras correspondientes a los parámetros indicados. Anestesia será inducida por baño de inmersión en 0.20% solución ácida ethyl3-aminobenzoato metanosulfónico (tricaine methanesulfonate, Sigma-Aldrich Inc., St Louis, MO) buferada con una solución 10 mEql-1 de bicarbonato de sodio (8.4%, Pro Care Animal Health, Dandenong, VIC). Las muestras sanguíneas (250–500 µl, ,1% body weight) serán colectadas para análisis hematológico, químico clínico y metabolómico colocando a la rana en recumbencia dorsal vía cardiocentesis con una jeringa de 1 ml y aguja 25 (Terumo Corporation, Binan, Laguna). El día 7, cada rana será eutanasiada por exanguinación cardiaca precedida con una inducción anestésica de acuerdo a lo descrito anteriormente. Las muestras de sangre serán colectadas para análisis de parámetros hematológicos y químico clínicos, así como los patrones metabolómicos.

6. **Diseño experimental 2:** Ranas sin infectar: En el inicio del experimento 2 (Día 0), *L.* vibicarius (n=25) y *L. Taylori* (n=25) serán anestesiadas para obtener las muestras correspondientes a los parámetros indicados, de acuerdo a lo descrito en el experimento 1. Las ranas serán expuestas a Bd mediante inmersión poco profunda en un baño de 25 ml de solución electrolítica diluida (mmol 1-1: KH2PO4 1, CaCl2.H2O 0.2, MgCl2 0.1) inoculado con 250 000 zoosporas por 24 horas, después las ranas serán devueltas a sus contenedores de mantenimiento. Durante el periodo de exposición, las ranas permanecerán en contenedores plásticos individuales y pequeños (50X100X150 mm3) con tapa para asegurar el continuo contacto de la superficie ventral con el inóculo.
Las ranas serán pesadas y se colectará un hisopado para PCR a los 10, 20, 30, 40, 50, 60, 75 y 82 días post-exposición. En el día 30 post-exposición, las ranas serán anestesiadas y se colectarán las muestras para análisis hematológicos y de química sanguínea, así como metabolómicos. En el día 75 post-exposición (correspondiente al día 0 de las ranas sin infectar del experimento 1), las ranas expuestas a *Batrachochytrium dendrobatidis* serán nuevamente anestesiadas para las pruebas ya mencionadas.

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En el día 82 post-exposición (correspondiente al día 7 de las ranas sin infectar del experimento 1), cada una de las ranas será eutanasiada mediante exanguinación cardiaca después de la anestesia y sometidas a una necropsia completa debidamente descrita en el apartado correspondiente.

7. **Análisis hematológicos y de plasma sanguíneo:** Las muestras sanguíneas de cada rana serán procesadas de acuerdo a los procedimientos estándares para los anfibios (Wright 2001, Young et al. 2012). Los frotis frescos serán fijados con metanol al 100%; 200 µl serán colectados en un tubo microcontenedor heparinizado con litio pediátrico (Becton and Dickinson, Franklin Lakes, New Jersey); y 150-200 µl serán colectados en un tubo de microcentrífuga de 1.0 ml ((Eppendorf AG, Hamburg), centrifugado (5,590Xg por 10 min) y el supernatante decantado y refrigerado a 4°C hasta su envío para su análisis en metabolómica. La sangre adicional (500-1000 µl) de la muestra final del día 7 (ranas saludables y sin infectar, experimento 1) y el día 82 (ranas expuestas a Bd, experimento 2) serán colectadas en un tubo de microcentrífuga de 1.0 ml, se deja formar el coágulo por 1 h, luego se centrifuga (5,590Xg por 10 min) y el supernatante se decanta y se congela a -70°C. Las células sanguíneas rojas (RBC), células sanguíneas blancas (WBC) y trombocitos serán contados manualmente en una cámara modificada de Neubauer a 400X de magnificación y usando Natt-Herrick´s como diluente (Wright 2001, Young et al. 2012, Natt & Herrick 1952). El diferencial de WBC serán contadas a una magnificación de 1000X con una tinción de Wright´s (Clinipure Wright's Stain and Wright's Buffer Concentrate, HD Scientific Supplies Pty Ltd, Wetherill Park, NSW) de los frotis sanguíneos. Sangre entera bien mezclada (5 µl) será colocado en un microhematocrito pediátrico (Becton and Dickinson, Franklin Lakes, NJ) y centrifugado (1126g for 2 min) para medir el volumen de paquete celular (PCV). La hemoglobina (Hb) se realizará manualmente usando el método de cianometahemoglobina modificado para especies con RBC nucleadas (Drabkin 1945, Melrose et al. 1995) y específicamente para anfibios (Young et al. 2012). El volumen corpuscular medio (MCV), Hb corpuscular media (MCH) y la concentración MCH (MCHC) fueron calculados mediante las fórmulas estándares usando los valores de Hb, PCV y RBC (Campbell & Ellis 2007).

Los analitos de plasma sanguíneo serán medidos en 100 µl de sangre entera usando equipo automatizado e incluirá: aspartato aminotransferasa (AST), ácido úrico (UA), creatinina quinasa (CK), glucosa, calcio, fósforo, potasio y sodio. Esta parte la realizaremos en colaboración con el Departamento de Patología y Microbiología del Colegio Atlántico de Veterinaria, Universidad del Príncipe Eduardo, Charlottetown, Prince Edward Island, Canadá. Pruebas t de muestras pareadas serán usadas para comparar las variables hematológicas y de plasma sanguíneo dentro de cada grupo experimental (saludables y *Batrachochytrium dendrobatidis*-expuestas) para cada especie.

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8. **Estudio anatomo-histopatologico:** Todos los animales serán examinados haciendo uso de los protocolos estándares de análisis por necropsia establecidos (Pessier y Pikerton 2003).
Se realizará el estudio histopatológico de todos los tejidos: piel (parte dorsal, ventral y lesiones), pulmones, corazón, estómago, intestino delgado y grueso, cloaca, páncreas, hígado, vesícula biliar, bazo, riñón, vejiga urinaria, gónadas, lengua, músculo esquelético, hueso, cerebro, nervios periféricos, ojo. La histopatología será examinada mediante microscopía de luz para determinar la presencia o ausencia de procesos patológicos y/o agentes etiológicos. Todos los hallazgos anatomohistopatológicos serán documentados mediante fotografías.

9. **Estudio exploratorio de perfiles metabolómicos:** Muestras de plasma obtenido de los especímenes de cada grupo, serán analizados mediante resonancia magnética nuclear de protones (1H-RMN) de acuerdo a Nicholson y otros (2007) para obtener perfiles metabólicos que permitan observar tendencias a nivel de metabolitos. En general, las muestras de plasma (aproximadamente 200 µL) serán diluidas adecuadamente con una solución salina 0.9% (400 µL) con agua deuterada (D2O). Cada muestra será colocada en microtubos de 300 µL y su espectro RMN de protones será medido por en el equipo Bruker 600 MHz que se encuentra en las instalaciones del CIPRONA. Muestras representativas de cada grupo serán seleccionadas para llevar a cabo mediciones de RMN de dos dimensiones con el objetivo de identificar los metabolitos presentes (Beckonert et al. 2007). El análisis de los datos se llevará a cabo incialmente por inspección de los espectros y además se llevará a cabo un análisis estadístico de componentes principales (PCA) para observar diferencias estadísticamente significativas entre grupos.

10. **Cultivo y aislamiento de *Batrachochytrium dendrobatidis*:** Los aislamientos de *Batrachochytrium dendrobatidis* serán realizados mediante el proyecto nuevo que se encuentra en proceso de inscripción "Lecciones de los sobrevivientes: Investigaciones ecológicas con poblaciones de ranas costarricenses sobrevivientes al declive poblacional para desarrollar estrategias útiles en la conservación de anfibios" en el Centro de Investigación en Estructuras Microscópicas.
El proceso de aislamiento se realizará usando protocolos establecidos por J. Longcore (Protocolo no publicado). De acuerdo a los resultados anteriores se elegirán las especies no amenazadas que muestran alta prevalencia o intensidad de la infección de *Batrachochytrium dendrobatidis*.
Se capturaran 20 individuos de *Lithobates vibicarius,* se mantendrán en cautiverio en el sitio, donde se realizaran hisopados de la piel para llevar a cabo el qPCR para determinar el estado de infección para cada anfibio individual.

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Los individuos que muestren altas cargas de *Batrachochytrium dendrobatidis* serán sacrificados acorde con las mejores prácticas de eutanasia, estos se usarán para el aislamiento de cepas de *Batrachochytrium dendrobatidis*. Cada aislamiento de *Batrachochytrium dendrobatidis* se mantendrá en solución de caldo triptona, y las muestras de cultivo serán inmediatamente criopreservadas y mantenidas en el CIEMIC, Universidad de Costa Rica.

QUINTO: El permisionario no puede ceder ni en modo alguno enajenar el permiso, pues el mismo es intransferible. Del mismo modo, cuando el investigador principal desee incluir nuevos co-investigadores o asistentes de investigación no autorizados en el Resuelve SEGUNDO de este permiso, deberá solicitarlo por escrito al Área de Conservación Arenal Huetar Norte del Sistema Nacional de Áreas de Conservación, con al menos 8 días de antelación. Además, no se autoriza el uso de este permiso y su respectiva licencia de colecta para otros fines académicos ajenos a las labores científicas, debiendo forzosamente solicitarse otros permisos y licencias adicionales para la realización de giras de campo, prácticas supervisadas, tesis de grado o posgrado y otras actividades similares que formen parte de los programas de estudios universitarios cursados por los estudiantes de la Universidad de Costa Rica.

SEXTO: El Sistema Nacional de Áreas de Conservación y el Área de Conservación Arenal Huetar Norte, se reserva el derecho de cancelar este permiso sin responsabilidad alguna para el Estado, cuando se compruebe que se ha incumplido el mismo.

SETIMO: El permisionario deberá rendir un informe escrito en idioma español, con los resultados de la investigación, ante el Área de Conservación Arenal Huetar Norte del Sistema Nacional de Áreas de Conservación, en la fecha del **trece de abril del año dos mil dieciocho.** *El incumplimiento de este requisito será sancionado con la imposibilidad, para los científicos en forma personal o para la institución que representa, de obtener nuevas autorizaciones, de estudios o investigaciones, hasta por un período de cinco años.*

Asimismo, cuando el Área de Conservación Arenal Huetar Norte lo considere pertinente, podrá solicitar copia de las bases de datos (digitales y/o impresas), imágenes o fotos de la información colectada en el campo, como apoyo a la información científica generada dentro de dicha área de Conservación. El permisionario deberá brindar cualquier tipo de información, derivada de este proyecto de investigación, que contribuya con los fines de conservación del ACAHN y del SINAC.

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Con el fin de asegurar la adecuada transferencia de los conocimientos derivados de este proyecto de investigación hacia las comunidades locales, el permisionario deberá desarrollar alguna iniciativa para la divulgación de los resultados hacia las comunidades localizadas en la zona de amortiguamiento del Parque Nacional.

OCTAVO: El permisionario deberá enviar una copia del informe final y de todas las publicaciones que se generen a raíz del presente permiso de investigación a la Biblioteca Nacional y otra copia al Área de Conservación Arenal Huetar Norte.

NOVENO: El permisionario deberá acatar las recomendaciones técnicas dadas por el ACAHN. Además deberá coordinar con la Administración del Área Silvestre Protegida con al menos ocho días de antelación a la fecha de ingreso y las facilidades que el Área Silvestre Protegida pueda, bajo sus posibilidades, ofrecerle. Deberá reportar, en la bitácora de investigación que para tal fin se encuentran en el Parque Nacional, su ingreso-salida y colecta, informar de las visitas y acompañantes sea co-investigador o asistentes, lugares de colecta y cantidades de material colectado con la firma del funcionario del PNAJCB.

DECIMO: El Área de Conservación Arenal Huetar Norte y el Sistema Nacional de Áreas de Conservación salva su responsabilidad sobre cualquier accidente o situación fortuita que afecte y/o ponga en peligro la integridad de las personas (investigadores, asistentes o acompañantes) que participan en el desarrollo de las actividades de la investigación.

DECIMO PRIMERO: Este permiso rige por un año, a partir del día trece de marzo del año dos mil diecisiete hasta el día trece de marzo del año dos mil dieciocho. Pudiendo ser renovado con el cumplimiento de esta resolución.

DECIMO SEGUNDO: Para el ingreso al **Parque Nacional del Agua Juan Castro Blanco**, se faculta en las áreas que son Patrimonio Natural del Estado y que según el Artículo 21 del Decreto Ejecutivo N° 38770 (con las reformas que introdujeron los decretos 38546 y 38295), cada investigador o asistente, deberá pagar la suma de **quince dólares ($15,00)** por persona, moneda de curso legal en los Estados Unidos de América, o su equivalente en colones (al tipo de cambio vigente para el día de la compra, según el Banco Central de Costa Rica), correspondiente al derecho de ingreso y permanencia para realizar la investigación dentro de

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dicha área silvestre protegida, por una estadía de hasta 12 (doce) meses continuos. La suma de diez dólares ($10,00), por una estadía de hasta 6 (seis) meses continuos y la suma de cinco dólares ($5,00), por una estadía de has 3 (tres) meses continuos. El depósito deberán hacerlo en la cuenta del Fondo de Parques Nacionales, creado por la ley de creación del servicio de parques nacionales N° 6084 del 24 de agosto de 1977. **Cuenta en dólares** N° 10002000 605009 0 **Cuenta en colones** N° 10001000 041220 5.

DECIMO TERCERO: Contra la presente resolución proceden los recursos ordinarios de revocatoria y / o apelación, de conformidad con los artículos 343 y siguientes de la Ley General de la Administración Pública, los cuales serán resueltos, el de revocatoria por el mismo órgano que lo dictó y el de apelación por el Consejo Nacional de Áreas de Conservación. Los recursos deberán interponerse ante el órgano que dicta ésta resolución dentro de los tres días tratándose de acto final y de veinticuatro horas en los demás casos, contados, en ambos casos, posteriores a su notificación.

DECIMO CUARTO: Notifíquese contra la presente al fax 2460-06-44.

Licenciado, Wilson Barrantes Chacón
Director Regional
Área de Conservación Arenal Huetar Norte
Sistema Nacional de Áreas de Conservación
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Teléfono: (506)2460.06.20 • Fax: (506)2460.06.44 • Apdo.: 11384-1000 San José, Costa Rica
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REPUBLICA DE COSTA RICA

El Sistema Nacional de Áreas de Conservación del Ministerio de Ambiente y Energía, solicita a las autoridades nacionales, funcionarios del Ministerio y a todo residente en el territorio nacional, le faciliten al portador del presente pasaporte científico toda colaboración que le sea posible para la realización de la investigación autorizada.

REPUBLIC OF COSTA RICA

The National System of Conservation Areas of the Ministry of the Environment and Energy requests to the national authorities, Ministrys's officials and all the residents in the national territory, to provide to the bearer of this scientific passport all the possible assistance to carry out of the authorized research.

Ministerio del Ambiente y Energía
Sistema Nacional de Áreas de Conservación

PASAPORTE CIENTÍFICO
Scientific Passport

Apellidos / *Last name* •• Nombre / *Name*: Alvarado Pedroza Gilberth

Nº Identificación / *Identification Nº*: 1-1167-0443

Nacionalidad / *Nationality*: Costarricense

Fecha de Nacimiento / *Date of Birth*: 19/3/87

Nº Pasaporte / *Passport Nº*: 03147

Costa Rica

País / *Country*

Universidad de Costa Rica

Profesión / *Profession*

Biólogo-Veterinario

Universidad o Centro de Investigación
University or Research Center

Título de la Investigación: "Inferencias enfermedad en los murciélagos sobrevivientes de Costa Rica Diagnosticando: Quitridiomicosis en el neotropico".

Área de Conservación donde se autoriza:

- [X] Área de Conservación Cordillera Volc. Central (**ACCVC**)
- [ ] Área de Conservación Tortuguero (**ACTo**)
- [ ] Área de Conservación La Amistad Caribe (**ACLA-C**)
- [ ] Área de Conservación La Amistad Pacífico (**ACLA-P**)
- [ ] Área de Conservación Osa (**ACOSA**)
- [ ] Área de Conservación Pacífico Central (**ACOPAC**)
- [ ] Área de Conservación Arenal–Tempisque (**ACA-T**)
- [ ] Área de Conservación Arenal–Huetar Norte (**ACA-HN**)
- [ ] Área de Conservación Guanacaste (**ACG**)
- [ ] Área de Conservación Tempisque (**ACT**)
- [ ] Área de Conservación Marina Isla del Coco (**ACMIC**)

Programa de Investigación

FOI-004-004

RESOLUCION DE INVESTIGACION CIENTIFICA Nº 005-2017- ACCVC-PI

El MINISTERIO DEL AMBIENTE, ENERGIA Y TELECOMUNICACIONES, Sistema Nacional de Áreas de Conservación, a través del AREA DE CONSERVACION CORDILLERA VOLCÁNICA CENTRAL, a las catorce horas del día Tres del mes de marzo del dos mil DIECISIETE.

Resultando:

PRIMERO: Que el día 16 de Marzo del dos mil diecisiete el señor **Gilberth José Alvarado Barboza cédula** 1-1167-0443, quién es funcionario de la Universidad de Costa Rica (UCR), en calidad de investigador principal solicitó un permiso de investigación, para realizar su investigación de título: **"Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el neotrópico."**, así mismo solicitó la emisión del pasaporte científico N° 03147 a su nombre.

SEGUNDO: Que el día 20 de Marzo del dos mil diecisiete, el funcionario Jorge Hernández Benavides, Coordinador de Investigaciones Científicas, realizó el análisis técnico correspondiente y otorgo el visto bueno para el desarrollo de la investigación denominada. **"Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el neotrópico."**.

Considerando:

PRIMERO: Que la Ley Orgánica del Ambiente (Ley Nº7554), Capitulo VII, en su Artículo 35, define como uno de los objetivos de las Áreas Silvestres Protegidas la promoción de la investigación científica, el estudio de los ecosistemas y su equilibrio, así como el conocimiento y las tecnologías que permitan el uso sostenible de los recursos naturales del país y su conservación.

SEGUNDO: Que la **Ley de Biodiversidad (Ley Nº 7788) en su Artículo 89: Fomento de programas de investigación, divulgación e información**; establece que el Ministerio del Ambiente y Energía y las demás instituciones públicas y privadas fomentarán el desarrollo de programas de investigación sobre la diversidad biológica.

TERCERO: Que con fundamento en los artículos uno y tres, de la Ley de Conservación de la Vida Silvestre, Ley número siete mil trescientos diecisiete del siete de diciembre de mil novecientos noventa y dos, declara que la Vida Silvestre está conformada por la Fauna Continental e Insular que vive en condiciones naturales, temporales o permanentemente en el territorio nacional y la flora que vive en condiciones naturales en el país; y que únicamente pueden ser objeto de apropiación particular y de comercio mediante las disposiciones contenidas en los tratados y convenios internacionales, en la Ley Nº 7317 y su reglamento, artículos 3 y 36 al 50 y otras normativas vinculantes.

CUARTO: Que la **Ley de Conservación de la Vida Silvestre (Ley Nº7317), en su Artículo 2**, define como **Estudio Científico:** *Toda investigación que aplica el método científico*; **Recolectar:** *acción de recoger, cortar, capturar o separar de su medio especies orgánicas, sus productos o subproductos*; **Recolecta Científica**: *la captura o extracción de animales o plantas, sus productos o subproductos, con fines de estudio científico*; **Recolecta Cultural**: *la captura o extracción de animales o plantas, sus productos o subproductos, con fines educativos.***Vida Silvestre**: *conjunto de la fauna continental e insular que vive en condiciones naturales, temporales o permanentes y de la flora que vive en condiciones naturales en el territorio nacional.*

QUINTO: Que la **Ley de Conservación de la Vida Silvestre (Ley Nº7317), en su Artículo 7**, expresa que el Ministerio del Ambiente y Energía, tiene como competencia, entre otros, el *promover y ejecutar investigaciones en el campo de la Vida Silvestre.*

SEXTO: Que la **Ley de Conservación de la Vida Silvestre (Ley No 7317)**, en sus **Artículos 3, 4, 5 y los Artículos del 36 al 51**, faculta al Ministerio del Ambiente y Energía para otorgar *permisos de investigación científica*, otorgar *licencias por recolecta científica*, a *establecer los requisitos por trámites y procedimientos* para otorgar dichos permisos, a *inscribir y registrar las investigaciones* y a otorgar *permisos de importación y exportación de flora y fauna*, entre otros.

SETIMO: Que según la Ley Nº 7317, Ley de Conservación de la Vida Silvestre, en el artículo 6 establece que la Dirección General de Vida Silvestre es el órgano competente en materia de planificación, desarrollo y control de la flora y la fauna silvestre.

OCTAVO: Que el **Reglamento de Investigaciones de los Parques Nacionales (Decreto Ejecutivo 12329-A)**, actualmente bajo administración del Sistema Nacional de Áreas de Conservación (SINAC); en sus considerándos establece: *a) que la investigación es uno de los fines fundamentales para preservar y proteger áreas naturales y juega un papel muy importante en la elaboración de los planes de manejo de los parques nacionales y áreas afines, así como para los avances de la ciencia en el área de los recursos naturales. b) que la investigación en los Parques puede resultar favorecida por la coordinación de sus funcionarios con organismos o personales especializadas. c) que tienen prioridad las investigaciones que ayuden a comprender y conocer mejor los recursos de los Parques, con el fin de manejarlos en forma correcta.*

NOVENO: Que la **Estrategia Nacional de Conservación y Uso Sostenible de la Biodiversidad**, establece dentro de sus políticas: el impulso a las investigaciones dirigidas a conocer el estado de las especies y ecosistemas de interés particular para la conservación.

DECIMO: Que las **Políticas para las Áreas Silvestres Protegidas** establecen que se debe: • *fomentar el desarrollo de la investigación básica y aplicada dentro de las áreas silvestres protegidas, de acuerdo con las necesidades identificadas por la institución;* • *autorizar aquellas investigaciones que se realicen en las áreas silvestres protegidas de conformidad con lo que establece la legislación vigente, la reglamentación interna del Área de Conservación y las prioridades de investigación identificadas por las áreas silvestres protegidas.*

DECIMO PRIMERO: Que el **Área de Conservación de la Cordillera Volcánica Central (ACCVC)**, tiene como uno de sus principios básicos la *coordinación, promoción, participación y facilitación del desarrollo de investigaciones básicas y aplicadas e inventarios de biodiversidad* que contribuyan a incrementar el conocimiento y entendimiento de la biodiversidad, sus ecosistemas y el componente geológico sobre el que se desarrollan.

DECIMO SEGUNDO: Que el **Area de Conservación de la Cordillera Volcánica Central** ha establecido un ***Programa de Investigación*** que tiene como objetivo hacer del ACCVC, un sitio de estudios científicos de primer orden, muy amigable a los usuarios y en donde los procesos científicos y la información obtenida, sirvan de eje central para su biodesarrollo y conservación a perpetuidad de la biodiversidad que se restaura y conserva en las 150.000 hectáreas terrestres de área silvestre protegida.

DECIMO TERCERO: Que el proyecto: **"Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el neotrópico.",** es considerado de interés por cumplir con los objetivos de las estrategias de biodiversidad e investigación y con los fines que persigue el ACCVC de ampliar el conocimiento de sus especies, ecosistemas, recursos geológicos y culturales.

DECIMO CUARTO: Que en el Manual de Procedimientos para realizar Investigación en Biodiversidad y Recursos Culturales en las Areas de Conservación, establecido por el *Comité Técnico de Investigación del SINAC* (decreto ejecutivo Nº28993-MINAE) y oficializado por el SINAC (oficios SINAC-DG-147 y DG-149, del 2002 y por el decreto ejecutivo Nº32553-MINAE) se establece como funcion de los encargados de los Programas de Investigación, entre otras, las siguientes: a) *Tramitar, evaluar y resolver los proyectos de investigación que se presenten ante su oficina regional, para realizar estudios científicos en su área de conservación. b) Elaborar las resoluciones respectivas y otorgar el pasaporte científico (licencia de recolecta científica). c) Recibir y analizar toda solicitud de permiso de investigación que se presente ante su oficina regional, para realizar estudios científicos en su área de conservación.d) Establecer acuerdos de Transferencia de Material cuando sea considerado necesario por la naturaleza de la investigación.*

DECIMO QUINTO: Que el día 16 de Marzo del dos mil diecisiete el señor **Gilberth José Alvarado Barboza cédula 1-1167-0443** en calidad de investigador principal cumplió con los requisitos de inscripción de investigaciones y recolecta científica establecidos en la Ley de Conservación de la Vida Silvestre (Ley Nº 7317) y su reglamento; con lo estipulado en el Manual de Procedimientos del SINAC y con lo solicitado por Programa de Investigación del ACCVC, para desarrollar su investigación titulada **"Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el neotrópico.",** por lo que lo procedente es aprobar este permiso.

Por tanto

Máster Rafael Gutiérrez Rojas, Director
Área de Conservación de la Cordillera Volcánica Central

RESUELVE:

PRIMERO: APROBAR EL PERMISO DE INVESTIGACION al señor **Gilberth José Alvarado Barboza cédula 1-1167-0443,** quién en calidad de investigador principal solicitó un permiso de investigación, para realizar su investigación de título: **"Infección o enfermedad en los anfibios sobrevivientes de Costa Rica: diagnosticando quitridiomicosis en el neotrópico."**, así mismo se autoriza la emisión del pasaporte científico N° 03147 a su nombre.

SEGUNDO: Este permiso es otorgado con el objetivo general de: **Determinar la respuesta inmunopatológica de *Lithobates vibicarius* y *Lithobates taylori* sometidas a infecciones experimentales con *Batrachochytrium dendrobatidis* mediante la caracterización anatomohistopatológica, clinicopatológica y de perfiles metabolómicos.**

TERCERO: En este permiso **Si** autoriza la recolecta científica de 50 ejemplares de *Lithobates taylori*

CUARTO: Este permiso es válido únicamente para el **Área de Conservación de la Cordillera Volcánica Central, específicamente en el Parque Nacional Volcán Poás y sus alrededores, en el Parque Nacional Braulio Carrillo y sus alrededores, Cerro Chompipe** y no faculta para hacer la investigación o recolectar en otras áreas de protección estatal o fincas particulares sin el respectivo permiso de quien está legalmente autorizado para otorgarlo.

QUINTO: El Sistema Nacional de Áreas de Conservación y el Área de Conservación de la Cordillera Volcánica Central autoriza los métodos de recolecta científica enumerados en el reglamento de la Ley de Conservación de la Vida Silvestre (Ley 7317) y los métodos autorizados en el Manual de Procedimientos del SINAC, así como aquellos que de previo hayan sido avalados por el Programa de Investigación. Se autoriza la colecta temporal de aves, anfibios, reptiles, mariposas y arácnidos, solamente con fines educativos).

SEXTO: El Área de Conservación de la Cordillera Volcánica Central se reserva el derecho de cancelar este permiso sin responsabilidad alguna para el Estado, cuando se compruebe que se ha incumplido el mismo o se han variado las actividades sin haber informado y obtenido autorización previa del Programa de Investigación.

SETIMO: El investigador no puede ceder ni en modo alguno traspasar el permiso, pues el mismo es intransferible.

OCTAVO: El Investigador deberá permitir a los funcionarios del Área de Conservación **de la Cordillera Volcánica Central** la verificación de las actividades cuando sea requerido y a acatar sus disposiciones cuando le sea indicado.

NOVENO: El investigador se compromete a mantener informados al Programa de Investigación y al ACCVC sobre el desarrollo de su investigación, presentará un **informe parcial el primero de mayo de 2018 y de resultados finales el día treinta y uno de Diciembre de 2019**.

DECIMO PRIMERO: EL Área de Conservación de la Cordillera Volcánica Central y el Programa de Investigación salvan la responsabilidad sobre cualquier accidente o situación que afecte y/o ponga en peligro la integridad de las personas (investigador, asistentes, acompañantes) que participan en el desarrollo de las actividades de la investigación tanto en laboratorio como en el campo.

DECIMO SEGUNDO: Este permiso rige a partir del día veintiuno de Marzo del dos mil DIECISIETE hasta el día veintiuno de Marzo del dos mil DIECIOCHO.

DECIMO TERCERO: Notifíquese contra la presente.

Firma:

SINAC - MINAE
AC. CORDILLERA VOLCÁNICA CENTRAL
ACCVC
DIRECCIÓN

Master Rafael Gutiérrez Rojas, Director
Área de Conservación de la Cordillera Volcánica Central

cc/ Jorge Hernández B, Coord. Programa de Investigaciones ACCVC
Lourdes Vargas F, Coordinador de Investigaciones Secretaría Ejecutiva SINAC

EVALUATION FORM

Author: ALVARADO BARBOZA, Gilberth José

Title: Experimental study and anatomo-histopathological, clinico-pathological and behavioral characterization of chytridiomycosis in *Lithobates vibicarius* in the highlands of Costa Rica.

Thesis submitted to the Postgraduate Program in Experimental and Comparative Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

Date: _____/_____/_____

Committee Members

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Al amor que me formó, al amor que me sostiene y al amor que me inspira.

ACKNOWLEDGEMENTS

I want to thank the two educating systems that allowed me to finish my academic cycle under a humanist and solidary-oriented perspective in Latin America. To my beautiful Costa Rica for investing in education instead of on weapons because, even though it is a small portion of land, it sets an example to the planet in the education and conservation fields. To Brazil, the South American giant, that keeps its arms wide open to the world, and for the great diversity of its people. I will never change sharing with people from all over the world, which showed me that it is in our differences where wisdom can be found.

Everything started in 2010, in an unbeatable scenario at Parque Nacional Iguazú, with a presentation card, and 9 years later, I keep learning from the human qualities and academic knowledge from Catão (I remember he did not want to be called Doctor). I cannot only talk about his professional excellence, but also the relationship with his students and the management of LAPCOM, which is something anyone could admire a lot. Thanks for always showing me how one can transcend in the professional scope and in human lives, where the essence of the human reason is based. With all that being said, science is produced in the path of life.

To María Forzán, for all her constant support, for always believing in this project and receiving me at the University of Prince Edward Island (UPEI) in Canada. Thanks for sharing all your knowledge in clinical and anatomical pathology in amphibians. I also thank Rafael Vanderstichel, Shannon Martinson, their family and pets for helping me with having a warm below-zero stay at that island.

To Robert Puschendorf for always guiding me and giving me suggestions to improve every day; without a doubt, you are a constant motivation source. Steven Whitfield, the *gringotico*, who has always collaborated throughout these years, together with Mark Wainwright and Luis Solano, for presenting the *Lithobates vibicarius* at the Children Eternal Rainforest, Costa Rica.

To Randall Jiménez, who has been, without a doubt, the best companion and friend during this 4-year adventure. Two PhDs that have merged and joined three countries, all the countless hours spent at the National Park and for sharing the unexplainable feeling of finding out a species that was believed to be extinct. Thank you for building the first enclosures, and for the several hours writing proposals and articles. Thanks for being the official laboratory data analyzer. You exceeded the

barriers of collaboration. Thanks Tania Chacón for introducing me to Randall and for always supporting us.

To Adrián Pinto, Catalina Murillo and Fiorella Fiatt for all your support on taking care and maintaining *Batrachochytrium dendrobatidis*, and for always having the inoculum ready in the precise moment. Thanks Federico Bolaños; you will always be the responsible for my work with these group of animals. I am now the same age you were at the moment we met. Thank you for helping me all this time. Gustavo Gutiérrez, I will be always grateful to you for opening the doors for me at Escuela de Biología and for trusting in my work; there is still a lot to do, and we keep moving on.

Daniel Briceño, I am truly thankful to you for your support during these last years at my project's most critical stage and with the formation of the Laboratory; thanks for all your unconditional help because it is nowadays a reality at Biology School. Thank you Viviana Lang and all the Biology School's crew, for being with me in this process; you have always ensured a full availability to help. Without a doubt, I have always felt at home there.

To Sara González and Leda González for the administrative support; with it, this project could persevere successfully. One of the key people in the construction and establishment of the laboratory was Fernando Fernández and all his crew: Jorge, Ricardo, César, Pablo, Edwin, Herney, Ruben, Ross, Alfonso and Juan, from the Central Maintenance Sector. Thank you for believing in this project, along with Sylvia Villalobos and Héctor Hernández from General Services Office, who have always been available to help in everything. Thanks Jorge Salazar from Experimental Station Alfredo Volio Mata for supporting and trusting in this project and let us make plans for our future in the station.

Laura Phillips and Karina Aguilar, thank you so much for your help in our first draft with the future laboratory installations. Randall Rodríguez, we will keep sharing insects and knowledge; thank you so much for saving us with the frogs' food. Pedro Murillo and Hannia Rodríguez, thank you for making me part of this experience of producing the documentary; even though this was not my first one, it will always be an honor to work with you. Juan Alberto Morales, Laura Alvarado and the Servicio de Patología crew from Escuela de Medicina Veterinaria at the Universidad Nacional; thank you very much for always being part of the team. Carolina Esquivel, thanks for your initial support with our frog behavior investigation.

To Eliana Reiko Matushima for being part of this project along these four years, and for always being able to cooperate and collaborate with your experience. Jorge Oyakawa, thanks for every minute given on those critical discussions and for always being able to support me in everything I needed. Josué Díaz Delgado, thank you for those two very intensive months of slides review and life reflections; thanks for being close and give your best in this project. Pedro Navas, thanks for always supporting since the very beginning and for the many conversations we had taking a cup of coffee. Catalina Ospina, thanks also for every single moment in this last stage, even though you were always present. Danny Fuentes (besides the fantastic illustrations of the frogs) and Daniela Castro, thanks a lot for every cup of wine shared and for making me feel at home during the last months while I stayed at your house.

To the rest of the LAPCOM team, thanks a lot for making me feel in family and showing me that Brazil is *pura vida*. Carlos Sacristan, Carol Ewbank, Gislaine Dalazen, Angélica Sarmiento, Marcelo Carvalho, Camila Molina, Cíntia Favero, Eduardo Machado, Fabiola Prioste, Pablo Cruz, Juliana Marigo, Kátia Groch, Marco Gattamorta, Ralph Vastreels, Roberta Zamana, Sândara Sguario, Silmara Rossi, Aricia Benvenuto, I am truly thankful to you for every detail, help and warm regards that you had with me. You are all welcome in Costa Rica.

To our biggest achievement in this process, the birth of a space that we call LAPECOM (Laboratorio de Patología Comparada y Experimental) in honor to LAPCOM. There are no words to express my gratitude to all the people that believed in this project two years ago, when nothing had even started. Thanks to all the people who have been part of it (that were previously mentioned) and that had collaborated throughout these last two years. I especially want to thank the students that joined us and helped every day to build this, so we reach what we already have. Thanks Tatiana Bolaños, José Sandoval, Gabriel Espinoza, Kimberly Castro, Josimar Estrella, Ana Belem Isaak, Pablo Aragonéz, Ana Catalina León, Oscar Ulloa, Juan Ignacio Abarca, Jimmy Barrantes, Oscar Rivera, Andrea Moya, Daniela Madrigal, Viviana Alemán, Miriam Chaverri, Julissa Gutiérrez and each of the people that have been at least for a short period; I will be thankful to you forever.

To my father, Mrs. Ana Isabel, Andrey, Jaqueline, Ismael and all my family (Alvarado, Barboza, Alemán and Laporte); thanks for always motivating me along this path. Thanks Emily Jiménez, Wayman Chipsen, Keylin Corella and Edwin León

for complying with the scholarship requirements, your friendship, confidence and support. I want to give special thanks to the woman that left everything aside four years ago to support the plans of another person; she stepped outside her comfort zone, and she is nowadays a completely successful professional who is leading the way in a new area for veterinary medicine in Costa Rica: the science of laboratory animals. Jilma, you are the bond of love that holds me; I admire you, and I am truly grateful to you for sharing your life with me.

Carrying out this project could not have been possible without the support of Sistema Nacional de Áreas de Conservación (SINAC), especially Sandra Díaz, Asociación Pro Desarrollo from Parque Nacional del Agua Juan Castro Blanco (APANAJUCA), Asociación Fuente Administradora de los Mantos Acuíferos del Cantón de Zarcero (AFAMAR) and COOPELESCA R.L. for authorizing me to work at the Pozo Verde sector. Thank you very much Douglas Vargas, your family and the Albergue Monterreal for always providing help to this project.

I want to thank the Oficina de Asuntos Internacionales y Cooperación Externa (OAICE) from the Universidad de Costa Rica for awarding a scholarship to me for my doctorate studies. Thanks Yamileth Damazio, Mauricio Saborío, Laura Agüero, Haydée Ramos and your team, who always kept an eye and supported this, thank you so much. I thank the Universidad de Costa Rica, the Ministerio de Ciencia, Tecnología y Telecomunicaciones (MICITT) and its Programa de Innovación y Capital Humano para la Competitividad (PINN), Amphibian Ark, Cleveland Metroparks Zoo and Cleveland Zoological Society for all the financial support to this project.

“Ninguém ignora tudo. Ninguém sabe tudo.
Todos nós sabemos alguma coisa.
Todos nós ignoramos alguma coisa.
Por isso aprendemos sempre”.

Paulo Freire

RESUMO

ALVARADO BARBOZA, G.J. **Estudo experimental e caracterização anátomo-histopatológica, clínico-patológica e comportamental da quitridiomicose em *Lithobates vibicarius* nas terras altas da Costa Rica.** 2019. 185 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

O fenômeno global de declínio das populações de anfíbios tem sido bem descrito e documentado. Há um consenso entre a comunidade cientifica de que o evento ocorreu simultaneamente em diversos países do mundo, sendo que o que distingue a natureza desse fenômeno é a sua ocorrência em áreas protegidas e intocadas. Na Costa Rica existe um grupo de sete espécies de anuros, *Lithobates vibicarius, Craugastor ranoides, C. taurus, Agalychnis lemur, A. annae, Ptychohyla legleri* e *Incilius holdridgei,* que se caracterizam por terem populações reduzidas ocupando áreas geográficas muito pequena. *L. vibicarius* é uma espécie endêmica de áreas de planalto da Costa Rica e oeste do Panamá. Na década de 90, acreditava-se que o *L. vibicarius* estava extinto devido ao *Batrachochytrium dendrobatidis* (*Bd*), porém, recentemente esta espécie recomeçou a recolonizar a Costa Rica se estabelecendo em dois locais restritos. Atualmente, uma dessas populações está bem inserida no Parque Nacional Juan Castro Blanco, aparentemente com um grande número de indivíduos adultos, jovens e girinos em suas estações reprodutivas e, ao que tudo indica, constituindo uma população saudável. O estudo dessas populações características permite conduzir diferentes avaliações sobre a susceptibilidade destas ao *Bd*, assim como análises inovadoras para obter informações para um programa de manejo. Antes de considerar um programa de translocação ou de reintrodução de *L. vibicarius*, é necessário verificar sua resistência e sobrevivência quando exposto ao *Bd*. Assim, usamos uma das cepas do fungo isolada em um dos locais com maior potencial para reestabelecer uma população desta espécie. O objetivo do presente trabalho foi construído na premissa de que diversas espécies de anfíbios da Costa Rica tiveram declínios populacionais causados pela quitridiomicose. Dessa maneira, por meio de estudos anátomo-patológicos, clínico-patológicos e comportamentais buscamos investigar as diferentes fases de infecção do *Bd* na espécie de interesse. Instalações foram criadas, assim como procedimentos padrões para assegurar o bem estar animal durante todo o processo

de infecção experimental. Os resultados obtidos mostraram que *L. vibicarius* foi competente para resolver os sinais clínicos em um período de duas semanas pós infeção e em oito semanas mais de 80% dos animais haviam eliminado o *Bd*, como sugerido pelas análises histopatológicas e moleculares. Não houve alterações anátomo-patológicas, hematológicas ou comportamentais diretamente associadas com a infecção experimental. Os resultados indicam que a infecção por *Bd* em *L. vibicarius* é auto limitante com baixa morbidade e sem mortalidade. Desta forma, os resultados aqui apresentados sugerem que não é possível atribuir o declínio dessas espécies a quitridiomicose baseado apenas nos diagnósticos da infecção, apesar da mortalidade observada em vida-livre na década de 90s. Estudos complementares são necessários para desvendar os diversos aspectos relacionados com esta mortalidade *in situ*

Palavras-chave: *Lithobates vibicarius*. *Batrachochytrium dendrobatidis*. Infecção experimental. Quitridiomicose.

ABSTRACT

ALVARADO BARBOZA, G.J. **Experimental study and anatomo-histopathological, clinico-pathological and behavioral characterization of chytridiomycosis in *Lithobates vibicarius* in the highlands of Costa Rica.** 2019. 185 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

The global phenomenon of the amphibian declines has been well described and documented There is a consensus made by the scientific community that this event occurred simultaneously in many countries around the world. What makes the nature of this phenomenon distinctive is that it occurs in protected and pristine locations. In Costa Rica there is a group of seven species of frogs with a low number of individuals in their populations and restricted to a very small geographic area; amongst them *Craugastor ranoides, Craugastor taurus, Agalychnis lemur, Agalychnis annae, Ptychohyla legleri and Incilius holdridgei*. Another species that belongs to this group is *Lithobates vibicarius*, which is endemic from the highlands of Costa Rica and western of Panamá. During the 90's it was presumed to be extinct and this was attributed to *Batrachochytrium dendrobatidis* (*Bd*), but recently started to recolonize and established relict populations in two sites of Costa Rica. Nowadays, a relict population seems to be well established at the Juan Castro Blanco National Park (JCBNP), since it appears to have a high number of adults, juveniles and tadpoles over its reproductive seasons and in appearance seems to be a healthy population. All these characteristics facilitate the use of individuals of this species population to obtain biological samples for different *Bd*-susceptibility evaluations, and innovative analysis to obtain robust information on how to implement a management program. Before considering a translocation program or the reintroduction of the species *Lithobates vibicarius*, it is necessary to verify its resistance and survival when exposed to *Bd*. Thus, we use one of the isolated strains in one of the most potential sites for the reestablishment of this species population. The objective of this study was built on the premise that many amphibian species from Costa Rica had population declines caused by chytridiomycosis. Thus, through anatomo-histopathological, clinico-pathological and behavioral studies, we seek to investigate the different phases of *Bd* infection in the species of interest. We established adequate facilities and standardized operating procedures in order to ensure animal

welfare and to obtain high quality generated data during the process of experimental infection. The results obtained showed that *L. vibicarius* was competent to resolve the clinical signs presented in a two week period and within 8 weeks, more than 80% of the animals had eliminated the *Bd,* as suggested by histopathologic and molecular analysis results. There are no indications of anatomo-pathological, hematologic or behavioral alterations directly associated with the experimental process of infection. Based on our results, *Bd* infection in *L. vibicarius* is self-limiting with low morbidity and no mortality. Thus, the results presented here suggest that it is not possible to attribute the decline of these species to chytridiomycosis based only on the diagnoses of the infection, despite the mortality observed in wild in the 90s. Complementary studies are needed to uncover the various aspects related to *in situ* mortalities.

Keywords: *Lithobates vibicarius*. *Batrachochytrium dendrobatidis*. Experimental infection. Chytridiomycosis.

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LIST OF ABBREVIATIONS

Bd	*Batrachochytrium dendrobatidis*
WWF	World Wide Fund
LPI	Global Living Planet Index
IUCN	International Union for Conservation of Nature
JCBNP	Juan Castro Blanco National Park
masl	meters above sea level
h	hour(s)
s	second(s)
l	length
w	width
h	high
ENSO	El Niño-Southern Oscillation
ICIBs	Intracytoplasmic viral inclusion bodies
PCV	Packed cell volume
RBC	Red blood cells
WBC	White blood cell count

SUMMARY

1 GENERAL INTRODUCTION

In the year 2000, the concept Anthropocene was first used by the Nobel Prize winner for Chemistry, Paul Crutzen. This proposal started to gain strength since its publication in some of the major journals since 2008 (Crutzen, 2002; Crutzen and Stoermer, 2000; Pievani, 2014; Steffen et al., 2011). The reason why this concept began to be implemented is due to the significant global impact human activities caused to the planet and the accelerated environmental changes that it has been subjected to (Glikson, 2013; Thomas, 2013; Zalasiewicz et al., 2011). In 2009, planetary boundaries were proposed as a conceptual framework to evaluate the fundamental procedures for the planet's stability and a safe human development field of action (Rockström et al., 2009). One of the limitations that causes the most uncertainty, and the one that set us out of the safe zone, is related to the biosphere integrity, in terms of its genetic and functional diversity and preservation (Rockström et al., 2009; Samper, 2009).

Nowadays, we are living an unprecedented biodiversity loss throughout human existence, never registered before (Barnosky et al., 2011; Pievani, 2014; Wake and Vredenburg, 2008). It has been suggested with substantial evidence that we are experiencing a massive extinction event, to which all our attention should be directed. Several scientists think that the main cause is global climate change (Alford et al., 2007; Li et al., 2013; Pounds, 2001; Pounds et al., 2006). Just like the global warming effect alters many other aspects, such as the destruction and modification of habitats that are provoking extinctions, most of them are directly related to human activities (Daszak et al., 2001; Daszak and Cunningham, 2003). The Global Living Planet Index (LPI) measures biodiversity levels through data compilation of several vertebrate populations of species; it also estimates the average change level throughout time. Currently, the global LPI is an important indicator of the ecological condition of the planet (WWF, 2016). From 1970 to 2012, the LPI shows a general decrease of 58% in the abundance of vertebrates (WWF, 2016).

Even though marine and terrestrial vertebrate databases have been enriched and updated with new information, the strongest decline has affected mainly freshwater species, which heavily influences other global vertebrate reductions (WWF, 2016). For the past 40 years, the effect has extended to one of the most

prominent vertebrate groups of these habitats: the amphibians (Hoffmann et al., 2010; Stuart, 2004). The amphibian population reductions started to be quite evident in some species that were being monitored in a systematic way at the end of the 80s; this especially in tropical areas of the world (Blaustein and Wake, 1995; Lips, 1999, 2005; Pounds and Crump, 1994; Wake, 1991). In 1989, the I World Congress of Herpetology was held in Canterbury, United Kingdom. In that moment, herpetologists of various regions of the world communicated and realized that the same population trends of several amphibian species were happening in different regions of the globe (Phillips, 1990). Naturally, the sites where most tropical amphibian species were located, became the focal points of population disappearances throughout a diversity of species (Hero and Morrison, 2004; Lips, 1998, 2005; Mendelson et al., 2006).

1.1 DECLINES AND DISAPPEARANCES OF AMPHIBIANS

After those first signs, we began to register the best documented massive amphibian decline for all vertebrate groups of the modern era (Mendelson et al., 2006; Scheele et al., 2019; Wake and Vredenburg, 2008). Currently, it has been established that around 40% of these amphibian populations have presented a phenomenon in decline, and by 2008, 36 species were already declared extinct by the International Union for Conservation of Nature - IUCN (Jiménez and Alvarado, 2017). Among the frogs that were declared extinct are *Craugastor escoces, Incilius holdridgei* and *I. periglenes.* These are frogs endemic to Costa Rica, and last seen in 1986 (first two) and 1989 (last mentioned). The golden toad (*Incilius periglenes*), which came from the cloud forests of Costa Rica in Central America (Crump et al., 1992; Pounds and Crump, 1994), together with the southern gastric brooding frog (*Rheobatrachus silus*), that came from the southeast of Queensland in the subtropical region of Australia, have become iconic cases of amphibian declines around the world (Blaustein and Wake, 1995; Hines et al., 1999).

Along with the climate change and the destruction of their habitats, one of the biggest causes associated with this amphibian population decline worldwide is the disease known as chytridiomycosis (Pessier et al., 1999). This disease is caused by a chytrid fungus called *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al., 1999), which is present worldwide (Adams et al., 2010; Berger et al., 2016; Matthew C Fisher et al., 2009; James et al., 2009; Rödder et al., 2009). *Bd* has been associated

with various massive death events and the severe reduction of amphibian communities, that globes different species and continents (Cheng et al., 2011; Johnson, 2006; Lips, 1999; Lips et al., 2008; Lotters et al., 2009; Rosa et al., 2013). This species of chytrid was first described in 1999, a year before the first chytridiomycosis cases were reported in Central America and Australia for this disease (Berger et al., 1998).

1.2 *Batrachochytrium dendrobatidis* AS A POTENTIAL PATHOGENIC AGENT

Chytridiomycosis is a cutaneous infection known for being a threat to amphibian populations in the wild (Berger et al., 2016, 1998) and in captivity (Pessier et al., 2014, 1999). This fungus is frequently located in the *stratum corneum* of the digit and superficial ventral areas (especially in the pelvic patch), and least frequent in their dorsal surfaces (Berger et al., 2005c, 1998; Pessier et al., 1999; Puschendorf and Bolaños, 2006). Its zoosporangium is the fungal structure that can be identified microscopically in the keratinized layers of the epidermis, as spherical structures These structures eliminate the motile zoospores (Longcore et al., 1999), which eventually get in contact with a susceptible host, penetrate the skin and begin an infection (Greenspan et al., 2012; Pessier et al., 1999). Thus, this fungus invades the keratinized areas of the skin of juvenile and adult individuals; they can also be found in the mouthparts of larvae (Berger et al., 2005a; Piotrowski et al., 2004).

Bd is a chytrid that colonizes the skin of amphibians and interrupts their physiology, causing electrolytic imbalances in the individual (Campbell et al., 2013; Voyles et al., 2009, 2007). Skin injuries are commonly present together with a hyperplasia and hyperkeratosis of the *stratum corneum* and *stratum granulosum* (Berger et al., 2005c, 1998). In terms of mild injuries in clinically normal amphibians, it is possible to locate a aggregation of organisms, in which the focal hyperkeratosis is observed; whereas in lethal infections, hyperkeratosis is usually distributed throughout the skin of the affected individual (Berger et al., 2005c). The thickness of the epidermis varies, including sometimes extensive thinning areas, which make possible to observe one or two-cell layers in some anurans (Berger et al., 2005c, 2000).

Other types of reported injuries include disorganized epidermal cell layers, spongiosis, erosions and occasional skin ulcerations (Berger et al., 2005c, 2005b,

2000, 1998; Pessier et al., 1999). An increase on the number of mitotic figures has also been reported (Davidson et al., 2003). Epithelial necrosis is frequently observed in *stratum basale* or *stratum spinosum* cells, in which these cells present a pyknotic nucleus and an enlarged and pale cytoplasm. Occasionally, this vacuolar degeneration leads to an epidermal detachment and ulcerations (Berger et al., 2000). Usually, it only shows a moderate inflammatory response, which slightly increases the amount of mononuclear cells in the dermis. Lymphocyte, macrophage and few neutrophil aggregations are located in the surface of the affected dermis, particularly in the ulcerated areas. At times, inflammatory cells are observed in the epidermis (Pessier et al., 1999).

Amphibians are the only recognized *Bd* vertebrate hosts. This pathogen shows low specificity, thus managing to affect a great number of amphibian species (Daszak and Cunningham, 2003). However, not all amphibian species might appear to be equally susceptible to chytridiomycosis; some species might resist it even if they have been infected by *Bd*. Examples of resistant species include two frogs that have been introduced by humans to different continents: *Xenopus laevis* and *Lithobates catesbianus.* These frogs could be seen as potential vectors of this fungal pathogen (Daszak and Cunningham, 2003; Davidson et al., 2003; Hanselmann et al., 2004; Lane et al., 2003; Mazzoni et al., 2003).

1.3 DISAPPEARING AND REAPPEARING FROGS IN COSTA RICA

In Costa Rica, around twenty species were considered missing and probably extinct in the 90s, and it has always been suggested to be a strong presence of *Bd* in practically all areas of the country, just like there has always been liability attributed to *Bd* for the surgence of this phenomenon (Bolaños, 2009). In Costa Rica, as well as in the neotropics, the most threatened amphibians are the ones that inhabit mountainous zones, and the most worrying aspect is that many of these regions correspond to pristine areas (Becker and Zamudio, 2011; Lotters et al., 2009; Puschendorf et al., 2009, 2006a). There are two amphibian groups that declined severely. One of them corresponds to the Central American Stream-breeding frogs, *C. punctariolus* (Hedges et al., 2008), the other is the harlequin frog group from the genus *Atelopus* (McCaffery et al., 2015; Puschendorf et al., 2005). Both frog species were located in fast-moving water streams in the highlands of Central America

(Savage, 2002). Alongside them was *Lithobates vibicarius,* one of the most common ranid that inhabited along the Costa Rican mountain ranges and the east of Panama (above 1 500 m.a.s.l.). This species was last collected in 1990 (Zoology Museum database, School Biology, University of Costa Rica), in Las Tablas Protected Zone in Costa Rica, near the border with Panama.

In the year 2002, we have the first reappearance of tadpoles for *Lithobates vibicarius* at the Children´s Eternal Rainforest. Afterwards, they were raised in laboratory conditions in the Tropical Science Center, and then, their species was confirmed. Currently, two relict populations might appear to be well established; the first one in the mountainous area on the eastern part of the Tilarán Mountain Range (Santa Elena Cloud Forest Reserve, Monteverde Reserve of Tropical Science Center, Children´s Eternal Rainforest, Alberto Manuel Brenes Biological Reserve of University of Costa Rica and Arenal Volcano National Park). The second population was located at western section of the Central Volcanic Mountain Range (Juan Castro Blanco National Park - JCBNP). In both areas, there might be some populations with a significant quantity of adults, juveniles and tadpoles; besides, they appear to be completely healthy communities (Alvarado et al., data unpublished). All these characteristics facilitate the use of individuals from this species population to obtain biological samples for the conducing different *Bd* evaluations and innovative analysis to obtain robust information about how to improve the health of the species.

Several groups of local people (NGOs) are making a significant effort to preserve the areas where some of these populations currently inhabit. Nonetheless, in the last three years of monitoring Juan Castro Blanco National Park´s population, we noticed that one of the lakes where these species reproduces was invaded by a human settlement; this lake is nowadays the habitat of various domestic animals. The other lakes that are located around this national park present the same risks because they are located in places where the agriculture and cattle industry are present. For these reasons, the possibility of losing these breeding zones at any moment cannot be ignored.

During the 70s, one of the last highest wetlands of our country was drained because of the livestock industry there, and unfortunately, this was perhaps the main breeding area for the vibicaria. We found out that two more lakes were drained in the last ten years; these zones are used nowadays for agriculture as well. Due to all these reasons, along with the presence of pathogenic agents in these areas, such as

Bd, this population faces a series of threats that may terminate their existence. It is risky to think that the few existing breeding places in the JCBNP may be sufficient to sustain this amphibian population; not to mention, the noticeable changes in our rainy season and the corresponding temperatures that are indispensable during the breeding cycle of this species. *L. vibicarius* represents a unique opportunity to understand why this species, thought to be extinct, has survived and recovered in this place after its drastic population decline in Costa Rica during the 90s.

1.4 ARE *Lithobates vibicarius* AND RELICT SPECIES OF AMPHIBIANS RESISTANT TO *Batrachochytrium dendrobatidis*?

Before considering a translocation program or the reintroduction of the *Lithobates vibicarius* species, it is necessary to verify its resistance and survivorship when exposed to *Bd*. Thus, we decided to use one of the isolated strains of *Bd* in one of the most potential sites for the reestablishment of this species population. This way, we could not only define, through pathological, clinico-pathological and behavioral characterization studies, the different phases of the *Bd* infection, but also we could validate the use of this species as a candidate organism for the application of techniques such as the reintroduction or translocation. The main objective is based on the assumed premise that many amphibian species in Costa Rica had population declines caused by chytridiomycosis.

The different studies on virulence and pathogenicity of *Bd* (Berger et al., 2005b; Matthew C. Fisher et al., 2009; Refsnider et al., 2015; Sun et al., 2011), peptides in the skin of these animals (Apponyi et al., 2004; Carey et al., 1999; Conlon, 2011; Conlon et al., 2011; Rollins-Smith, 2009; Rollins-Smith et al., 2002), and their microbiome (Jiménez and Sommer, 2017; Kueneman et al., 2014; Rebollar et al., 2016; Walke and Belden, 2016) are amongst the most detailed. However, host-pathogen studies, where individual response as a host is evaluated, are still scarce. The difficulty of establishing adequate facilities and standardized operating procedures, as well as the daily maintenance of the animals, act as challenging obstacles in the development of experiments where all variables involved can influence the quality of the obtained results. Integral host evaluation is a fundamental part of formulating reliable conclusions on health impact (Fonzar and Langoni, 2012; Gervasi et al., 2013; Peterson et al., 2013; Searle et al., 2011; Van Rooij et al., 2015;

Voyles et al., 2011; Young et al., 2014). The presence of lesions, along with the etiological agent, also become crucial in order to determine the occurrence of the disease (Berger et al., 2005c; Brannelly et al., 2016).

1.5 A MAIN CHALLENGE: CREATING AND ESTABLISHING THE EXPERIMENTAL INFECTION FACILITIES.

Along with the research task itself, emerged one of the greatest challenges of this work process: the creation of a laboratory that encompasses the best conditions to maintain and execute the experimental infection process. The design of animal facilities, together with its adequate housing and management, are essential to reach the highest animal welfare standards, the quality of this investigation, animal production, any educating process that involves animals, staff health and security measures. An adequate program must provide the corresponding environments, housing and proper husbandry of any animal species, taking into consideration their physical, physiological and behavioral necessities, that allow them to grow, mature and reproduce normally, while guaranteeing their health and welfare.

Amphibians are poikilothermic animals, and their core temperature varies with the environmental conditions that surround them; this limits their ability to metabolically maintain their core temperature. The staff that would work with poikilothermic and aquatic animals must be familiarized with the handling conditions they require. Like in terrestrial systems, the microenvironment of an aquatic animal corresponds to the physical environment that surrounds it with an enclosure, such as the tank. This contains all the resources animals are in direct contact with; besides, it provides the boundaries of the animals' immediate environment. These microenvironments are characterized by many factors, including the quality of water, illumination, acoustic, vibrations, temperature and humidity. The physical surroundings of a secondary enclosure, such an experimental room, represent a macro environment. The conditions of microenvironments can directly affect the physiological and behavioral procedures of animals and might alter significantly their susceptibility to diseases (National Research Council, 2011; Schmeller et al., 2011).

Husbandry conditions and experimental procedures were performed by the Laboratory of Experimental and Comparative Pathology of the School of Biology of the University of Costa Rica (Fig. 1). This laboratory has two structural independent

experimental rooms, and they were created exclusively for the development of this project. These macro environments or experimentation rooms register a mean temperature of 19.22 ± 1.12 °C and a mean relative humidity of 92.74 ± 6.70 %. The data obtained from these two variables was measured and registered every hour with an Arduino microprocessor, together with temperature and humidity sensors. The walls of the macroenvironments were built with a fiberglass layer of 10 cm thick to isolate the acoustic and enviromental vibrations. The paint on the walls has a high resistance to humidity; the ceiling was also selected based on its high capacity of acoustic insulation and moisture resistance. The entrance of natural light was completely blocked at the door (Fig. 2).

Frogs were placed in individual transparent glass tanks (50l x 25w x 35h cm, 37.85 L). Also, they have been exposed to a 12 hour automated photoperiod, which was made up by led hose lights (IP67 Sylvania) and a timer, located approximately 25 cm above the tanks. Inside each tank, there are two levels: a first level of 8 cm of water; above, it has a 10 cm high platform (23 x 30 cm) that constitutes the second level that covers 60% of the water surface. This platform is lined with natural fiber paper towel as substrate. In the back left quadrant of the platform, there is a plastic flower pot (13 diameter x 13h cm) with an opening cut (10h x 7w cm), that works as shelter. The plastic pot is inverted, and the top is used by the animals as a third level (Fig. 1). Each tank is fed water through individual pipes that are connected to a filter tank and a draining system with individual stopcocks. Also, there are water mist nozzles installed over every platform that are regulated by solenoid valves that activated at 15.00, 17.00 and 19.00 h for 30 s (Fig. 3, 4). The enclosures are arranged in functional modules of 24 units that have main water connections for facilitated cleaning, an automated misting system, as well as the main collector drains (Fig. 5, 6).

Water used for husbandry and in experimental activities was first passed through two types of filters, a conventional one as a physical method (G0058-02, Purefer), and one with a micro-granular carbon cartridge (UDF- GAC- 2in1, Purefer), in order to reduce the presence of microorganisms and to eliminate components like chloride in the water. The main water tank is located inside the laboratory, so there are no changes in water temperature (Fig. 7). Every week, we replaced the paper towel and completely cycle out the old water with any organic material it may contain. The frogs were fed every 48 hours with adult crickets exclusively - *Acheta domestica*

(one cricket if the individual is < 30g; two crickets if the individual is ≥ 30g). These crickets were raised in the laboratory and maintained on a strict and permanent *ad libitum* diet of chicken feed (Ponedora 18% Golden, Mundi Vet – Dos Pinos), carrots, broccoli and oranges. Any cross-contamination between the infected and control animal modules was prevented by using individualized protection equipment for each division; for instance, wearing new gloves when having contact with each tank, and always working with the control group a day before any experimental procedure on the exposure group and two days before maintenance.

It was mandatory to adapt the previously described conditions in order to ensure the animal welfare and to obtain high quality generated data during the process of experimental infection. The idea was to provide the best appropriate conditions not only for the *Lithobates vibicarius,* but also for the *Batrachochytrium dendrobatidis*, with the objective of eliminating the influence the environment could have over the host-pathogen interaction on the experimental infection and this way guaranteeing the reproducibility of this process.

The corresponding housing strategies for a specific species must be developed and implemented for the animal care and husbandry, along with the user and veterinary advisory. Every step must be carefully revised by the Institutional Animal Care and Use Committee. The housing must provide healthy conditions and welfare to the animal by being consistent in its use. Seeking out counseling is very important when housing new species or when there are specific requirements associated with animals and their use. Objective evaluations must be conducted in order to demonstrate suitability in environment, housing and animal management. In addition, it is essential to state the standardized operational procedures for the laboratory, in order to ensure coherence at the moment of managing and caring for the animals.

Our biomodel, *Lithobates vibicarius* is a frog with a historical distribution range that covered the high mountains of Costa Rica in their entirety (Savage, 2002). That range started decreasing until it disappeared, placing the species in a critical endangerment by the IUCN in 2004. However, this species has recovered a great deal since then and began a process of re-evaluation to be now a vulnerable species, according to the IUCN in 2013 (IUCN SSC Amphibian Specialist Group & NatureServe, 2013). This species stands a rare opportunity to evaluate aspects of

defense mechanisms a host activates when exposed to an endemic strain of *Bd* from Costa Rica.

Fig. 1. Panoramic view of the experimental area of the Laboratory of Experimental and Comparative Pathology (LAPECOM), School of Biology. University of Costa Rica. A. Main water tank and insect production unit. B. Working area. This laboratory was designed and built based on the project that represents these thesis.

Fig. 2. Animal experimental room with two functional modules of the Laboratory of Experimental and Comparative Pathology (LAPECOM), School of Biology. University of Costa Rica. A. View towards the back B. View towards the front. This laboratory was designed and built based on the project that represents these thesis.

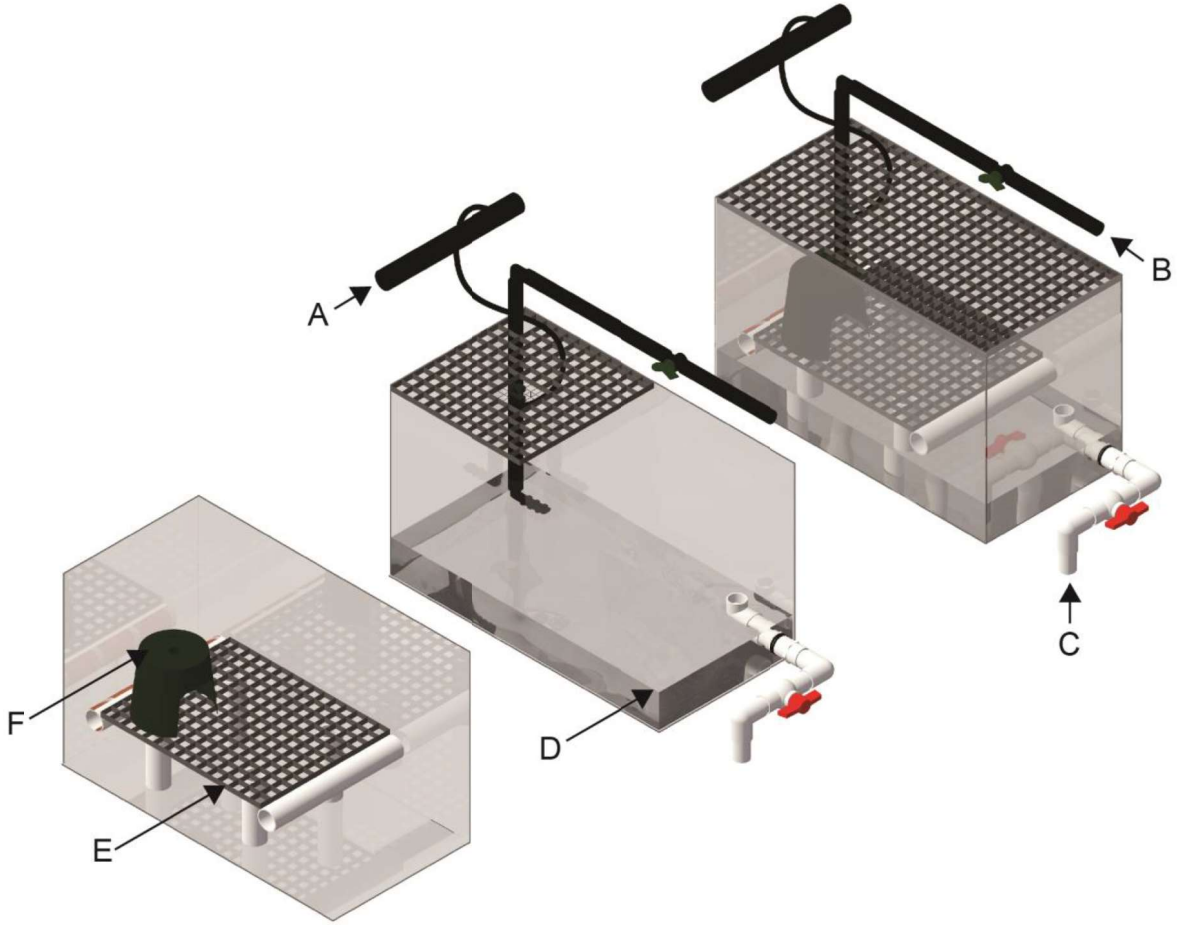

Fig. 3. Microenvironments designed exclusively for *Lithobates*. A. Pipeline for water misting system. B. Pipeline for water washing system. C. B. Drainage pipeline. D. First level. E. Second level. F. Third level.

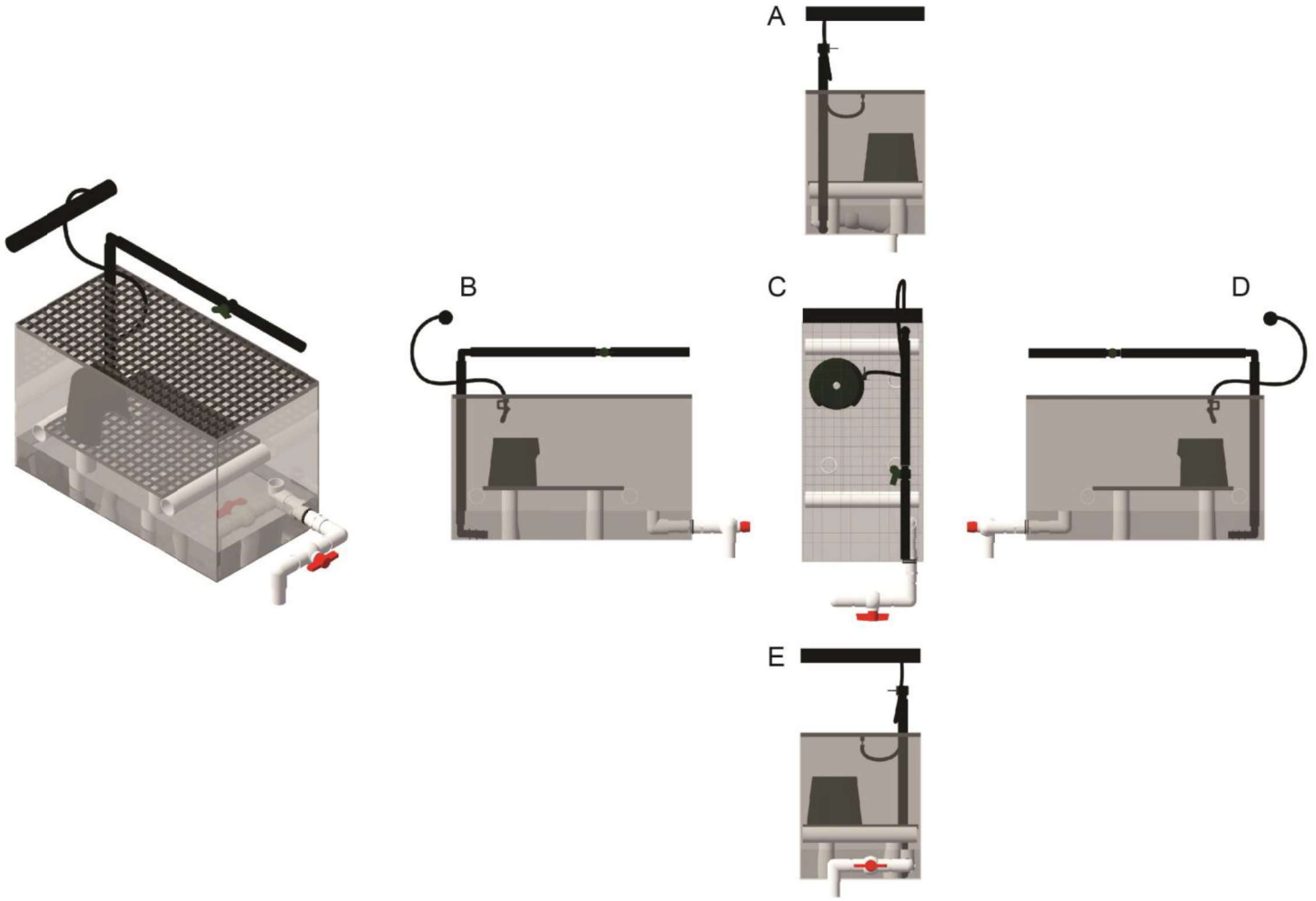

Fig. 4. Views of the microenvironment designed for *Lithobates*. A. Back view. B. Left side view. C. Top view. D. Right side view. E. Frontal view.

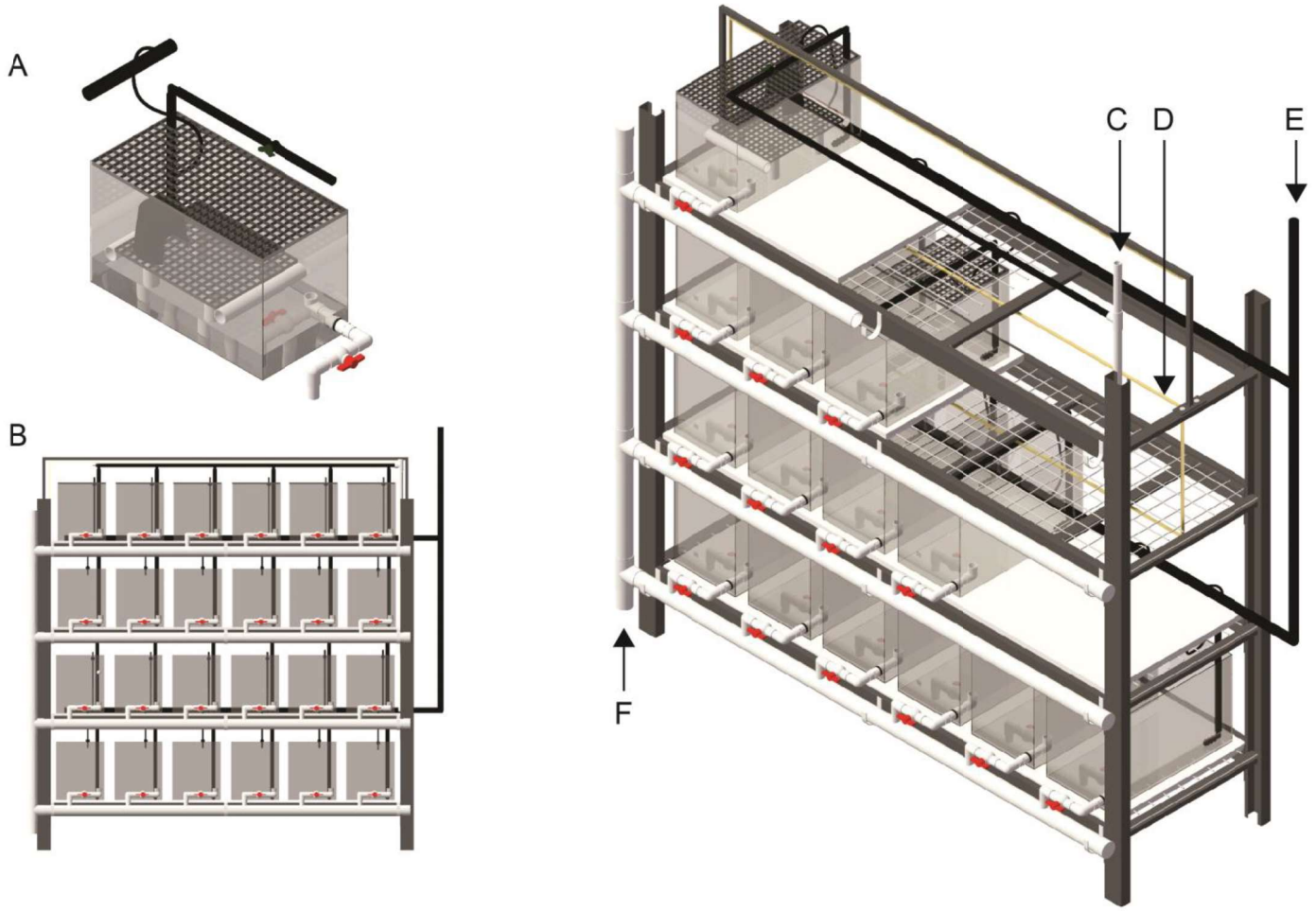

Fig. 5. Functional module designed for *Lithobates*. A. Complete primary enclosure. B. Functional module frontal view. C. Main pipeline for the washing system. D. Led Lights. E. Main pipeline for the automated misting system. F. Main collectors drains**.**

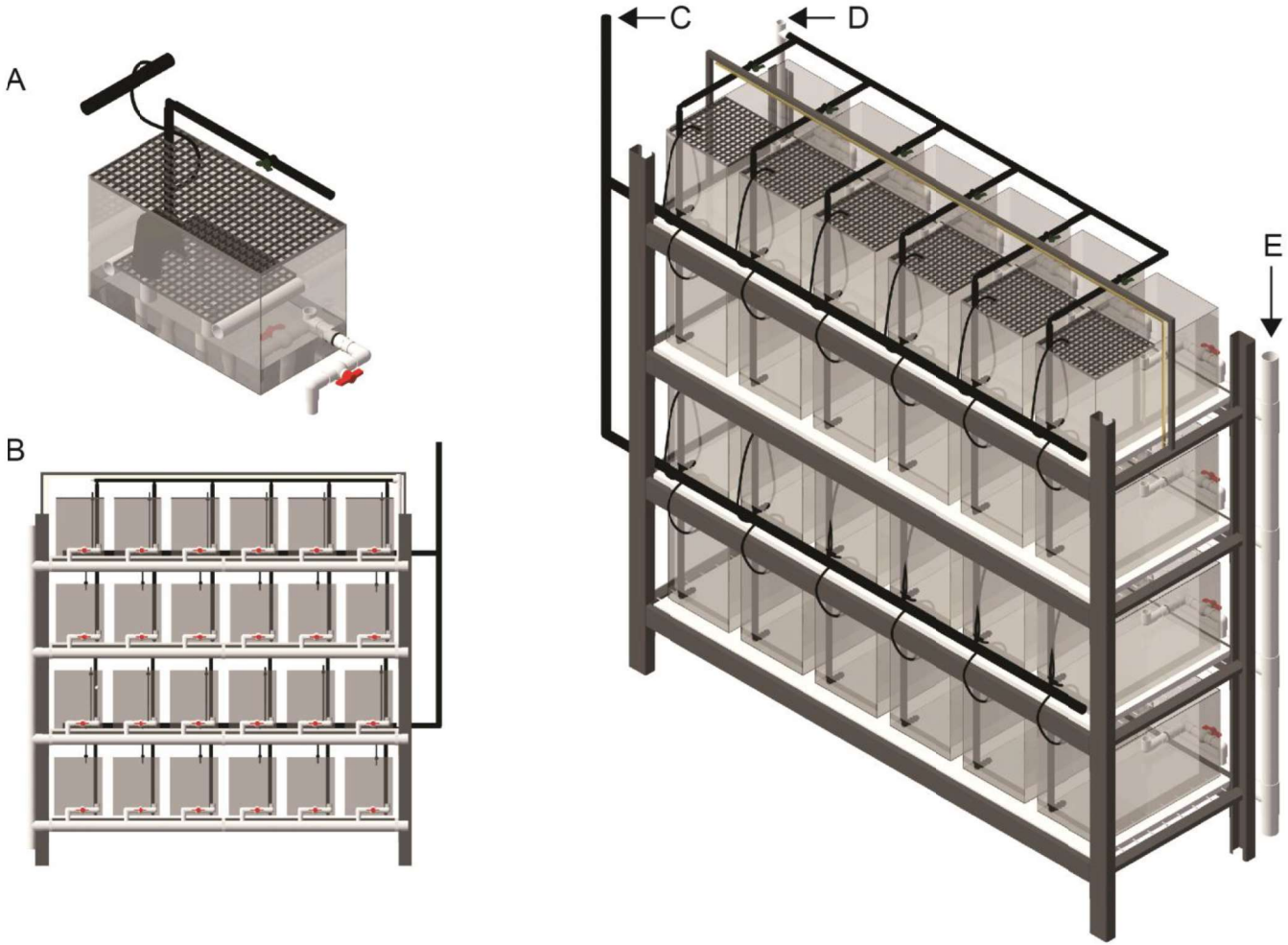

Fig. 6. Functional module designed for *Lithobates*. A. Complete primary enclosure. B. Functional module back view. C. Main pipeline for the automated misting system. D. Main pipeline for the washing system. E. Main collectors drains**.**

Fig. 7. A. Washing area and B. Filtering, storage and distribution water system of Laboratory of Experimental and Comparative Pathology (LAPECOM), School of Biology. University of Costa Rica. This laboratory was designed and built based on the project that represents these thesis.

This paper will be submitted to Biological Conservation - Perspectives

2 *Lithobates vibicarius:* A FROG THAT TELLS THE STORY OF A GLOBAL CATASTROPHE.

Authors: Gilbert Alvarado, Randall Jiménez, José Sandoval, Kimberly Castro, Yoryineth Méndez, J. Alan Pounds, Mark Wainwright, Robert Pushendorf, Steven Whitfield, Federico Bolaños & José Luiz Catão-Dias.

2.1 HIGHLIGHTS

- Costa Rica represents, in a small scale, the global amphibian decline.
- *Lithobates vibicarius* represents a historical process shared with various frogs around the world.
- *Lithobates vibicarius* associates concepts related to both *Lazarus* species and umbrella species.
- *Craugastor escoces* opens an ethical debate on the final destination of a species.
- *Ex situ* conservation must be completely associated with an *in situ* program.

2.2 ABSTRACT

The global panorama of the amphibian declines has been well described and documented There is a consensus made by the scientific community that this phenomenon occurred simultaneously in almost all countries. What makes the nature of this phenomenon distinctive is that it occurs in protected and pristine locations. Costa Rica and *Lithobates vibicarius* both give us the opportunity to represent a global process. We have documentation of this species from the 60s to present day for the discussion of concepts associated with conservation in an applied and practical manner. The importance of museums, the reparation process of some species of frogs in the past 15 years, the dynamic in species conservation status as stated by the IUCN, the “decade of Silence of the Frogs” that took place in the 90s, the ever-relevant role of species’ natural history for our understanding of their

ecological dynamic, the direct anthropogenic impact, events like ENSO, the rediscovery of an “extinct” species and another not seen in several years and in critical danger, are all events associated with our conservation program and the investigation carried out on the species. The paper also presents an ethical debate regarding the final destination of a *Lazarus* species and the reevaluation of the efforts for and importance of ex situ conservation. Costa Rica has been the epicenter of one of the main amphibian declines and the Green-Eyed Frog stands as the embodiment.

Keywords: Amphibian conservation, *Lithobates vibicarius*, *Lazarus* species, *Ex situ* conservation.

2.3 INTRODUCTION

Probably the best documented massive decline in a group of vertebrates recorded in modern age corresponds to that experimented by the amphibians (Hoffmann et al., 2010; Mendelson et al., 2006; Scheele et al., 2019; Stuart et al., 2004). By the end of the eighties and early nineties, there was a clear establishment of the phenomenon of population decline and loss amongst amphibians in different places around the world, for which no clear causality had been determined (Alford et al., 2007; Blaustein et al., 2011; Blaustein and Wake, 1995; Lips et al., 2005; Longcore et al., 2007; Phillips, 1990; Pounds et al., 2006; Wake, 2012, 1991). The pinnacle of this documentation took place in the first decade of the 2000s when 36 species of frogs had been declared extinct worldwide by the IUCN (Jiménez and Alvarado, 2017). This became further important by the large group of species declared critically endangered (CR), many with the expectancy of never being seen again (IUCN SSC Amphibian Specialist Group & NatureServe, 2013). The phenomenon was so alarming it became one of the greatest examples of biodiversity crisis any current generation has experienced (Barnosky et al., 2011; Pievani, 2014; Wake and Vredenburg, 2008).

Of the 36 species, two of them became emblems for the problem; golden toad (*Incilius periglenes*) of the cloud forests of Monteverde, in the northern mountains of Costa Rica, and southern gastric brooding frog (*Rheobatrachus silus*) southeast of Queensland in the subtropical region of Australia (Blaustein and Wake, 1995). Both species had populations under constant studies when individual count dropped

dramatically in three years to the point of registering a single individual at the time of the last year of monitoring; this happened in 1981 for the Australian species and then in 1989 for the Costa Rican species (Crump et al., 1992; Hines et al., 1999; Pounds and Crump, 1994). These are only two examples of species with rapid population disappearance, so unexpected researchers were left almost barren of a chance to plant an immediate hypothesis and take action (Hero and Morrison, 2004; Lips et al., 2005).

The declaration of extinction (EX) is the worst status to be assigned to a species by the IUCN. It assumes the species will never be seen alive again. However, eight of the 36 species of frogs have been observed in natural conditions around the world (Jiménez and Alvarado, 2017). The second worse position in conservation status for a species is critically endangered (CR); many of the cataloged amphibians around the world received this condition. During the last official evaluation done in Costa Rica in 2014, of 201 reported species, 12% (24 species) were cataloged to be in critical endangered status, as in, species with an incredibly high risk of extinction in the wild as per the criteria established by the IUCN (Chaves et al., 2014a).

The pronouncement of the conservation status of a species does not act as a permanent and rooted label. The examples mentioned of species thought to be extinct only to be rediscovered and placed back on the list in time are very clear, the known Lazarus species (Flessa, 1983; Meijaard and Nijman, 2014). The same goes for species that weren't declared extinct but were thought to be and were then cataloged in critical endangered. There are several species that reappeared, in the case of Costa Rica, there´s documentation of seven species now placed under threatened when some were thought to be practically extinct in the nineties (Whitfield et al., 2017). Of the nine species, the one that has had a better recovery story is Green Eye Frog, *Lithobates vibicarius* one of the most abundant species in the high mountains of our country before the eighties (Savage, 2002). After having been considered extinct in the nineties, it was first evaluated as critically endangered (CR) until 2013 where it was reevaluated as a vulnerable species (VU) by the IUCN (IUCN SSC Amphibian Specialist Group & NatureServe, 2013). It is important to underline that this species is currently considered to possibly be extinct in Panama; as part of the historical distribution of the species corresponds to the west mountain zone in this country (Hillis and de Sá, 1988).

2.4 REAPPEARANCES AND HISTORICAL RECORDS. VIBICARIA FROG AS A MODEL SPECIES.

The institutions that have most helped in the documentation of amphibians are the natural history museums from around the world. For centuries, the collections inside these museums have provided the essential databases to formulate a historical perspective of distribution, diversity and general biology of species in space and time. These collections also have critical data for our understanding of the dynamics of ecosystems and any decision making regarding the conservation of biodiversity (Alvarado et al., 2014; Cottril, 1995).

The Museum of Zoology of the University of Costa Rica contains specimens of amphibians cataloged in the 60s. *L. vibicarius* stands as one of the species that had been observed and cataloged before the Museum had obtained any register for the country. The data obtained from these registers details this species as one of the most common frogs above 1400 m.a.s.l. with a spatial distribution comprised mainly of the central and east of Costa Rica's Central Mountain Range. However, the presence of this species is also well known along the Tilarán and Talamanca Mountain Ranges on its Costa Rican and Panamanian side (Fig. 1).

The population declines and losses known for this species reached a peak in the late eighties and early nineties. Amongst the best-documented cases were those of Monteverde and the Las Tablas Protective Zone in the limit between Costa Rica and Panama (Crump et al., 1992; Lips, 1998; Pounds and Crump, 1994). After these reports, there is approximately a decade of species disappearance not only in Costa Rica but around the world. The most alarming part was that at that moment, the phenomenon could not be associated with any evident cause; along with this, the occurrences were taking place in protected, pristine areas (Laurance et al., 1996; Lotters et al., 2009; Puschendorf et al., 2006b, 2006a; Ron et al., 2003; Whitfield et al., 2007). All of the mentioned generated the “decade of silence of the frogs”; with no real scientific explanation, many unifactorial hypotheses were established always to be outweighed by a multifactorial outlook (Blaustein et al., 2011; Blaustein and Kiesecker, 2002; Collins and Storfer, 2003).

2.5 REAPPEARANCE AND LOCATION OF *L. vibicarius* IN NEW SITES.

In the case of *L. vibicarius,* like other species around the world, disappeared from protected areas and populations that were being monitored at the time. There is documentation of populations that stopped being seen in the 90s in the central and western part of the Central Volcanic Mountain Range, Tilarán Volcanic Mountain Range and the periphery of the Talamanca Mountain Range. However, in the western part of the Central Volcanic Mountain Range, there was never constant and systematic monitoring of the amphibian populations. In 2002, in a central location within the eastern part of the Tilarán Volcanic Mountain Range, specifically in the Children´s Eternal Rainforest, samples of tadpoles were found and transported to laboratory conditions; this finding confirms the reappearance of *L. vibicarius*. This species is the first to reappear from a select group that has been cataloged as relict frogs of Costa Rica (Whitfield et al., 2017). After the reappearance of the Vibicaria frog, more reappearances for other species were being reported, including new reports for *Incilius holdridgei* and *Craugastor escoces* declared extinct by the IUCN in 2004. These two complement the reappearance of eight species of amphibians that had been officially declared extinct in the world (Abarca et al., 2010; Jiménez and Alvarado, 2017).

In order to evaluate the species status we have decided to establish three categories for the populations of *L. vibicarius* parting in 2002. First, we have *established* populations (ES) that correspond to those where all life stages have been observed (egg, tadpole, metamorph, juvenil, adult). The second category is comprised of *non-established* populations (NE) where only adults have been observed and in conservative numbers for most cases. The last category is composed of the cases where *only one individual* has been reported (SR) (Fig. 2). In the past years, *L. vibicarius* has allowed us to detail the west part of the Central Volcanic Mountain Range in the Juan Castro Blanco National Park, a protected site devoid of any register of a previous systematic search for amphibians. However, there are accounts of a decrease in population number for this species from residents of the outskirts of the park. The frog is reported in the periphery, with a later reduction of its populations mainly in the nucleus of the protected area; in an interesting way, the years reported to have been a decrease in population dynamics correspond to the end of the decade of the eighties and the beginning of the nineties.

The species was documented in Juan Castro Blanco National Park in 2007 (UCR 20246) and later in 2012 in another sector (Castro-Cruz and García-Fernández, 2012). Since 2013, we have determined nine sites with bodies of water (lagoons, wetlands or swamps) that are used in this species' reproduction. We decided to separate the territory in five sectors that have been monitored. The sectors were established according to geographic barriers and are separated by an average of two 2.0 Km in a straight line. (Fig. 3).

2.6 NOTES ON THE NATURAL HISTORY OF *L. vibicarius.*

There are two reproductive events that occur throughout the year in the Juan Castro Blanco National Park. A first event takes place in May and the second begins around October, corresponding to the time of greater establishment and precipitation in the areas of study respectively. The reproduction that takes place in the second half of the year is notably more efficient based on the increasingly higher number of egg masses, tadpoles, and froglets in the reproduction ponds. From the beginning of our monitoring, 2014 stands as an exceptional year in terms of being one of the warmest in Costa Rica due to the effect of the ENSO (El Niño Southern Oscillation) phenomenon, thus progressing into the most intense drought in the past 30 years (IMN, 2014). That year the reproduction ponds dried out completely, putting a damper on reproduction for the animals near the focal sites.

Despite the existence of a pattern in reproduction amongst the observed animals since 2013, it is also possible to find egg masses and tadpoles in the form of isolated events between April and December. In the same manner, it is not uncommon to find adults in distant locations during the same time frame. A phenomenon that has drawn our attention is the massive number of tadpoles that swim in aggregated regular form with complete synchrony. Both observations of this phenomenon consisted of a main and singular group that moved throughout the pond during the second period of reproduction in one of the studied sectors.

2.7 RISKS IN THE CONSERVATION OF LAKES USED AS REPRODUCTION SITES FOR *L. vibicarius.*

The reported sighting for *L. vibicarius* are within National Parks or protected areas, in the eastern part of the Tilarán Volcanic Mountain Range, (Santa Elena Cloud Forest Reserve, Monteverde Reserve of Tropical Science Center, Children´s Eternal Rainforest, Alberto Manuel Brenes Biological Reserve of University of Costa Rica and Arenal Volcano National Park). In the case of the western part of the Central Volcanic Mountain Range, the Juan Castro Blanco National Water Park and its outskirts, in particular, some of the ponds are found outside the protected areas by law. Local organizations (ONGs) make an effort towards preserving some of the sites inhabited by the species currently. However, we have documented how one of the main reproduction ponds was invaded by a human settlement and is now designated to satisfying the needs of several domestic animals. With the exception of two sectors (Sectors Pozo Verde and Ocotea), the remaining three sectors are located in the periphery of the National Park and they have a high risk of suffering the loss of ponds because they are also found in places dedicated to farming and agriculture. This means that there are five bodies of water currently used for amphibian reproduction with a high probability of being eliminated on account of anthropogenic impact at any moment.

During the sixties, one of the largest wetlands of the high mountains of Costa Rica was drained in order to be used for dairy cattle. The site was probably one of the main reproduction sites for the species; to this day, a small remnant pond at the far end of the terrain still meets that purpose. We found two more ponds that have been drained in the past ten years for agricultural purposes. All of these causes in addition to pathogenic agents like *Batrachochytrium dendrobatidis* (*Bd*), the remarked populations wrestle with a series of threats that could terminate their existence (Alvarado et al., data unpublished).

2.8 *L. vibicarius* INCITES THE SEARCH AND REAPPEARANCE OF OTHER SPECIES.

The Green Eye Frog is one of the animals that rekindled a sense of hope that had been lost by the scientific community and general society, after its reappearance in 2002. It also promoted greater interest in monitoring of a National Park we did not know a whole lot about at a national level and further less internationally, the Juan Castro Blanco National Park. In the frame of this monitoring that continued into the

second semester of 2016, we found a singular specimen of *Craugastor escoces,* a species that had not been observed since 1986, and declared extinct in 2004 by the IUCN (Jiménez and Alvarado, 2017). Most recently, the continued monitoring has helped with further obtaining reports of species placed under critically endangered (CR) on account of not being seen since 1960, an example of this is the tree frog *Isthmohyla rivularis* (Jimenez et al., 2019).

This event reminds us the function that some of these species play in the design of a conservation strategy that benefits other co-occurring species and that can be promoted as a frame of work for the planning of conservation under the concept of Umbrella species (Branton and Richardson, 2010). Now, the Vibicaria frog is not only responsible for urging the conservation of an unknown site for science, but also for the rediscovery of a species declared extinct by the IUCN. This phenomenon reflects one of the few positive sides that the disappearance of amphibian populations worldwide has, which is that as we verify the number of species for that taxon, we can update our bases as it has probably doubled since the second half of the eighties, currently reaching approximately 8000 species (Amphibianweb, 2019).

This significant increase demonstrates the revolution caused by molecular techniques in taxonomy (Storfer et al., 2009). However, a great part of the impact in the number of new species comes from the exploration of new sites in search for species reported as extinct or in critical endangered by the IUCN. The documentation of the “decade of silence for frogs” and the declarations made by the IUCN at the beginning of the century, allowed the movement of important resources for investigation and *in situ* conservation of this group of vertebrates (Kohler et al., 2006).

2.9 *Lazarus* SPECIES COMPEL US TO MAKE A DECISION TOWARDS THEIR CONSERVATION.

The reappearance of *C. escoces* opened the debate about where the individual should end up. We thought of three options regarding the course of action that researchers should take with individuals of Lazarus species at the point of rediscovery and will then discuss the implications for the conservation of these species (Fig. 4). Upon finding a species declared extinct and after having collected samples (e.g., blood and skin) through non-lethal methods to gain knowledge about

the health status of the animals for future conservation actions (if applicable), researchers are faced with a critical ethical problem about what to do with the animals. At this point, researchers could a) collect some individuals, under strict permits, in order to store them in museums as voucher specimens, while not falling into the trap of over-collecting, b) keep some individuals in a conservation *ex situ* program under adequate conditions in order to create a breeding program for reintroduction and to ensure the species survival, or c) temporarily capture individuals following ethical guidelines in order to take adequate high-resolution photographs and videos and genetic samples (e.g., skin, hair, saliva) and then return the individuals to their habitat.

We believe the first option is not beneficial to conservation, because it comes at the cost of the lives of valuable individuals of seriously threatened species; this can contribute to the risk of extinction. However, the collection of specimens is commonly used in science to confirm a species' reappearance and for other scientific purposes (e.g., phenotype and evolutionary studies), but only if specimens are well-preserved (Alvarado et al., 2014). We hope that this option will not be considered solely to obtain personal gratification in announcing the rediscovery of a Lazarus species to the public and scientific community, especially by amateur researchers.

On the other hand, we believe the second option may be beneficial for conservation because reintroductions can lead to species recovery in the wild. However, this option comes with uncertainty regarding the success of the breeding program and reintroductions and requires commitment, time, and financial resources (McGowan et al., 2017). Nevertheless, it can provide crucial information about the biology and ecology of the species (e.g., reproductive behavior, longevity, and growth rate) and can be used to fortify critical data for IUCN Red List criteria. Lastly, if an animal died in captivity, it could be converted to a voucher specimen. Furthermore, this option must be complemented with efforts to determine whether the Lazarus species continue to survive with a small population and in environments that protect them against a specific threat; this can help the success of reintroductions (Tapley et al., 2015). This was the option for the female of *C. escoces* that was found in Costa Rica, and we have undertaken to locate more individuals for our captive-breeding program with the support of public institutions and private organizations (Jiménez and Alvarado, 2017; Zippel et al., 2011a).

The third option may be beneficial to conservation; we can use adequate photographs and videos and genetic samples to document a species' reappearance instead of converting an individual to a voucher specimen, and the safe return of the animals to their habitat gives them the opportunity to mate and reproduce (Minteer et al., 2014). In the case of *C. escoces*, *in situ* conservation cannot by itself ensure the species survival. There are a series of threats already mentioned that require the application of an *ex situ* conservation program (Jiménez and Alvarado, 2017).

2.10 ESTABLISHMENT OF *ex situ* CONSERVATION PROGRAMS.

As the status and the level of threat for many species of amphibians became clear due to the documents collected by specialists from the Worldwide Amphibian Assesment that took place in 2004, we started to become aware of a need to develop *ex situ* conservation programs. This is how many projects around the world have come to be supported by international organizations that help with budgets and logistics (Zippel et al., 2011b). In this way, the Laboratory of Experimental and Comparative Pathology is born for the establishment of an *ex situ* conservation program for *L. vibicarius* that accompanies the *in situ* conservation efforts and that serves as a model and support for the other species that have reappeared in Costa Rica. Even though there has been an immense effort on the conservation of amphibians provided by zoos during the past 20 years, there are still many species that are not a priority for *ex situ* conservation. It is critical we develop infrastructure, abilities, and knowledge in the country of inhabitance of the different species. This along with a record of all efforts made towards the conservation, is important evidence for the planning of a global strategy (Dawson et al., 2016).

There has been an increase in amphibian collections due to the continued decrease in populations and overall species disappearance. A worldwide evaluation in the status for vertebrates stated that captive breeding has played an important role in the recovery of 17 out of 68 species (Hoffmann et al., 2010). However, captive breeding is a costly process that is encompassed with technical difficulties that can lead to issues in terms of hybridization (Crawford et al., 2013); also, there is the effect there may be on learning and the development of practical abilities vital for survival in the wild. The establishment of *ex situ* conservation programs can turn out to be challenging which is why it should not be considered a mere option in case of

emergencies, rather a preventive method that should be placed upon before species reach that point of no return (Harding et al., 2016).

We have known the limitations associated with breeding in captivity for more than three decades now, along with other highly problematic aspects such as the high costs it generates, the establishment of self-sufficient captive populations and the maintenance of an administrative continuity. *Ex situ* conservation tends to be an improvised and a pre- maturely used resource as it seems to be the solution despite there not being an exhaustive search for alternative methods for conservation (Harding et al., 2016). Demonstrating that a species' population is in decline should not be sufficient motive for justifying captive breeding as an appropriate means for recovery. The repercussions that the environment in captivity would have on the species' genotype and phenotype, could be irreversible. From our standpoint, *ex situ* conservation is not justifiable unless associated with an *in situ* conservation program (Snyder et al., 1996).

The inclusion of health programs is vital as a form of scientific backup if they are also applied by highly qualified personal. In addition, it is also necessary the creation of animal welfare programs that provides high standards for animals and a guarantee of adequate infrastructure, the development and application of the best procedures for operation, along with qualified personnel. It should be mandatory for current *ex situ* conservation programs to be evaluated and authorized by Institutional Animal Care and Use Committees (IACUCs) that guarantees the operation requirements and manages their completion.

The international web of collaboration and the facilities that we have regarding global communication are of the essence, as is the establishment of standardized methodologies for the attainment of data and the development of standardized operation procedures. All of the mentioned items are requirements we should have at the time of deciding to establish an *ex situ* conservation program for amphibians or any other taxon.

2.11 CONCLUSIONS

Amphibians have been the best example in recent history of the crisis many terrestrial vertebrates experience (Wake and Vredenburg, 2008). The use of these animals as ecosystem health indicators due to the sensitivity of the molecule

transportation system throughout the skin, has positioned them to be one of the first to perceive eco-physicochemical changes in the environment (Hopkins, 1991). The nineties has represented the “decade of silence of the frogs” where a single phenomenon swept the unprepared scientific community, and for what we still do not have clear and concise explanations (Blaustein and Wake, 1995; Wake, 1991). However, that catastrophic episode has provided the necessary pressure and vision we needed in order to imagine the different approaches we could take using the tools we have available for conservation (Beebee, 2005; Griffiths and Pavajeau, 2008; Langhorne et al., 2013; Scheele et al., 2014).

A decade of disappearances where the community responsible for conservation has only begun to organize itself, systematize and prioritize the needs of amphibian populations around the world (Ibáñez et al., 2010; McGowan et al., 2017). The first decade of the 2000s, when a lot of the work done gets reverted with the re-discovery of species of frogs that were thought to never be seen again (Abarca et al., 2010; Chaves et al., 2014b; Jiménez and Alvarado, 2017; Whitfield et al., 2017). Years when new molecular technologies arose for use in the taxonomy of species, along with an increased investment in searches for species thought extinct. All these have given way to a substantial increase in new species of amphibians around the world, what can be called the paradigm of disappearances in the increase of new species.

This phenomenon of reappearances presents a new opportunity, once we have species living in the same conditions we thought caused its extinction, the so called “survivors of the decade of silent of the frogs” (Whitfield et al., 2017). The search and establishment of new systematic monitoring gave us the opportunity to explore new sites and with that certain species that had been lost for a while start to show up; the *Lazarus* species of the modern age (Meijaard and Nijman, 2014).

All of these species pose as motive for resurgence of ethical matters such as up to what point should we intervene with their preservation, the generation of data necessary for science and support the conservation of the different species. One of the tools applied to these animals has been *ex situ* conservation that has generated a whole movement worldwide for the establishment of centers with this objective. Every *ex situ* program should have as final object to reintroduce the animals, for this it is completely indispensable to write protocols for reproduction that also guarantee the genetic and ethological quality of the animals in the wild; all of which are

extremely complex and expensive processes (Griffiths and Pavajeau, 2008; Harding et al., 2016; Zippel et al., 2011a). However, most complicated bit is being able to maintain these programs in a long term perspectives, under different administrations and social, economical and political changes the any country may endure.

Thirty years of change in a group of animals has definitely not been enough for the scientific community and conservationists to generate robust technical criteria for making decisions on all we have encountered (Greenberg et al., 2016; Mendelson et al., 2019). The Green Eyed frog *L. vibicarius* is a species that has showed us, in a short interval, what has happened during the years to many other species from different taxa and what could or is happening to animal populations around the world. The importance of systematization in our field work, revision of historical records and a methodic search of these and new places, is displayed with the re- discovery of an extinct species. This shows us the need for a prevailing knowledge of the biology at hand with a focal species and the immediate risks it presents for the species' conservation.

A daunting story, a story of hope that ultimately ends in a story of greater responsibility. The reappearance of a species should fill us with immense joy, but also a sense of duty because a second act of extinction this time, would be documented and directly witnessed by us.

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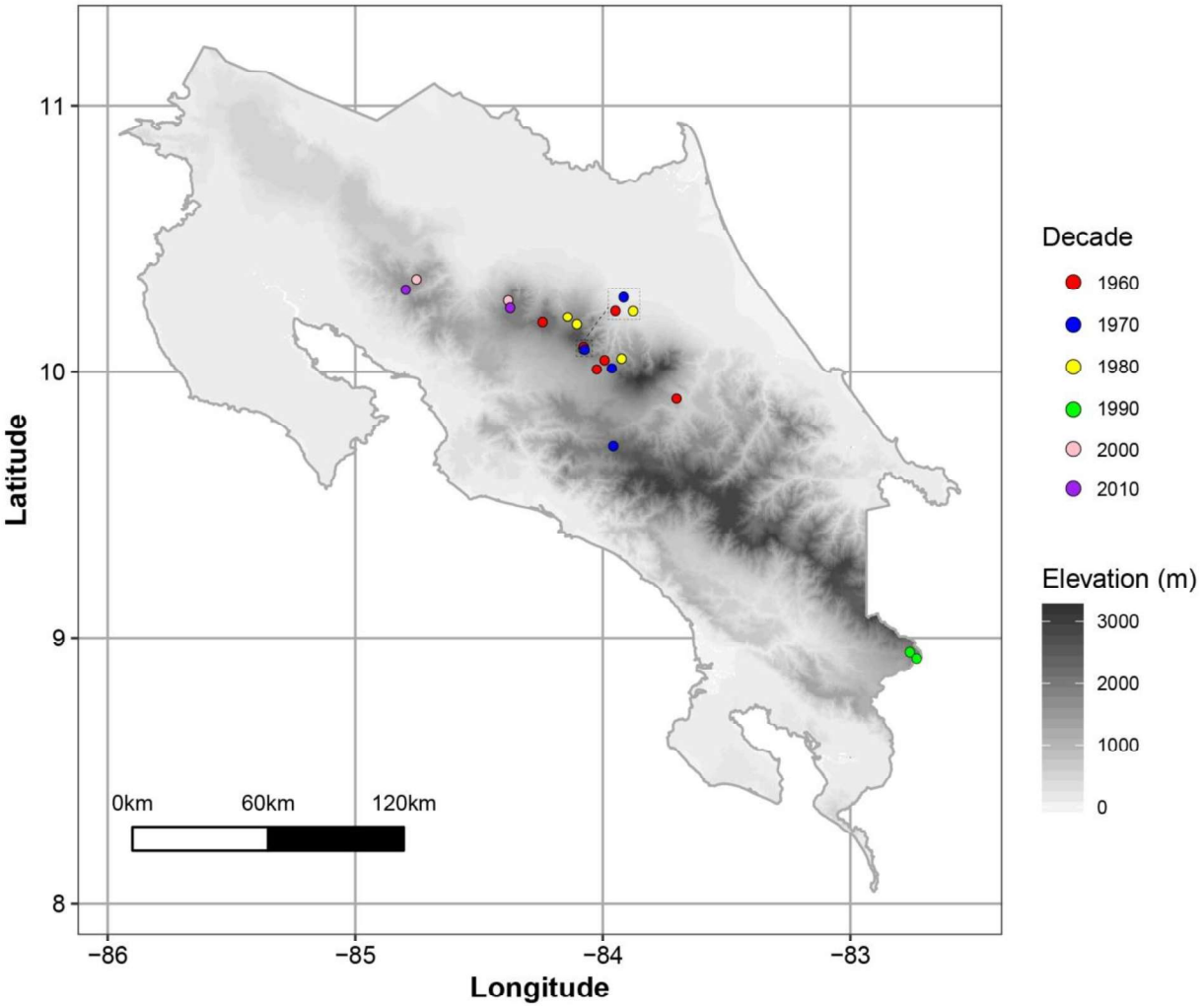

Fig. 1. Historical distribution of *Lithobates vibicarius* corresponding to the specimens collected and deposited in the Zoology Museum of the School of Biology of the University of Costa Rica since the early 60s. The last specimens collected were deposited by Karen Lips in 1990 from the Las Tablas Protective Zone in the Southeast of the country. There are new records up to 2002 of specimens deposited by Alan Pounds collected in Monteverde, Costa Rica. Green Eye Frog was last seen in the late 80s in the central part of the country and in 1993 in the Southeast of the country on the border with Panama.

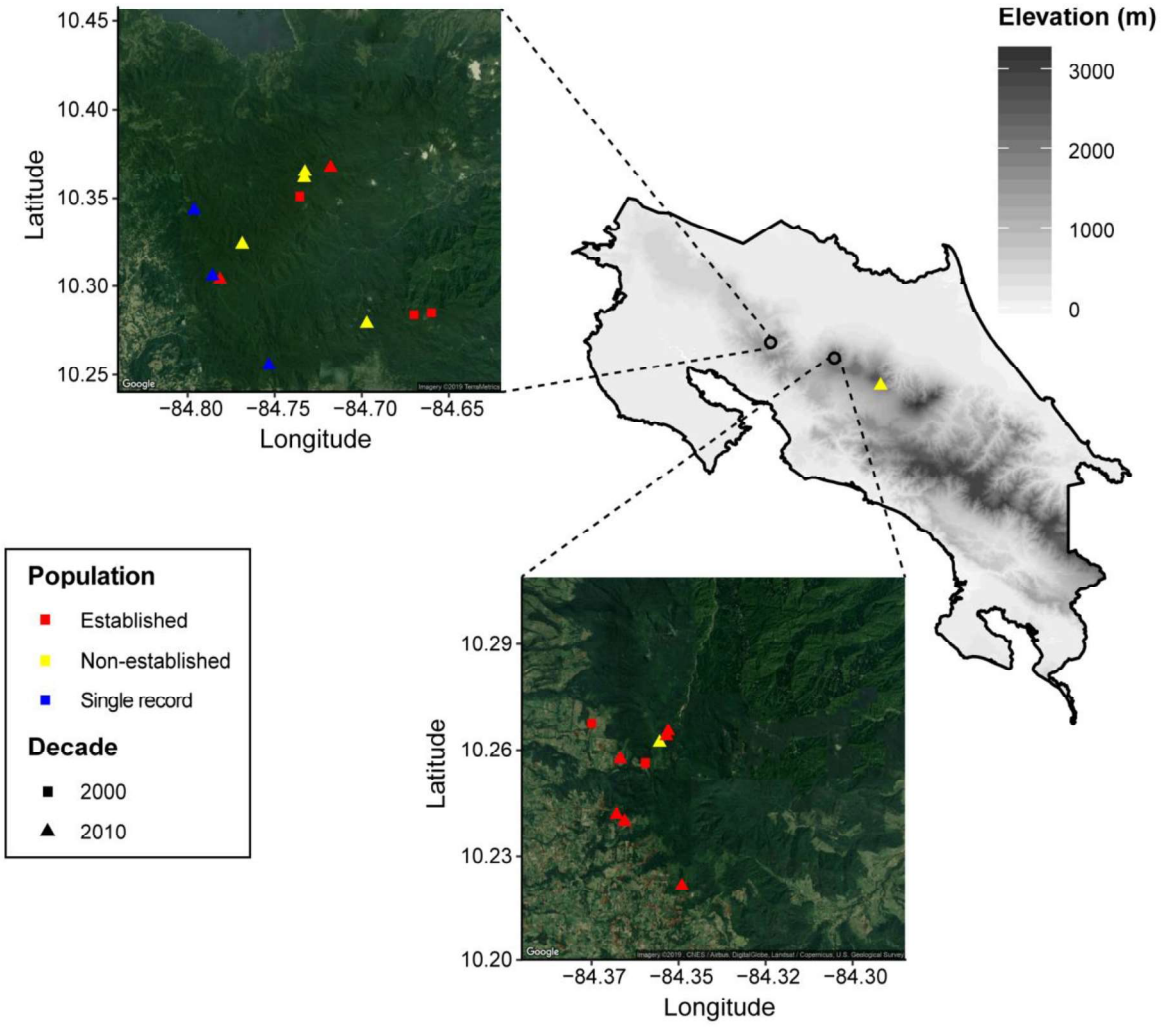

Fig. 2. Metapopulations and decade in which they were seen for the first time from the eastern section of the Tilarán Mountain Range (Santa Elena Cloud Forest Reserve, Monteverde Reserve of Tropical Science Center, Children´s Eternal Rainforest, Alberto Manuel Brenes Biological Reserve of University of Costa Rica and Arenal Volcano National Park) and western section of the Central Volcanic Mountain Range (Juan Castro Blanco National Park). Only two natural blocks with established populations of *Lithobates vibicarius* present today. Source of information: authors and Morera Chacón and Sánchez Porras (2015)

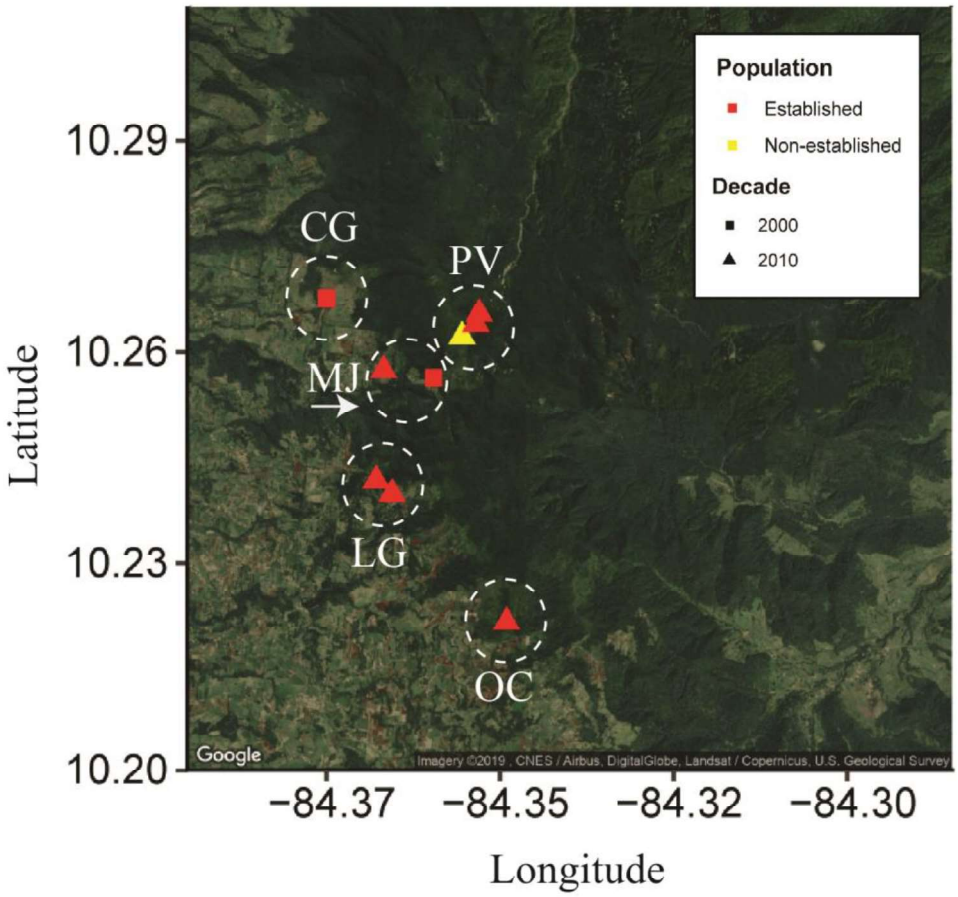

Fig. 3. Metapopulations and decade in which they were seen for the first time establishing study sectors of *Lithobates vibicarius* associated with reproduction centers of the Juan Castro Blanco National Park in the systematic monitoring carried out since 2013. PZ: Pozo Verde, MJ: Los Monjes, CG: Congo, LG: Lagunillas, OC: Ocotea.

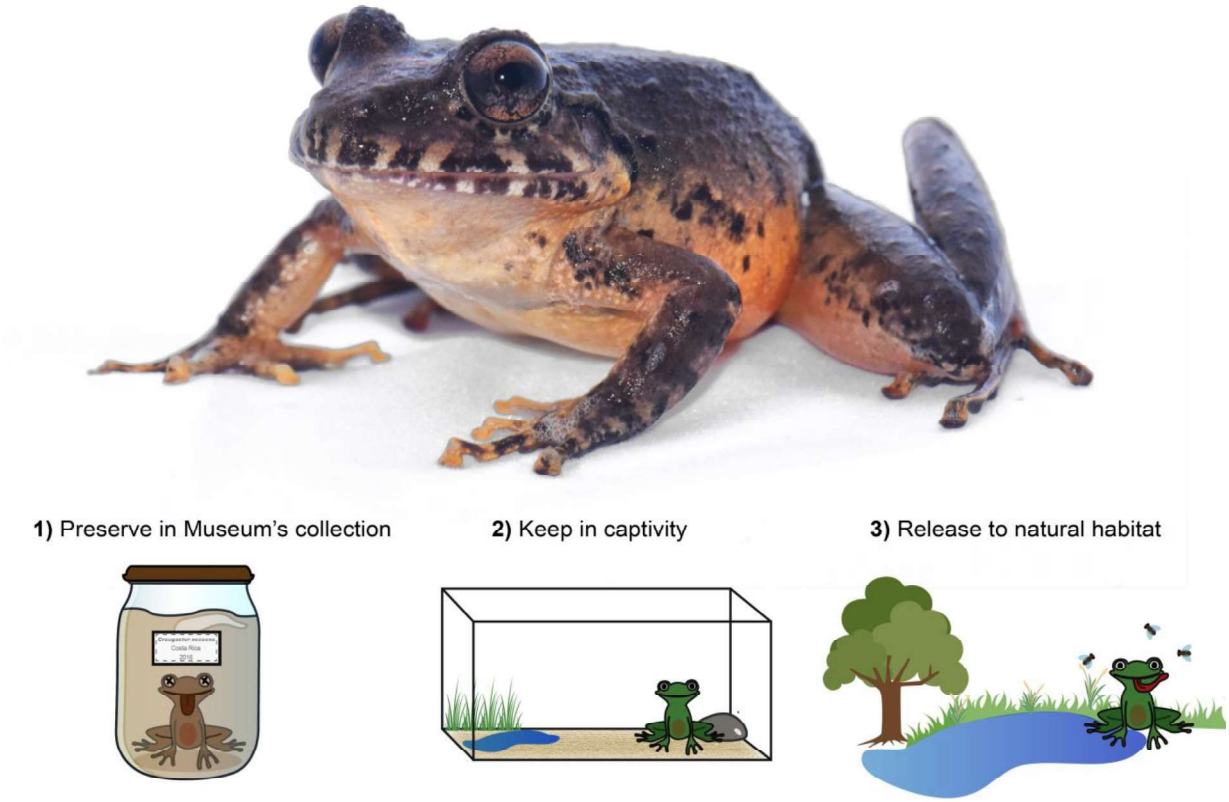

Fig. 4. Existing possibilities for the critical, ethical and responsible decision on the final destination of an individual considered as a Lazarus species. Real situation experienced by two authors in September 2016 when an individual of *Craugastor escoces* was found. Ilustrations by Tania Chacón-Ordoñez.

This paper will be submitted to Scientific Reports.

3 EXPERIMENTAL EXPOSURE INDICATES THAT THE ENDEMIC AMPHIBIAN CHYTRID POSES A LOW RISK FOR THE REAPPEARED POPULATION OF *Lithobates vibicarius* FROGS OF COSTA RICA.

Authors: Gilbert Alvarado, Josué Díaz, Tatiana Bolaños, Randall Jiménez, Jilma Alemán-Laporte, José Sandoval, Kimberly Castro, Fiorella Fiatt, Juan Ignacio Abarca, Catalina Murillo, Adrián Pinto, Juan Alberto Morales, María Forzán & José Luiz Catão-Dias.

3.1 ABSTRACT

The amphibian population declines are among the most alarming phenomena of the Anthropocene One of the suggested culprits is *Batrachochytrium dendrobatidis* (*Bd*); however, its pathogenic effects solely are not enough reason for the species' disappearance. The green-eyed frog (*Lithobates vibicarius*) is a Neotropical frog of high altitudes, thought to have become extinct in the 90's, the "decade of silence for frogs". After its reappearance in 2002, populations seem to be increasing. In the laboratory, we have provided optimal conditions for both the host and the pathological agent with the objective of evaluating host response to an infection. The strain used is in circulation within the region inhabited by the frog. The model species was able to resolve the clinical signs presented in a two week period and within 8 weeks, more than 80% of the animals had managed to eliminate the *Bd.* There are no indications of anatomo-pathological or hematologic alterations directly associated with the experimental process of infection. Based on our results, it is suggested that *Bd* infection in *L. vibicarius* is self-limiting with low morbidity and no mortality.

Keywords: *Batrachochytrim dendrobatidis*, experimental infection, pathology, hematology, chytridiomycosis.

3.2 INTRODUCTION

Amphibian populations started disappearing towards the end of the eighties, many for which there was systematic monitoring for around the world [1–4]. In Costa Rica this happened with the emblematic “golden toad” *Incilius periglenes,* in the cloud forests of Monteverde [5,6], the phenomenon was also well documented for many other species near the southern border of the country, amongst them *Lithobates vibicarius* [7]*.* During the nineties, several species vanished from the sites they lived in, under seemingly usual conditions. Since 2002, various species of amphibians thought to have become extinct reappeared in Costa Rica [8]; these included the *Incilius holdridgei* [9]and *Craugastor escoces* [10]. Other species with relict populations like *Craugastor ranoides* [11,12] and *Craugastor taurus* [13] are rediscovered. This is a reoccurring phenomenon for populations around the world [10].

Chytridiomycosis is a disease caused by *Batrachochytrium dendrobatidis* (*Bd*) in the skin of amphibians, mainly reported in anurans [14–19]. This etiological agent has been responsible for the recent decline of more than 500 species around the world [4]. This chytrid was described 10 years after the major declines were reported in the highlands of central Costa Rica [20]. *Bd* has been cataloged as one of the most severe threats endemic amphibians from around the world should face [4]. The evidence of its presence on a global scale and in historical ranges over 100 years have been well documented [21–26]. The distribution of *Bd* in Costa Rica is broad and reported in well enough detail that the establishment of host risk for vulnerable areas is a prioritized objective [27]. The risk of disease is one of the key elements in establishing the decline of an animal population in the wild [28,29].

The different studies on virulence and pathogenicity of *Bd* [30–33], peptides in the skin of these animals [34–39] and their microbiome [40–43] are amongst the most detailed. However, defiance studies where individual response as a host is evaluated, are still scarce. The difficulty of establishing adequate facilities and standardized operating procedures, as well as the daily maintenance of the animals, act as challenging obstacles in the development of experiments where all variables involved can influence the quality of the results obtained. Integral host evaluation is a fundamental part of formulating reliable conclusions on health impact [19,44–49]. The presence of lesions along with the etiological agent also becomes crucial in order to determine the continuance of the disease [17,50].

Lithobates vibicarius is a frog with a historical distribution range that covered the high mountains of Costa Rica in their entirety [51]. That range started decreasing

until it disappeared, placing the species as critically endangered by the International Union for Conservation of Nature (IUCN) in 2004. However, the species has recovered a great deal since then and began a process of re-evaluation as a vulnerable species with the IUCN in 2013 [52]. The species stands a rare opportunity to evaluate aspects of defense mechanisms a host activates when exposed to an endemic strain of *Bd* from Costa Rica. The assessment were carried out using clinical and anatomical pathology indicators with the objective of determining the danger the presence of this etiological agent represents for *L. vibicarius* in country's highlands.

3.3 RESULTS

We did not find significant differences of the weight in male and female frogs between experimental groups and control group for each experimental day. (Table 1). Males presented no differences in weight between the start of the experiment and either day 14 or day 56. Males had an increase in weight between collected day and day 0 of exposure to *Bd* (Table 2) Females experienced an approximate increase of 20% of their weight between day 0 and day 14 post-exposure (Table 2; Fig. 1).

In regards to clinical signs or medical conditions, none of the animals presented anorexia throughout the experiment. The physical condition remained constant for every animal for the entirety of the experiment and, at the moment of euthanasia, 31/36 animals presented ideal body condition. The rest of the animals were slightly above the ideal range. The frogs consumed their food immediately; therefore, we can guarantee they had proper nourishment. On day three, a light reddening (erythema) had occurred on the pelvic skin, the medial aspect of the posterior extremities, and the plantar areas. Erythema was most intense on day seven, 17 of 24 individuals presented moderate or marked erythema. Some of the animals had noticeable edema. Only one of the individuals in the control group showed signs of moderate erythema, and in the rest of the animals, erythema was absent or mild (Fig. 2). Erythema progressively decreased until complete resolution on day 10 in every animal. One of the animals in G56 died suddenly 12 h before the experiment end with no previous clinical signs of disease.

One animal was *Bd* PCR positive before treatment with itraconazol; however, all the animals were *Bd* PCR negative one day after the treatment, a week before infection, and on day 0 of infection. Based on the previous information, we considered all animals for evaluation in the process of experimental infection *Bd* negative. All of the animals in the control group remained *Bd* PCR negative throughout the experiment. Only one frog, from group G56, was negative on day 7. On day 14, three animals resulted in a negative test. On day 28, 50% of the animals were negative and by week 8, 80% of the animals had cleared *Bd* infection. The peak of infection is after one week (Fig. 3). The animal found dead had been negative since day 14 and its erythema was classified as mild.

In the variables considered for the hematology and the total proteins, no significant changes were observed between the control group and the infected group. There were also no changes in the indicators over time during the experimental process. The mean for all variables were always within the established reference intervals (Table 3; Supplementary Table S1).

No significant gross findings other than hepatic alterations were seen on necropsy examinations. Females had proportionally smaller livers than males. All males in the three groups had some degree of hepatomegaly and mildly enhanced reticular pattern (9/9 control, 8/10 G14, /9 G56). Only in one case hepatomegaly was considered marked. All females had an abdomen full of mature oocytes. Large intracoelomic fat-bodies were readily evident in 70% of the animals (25/35; 9/12 control, 9/12 G14, 7/11 G54) (Fig. 4).

Microscopically, all cutaneous findings were regarded as minimal to mild. Epidermal exocytosis, epidermal hyperplasia (acanthosis), congestion, edema, and lymphoid nodule hyperplasia, were seen in more than 50% of the infected animals (Table 1; Fig. 5). To a lesser extent, some of these findings were noted in some of the control frogs. Fungal elements compatible with empty zoosporangia were only detected microscopically in one frog on day 56 (this animal also had PCR-positive result), and the morphological changes associated with these structures were considered mild. The frog that died suddenly had necrotizing dermatitis with presumptive intracytoplasmic viral inclusion bodies (ICIBs)

Additional microscopic findings were noted in internal viscera of infected and control animals. These are recorded in detail in Supplementary Table S2, Figure S3. In the liver, more than 90% of the frogs had either hypertrophy and hyperplasia of

melanomacrophage centers, moderate to marked hepatocyte microvacuolar change with glassy cytoplasm, and/or nodular histiocytic aggregates. Hematopoiesis was a consistent finding in liver and kidney. Mucosa-associated lymphoid nodules were readily evident throughout the digestive system, including the tongue (17/36 frogs). Parasitic infestations, largely represented by intestinal nematodiasis, were observed in more than 50% of the animals in both groups. The frog that died suddenly had evidence of acute systemic infection, most likely of viral origin and complementary studies are in advanced.

3.4 DISCUSSION

With the results of this study, we are demonstrated that, under our experimental conditions, *Lithobates vibicarius* is no susceptible species to the *Bd* endemic to Costa Rica. In the laboratory, we recreated optimal conditions for the host and the etiological agent in terms of temperature and humidity [51,53]. Studies focused on the integral evaluation of hosts during the process of infection are scarce because of the challenge achieving adequate conditions for the animals represents [45,47,54,55]. The conditions the animals are in will determine their survival. In consequence, it is essential to establish the possible causes of lesions and death of animals because one of these outcomes is not sufficient for the assignment of responsibility to the presence of an etiological agent in the experimental process [56,57].

The strain of *Bd* used was genetically evaluated in recent studies. In Costa Rica, there has been isolation of the strain in different sites and the genotypic characterization seems to indicate a high resemblance, this means it is most likely the same endemic strain that is circulating in the country [58]. The strain used was chosen because it had its origin in *Lithobates taylori,* a species closely related to the species being evaluated in our work. Also, the strain was obtained from a site that, with the exception of a single observed individual of *L. vibicarius* in 2013, does not have registers since the 80s of the established population. The Cerro Chompipe area of the Braulio Carrillo National Park was one of the places with the most numerous populations of *L. vibicarius* of the 80s (Alvarado et al., unpublished data). Also, it corresponds to one of the traditionally monitored sites since the 70s [51,59].

The evaluation of a complete clinical scope is important because this way we will be able to establish characteristic patterns in which a certain disease can

manifest itself, along with knowing which ones can be clinically diagnosed in the host [57]. The most evident manifestation in the animals was cutaneous erythema with a moderate to severe edematous appearance in some cases. The erythema would be distributed on areas that would be in contact with the substrate, on the ventral aspect of the animals. This did not occur in the same manner for the control group; this indicates that the infection could take place and generate a reaction with clinical signs within the first eight days. However, these signs are nonspecific and attributed to any condition associated with generalized cutaneous bacterial infection in frogs [60]. Two weeks was enough time for the animals to completely recover from the described condition.

The animals were collected within a considerable time frame due to the collection plan presented under strict ethical criteria for obtaining permits; we are performing a medium term, parallel, capture/ recapture monitoring. The residence of the frogs in the laboratory for ten months before initiating the experiment, allowed us to develop the standardized operating procedures needed for the maintenance and processing for obtaining samples from the animals. The mentioned time also favored a slight increase in weight for males. For the females, the increase in weight due to the increased abdominal circumference is a constant throughout their stay in the laboratory. In addition, the laying of egg masses on the substrate and in the water presented itself in repeated occasions for four of the seven females used in the study. Oogenesis and the presence of fat bodies are probable indicators of proper maintenance of the animals, once it result in the use of energy for reproduction or to establish a fat reserve [61,62]. Females are able to completely recover their oocyte production until reaching a maximum in a period of approximately three to four weeks. Amongst the observed behaviors, present in more than 50% of the individuals was dermatophagia, a condition in which, in this case, they consume 100% of the skin they shed. The consumption of skin by the same individual that produced it is a phenomenon described before for other species of ranids [63]. The shedding happens with a frequency of approximately three weeks.

It is important to emphasize that an animal was only considered for the experiment if the PCR- test resulted in negative on all three occasions: a day after treatment with Itraconazole, a week before day 0, and on day 0. The animals reached a peak of infection on day 7 and then on, the number of positive animals begins to decrease. On day 56 (eight weeks), we only had two positive individuals for

the PCR- test. This is consistent with the periods of 10 and 15 weeks in order to get all negative individuals; these ranges were established in an experimental infection for *Leiopelma hochstetteri* and *L. pakeka* respectively, species endemic to New Zealand [54]. We also must consider that, mild infections might not be detected because of the intensity or the location of the *Bd* structures on the epidermis [64,65]. However, our results are consistent in terms of positive or negative individuals through time. The experimental husbandry conditions taken during the first 14 days of infection could have collaborated in the maintenance of a high number of infected individuals thus increasing the possibility of a constant reinfection of the animals; this because we did not allow the water in the tanks to circulate, and the substrate was not cleaned out. After day 14, the maintenance standards changed to a weekly replacement of water and substrate; this could explain the decline in infected animals that came with the transition. Another point to consider is that on day 28 of the experiment, all the animals had gone through ecdysis at least once. Dermatophagy is a behavior that could also reduce the quota of *Bd* in the enclosures; as a result, this prevents the return of the *Bd* structures, present in the dead skin, to the environment. When consuming their skin, the animals transfer it directly to their mouth without it coming into contact with the substrate. These results are consistent with the low number of positive individuals found in a parallel capture/ recapture study done with individuals living in the wild (less than 5%; Alvarado, unpublished data). To our knowledge, no other Neotropical amphibian with marked population decline history had previously been faced with the endemic strain of *Bd* circulating in their region, in laboratory conditions and have a subsequent, cleared infection report. Together with the genus *Leiopelma*, *L. vibicarius* is the new species reported that resolve *Bd* infection; however, the studies cannot be compared directly as strains of different origin were used in the experiments. We must also consider the scarce o null probability of using an inoculum with the load of zoospores that the animal would be exposed to in natural conditions. In the same way, it is improbable the processes of exposure to the inoculum and the conditions for reinfection used during the first two weeks of experimentation. The temperature used in the experiment (a mean of 19°C), and 100% humidity in the tank are the ideal conditions for both the frogs and the *Bd* (4–25°C, reported as ideal) [53,66]. The importance these two variables have on the response the host and the etiological agent, has been described [67–70]; because of this, the purpose of the chosen settings was to minimize the influence they could

have on the results of the experiment. The clearance of the *Bd* infection observed in the laboratory, occurs at temperatures that are biologically favorable for both host and etiological organisms. This allowed us to recreate conditions for the sites that *L. vibicarius* inhabits, and the latitudinal ranges where most declines in frog populations in Costa Rica and other places around the world, have been reported [51].

Fungal pathogens have the quality of evading the host's immune system, and possibly inhibit antifungal defenses. There are different tests that demonstrate an active suppression of *Bd* on the host's part [15]. When an evasion of host immune recognition system is detected, two causes have been suggested: ineffective activation of adequate immune pathways and the inefficiency of antibody production [19]. It was determined that the appearance of soluble elements in the supernatant of a *Bd* culture, inhibits the proliferation of lymphocytes and induces apoptosis. However, the same researchers later present proof of lymphocyte proliferation and the abundance in the cells of the spleen could happen because of multiple, repeated exposures and the use of temperature as a treatment for the infection [71].

It was not possible to determine a clear tendency in quantity and types of cells the blood tests affected. Hematology is not a commonly used tool because of the difficulty in taking samples and the obtaining the volume required. One of the reasons we chose *L. vibicarius* as a model species is, beyond its apparent recovery in population numbers, its size and the opportunity it provides to obtain such a biological sample. It was not possible to demonstrate the compromise of white blood cells, as was presented by others authors; however, they do not manage to determine a clear mechanism for resistance and elimination of the infection [47]. The absence of changes in the hematology between groups and throughout the study is an indicator of stability in terms of nutrition and maintenance of the animals. We did not obtain a high N:L index suggestive of an activation of a hematopoietic stress response in either infected or control groups.

Necropsy continues to be the most important tool in the determination of cause of death and anatomical alterations in animals [56]. All microscopic findings described were minimal or mild in intensity with the exception of the animal that died and presented considerable lesions in their lungs and kidney. The presence of structures compatible with *Bd* was difficult to determine probably because of the location and intensity of infection when present. In other studies, characteristic lesions in the skin of animals that are also related to chytridiomycosis like epidermal

hyperplasia or hyperkeratosis, resulted in mild intensity and were presented in some of the control animals. It was not possible to determine a specific pattern to the presence of *Bd* in the skin of animals. Regarding the liver, an enhanced reticular pattern corresponded to the only microscopic finding observed. The difference in size between males and females might be due to the space utilized to keep oocytes in the coelomic cavity of the animals, or it could be because of the mobilization of glycogen for the formation of oocytes.

The presence of cofactors in animals living in the wild, is a variable we could not eliminate; because of this, it was fundamental to look for possible microscopic findings in order to elucidate their relation, or lack thereof, with a *Bd* infection. The presence of parasites was a constant in many individuals with inflammatory reactions; however, we believe this phenomenon is independent of the experimental process. Even considering the presence of cofactors and their influence on the host immune system, the infected animals managed to survive and eliminate it in the great majority of cases. The only animal that died had first resulted in three negative results for the PCR test and its cause of death is allegedly associated with a viral etiology still under investigation.

The opportunity provided by *L. vibicarius*, a threatened species, to study its response to infection by a circulating and endemic strain of *Bd* of the same habitat, demonstrates that the host can resolve the infection within the first weeks when in optimal laboratory conditions for both the host and the etiological agent. The laboratory and experimental conditions act as further challenges for the host by placing it in an even more vulnerable position. These results coincide with the previous results that show coexistence of *Bd* and animals in the wild that seem completely healthy in appearance [8]. This study reaffirms the urgency for the establishment of susceptibility in animals in order to correct the erroneous presumption of imminent risk for all species in terms of presence of *Bd* in the organisms or the region they inhabit. This information is relevant and important for the decision making and establishment of priorities in species conservation.

3.5 METHODS

3.5.1 Ethics statement

This study was developed in strict accordance with the recommendations cited in the Guide for Care and Use of Laboratory Animals of the National Research Council of the National Academies of Science [72]. The protocols used in the investigation were approved by Institutional Animal Care & Use Committee of University of Costa Rica (CICUA 028-15) and Ethics Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil (N°2657080715). The project was approved by the Biodiversity Commission of the Vice-Rectory for Research of the University of Costa Rica (VI 6457-2016 Resolution N°69). All field work, including collection of frogs from the wild, were authorized and all the animals were collected under the permission of the Arenal Huetar Norte Conservation Area (ACAHN) of the National System of Conservation Areas of Costa Rica (SINAC-ACAHN-PI-R-001-2017) of the Ministry of Environment and Energy of the Government of Costa Rica.

3.5.2 Field collection of study individuals

We collected 36 clinically healthy individuals of the green-eyed frog, *L. vibicarius,* from the Juan Castro Blanco National Park and its periphery. The collected frogs did not have any grossly noticeable skin alteration. These frogs were captured between May and November of 2017 in six different expeditions and in four different geographic locations based on existing monitoring efforts that were initiated in 2013 (Alvarado, unpublished data). The sectors established have geographic barriers and are separated by an average of 2.0 Km in a straight line. A mean of nine animals were collected in each sector in at least two different dates with the conditions of no more than five individuals per visit and three or fewer females per site. All measures taken regarding the collection of the animals met the standard of least impact on the populations in the wild and abide by the agreed permits granted by the Costa Rican government. The animals were captured manually starting at 19.00 h, each individual was manipulated with single use nitrile gloves. They were placed and transported in semi-transparent plastic containers (9l X 9w X 7h cm, 0.5 L) with a natural fiber paper towel lining drenched in Amphibian Ringer's Solution following established protocol [73]. The animals were transported to the laboratory between 19.00 and 06.00 h.

3.5.3 Animal husbandry

Maintenance and experimental procedures were performed by the Laboratory of Experimental and Comparative Pathology of the School of Biology of the University of Costa Rica. The laboratory has two structurally independent experiment rooms. These macroenvironments or experiment rooms register a mean temperature of 19.22 ± 1.12°C and a mean relative humidity of 92.74 ± 6.70 %. Data regarding these two variables was measured and registered every hour after entry with an Arduino microprocessor in conjunction with temperature and humidity sensors. The animals were placed in individual transparent glass tanks (50l x 25w x 35h cm, 37.85 L). Also, they were exposed to a 12 h automated photoperiod, accomplished by combining led hose lights (IP67 Sylvania) to a timer located approximately 25 cm above the tanks. Inside each tank, there are two levels: a first level of eight cm of water; above it a 10 cm high platform (23 x 30 cm) that constitutes the second level and has coverage of 60% of the water surface. This platform is lined with natural fiber paper towel in place of substrate. In the posterior left quadrant of the platform, there is a plastic flower pot (13 diameter x 13h cm) with an opening cut (10h x 7w cm), which acts as shelter. The pot is inverted and the top is used by the animals as a third level. Each tank is fed water through individual pipes that connect to a filter tank, and a draining system with individual stopcocks. Also, there are water mist nozzles installed over every platform that are regulated by solenoid valves that activate at 15.00, 17.00 and 19.00 h for 30 s.

Water used for husbandry and in experimental activities was first passed through two types of filters, a conventional physical method (G0058-02, Purefer) and a micro-granular carbon cartridge (UDF- GAC- 2in1, Purefer) in order to reduce the amount of microorganisms present and to eliminate components like chloride in the water. The principal water tank was located inside the laboratory so that there are no changes in water temperature. Every week we replaced the paper towel and completely cycle out the old water with any organic material present. The frogs were fed every 48 h with adult crickets (*Acheta domesticus*) exclusively (one cricket if the individual is < 30g; two crickets if the individual is ≥ 30g). These crickets were raised in the laboratory and maintained on a strict and permanent *ad libitum* diet of chicken feed (Ponedora 18% Golden, Mundi Vet – Dos Pinos), carrots, broccoli, and orange. Any cross-contamination between the infected and control animal modules was

prevented by using individualized protection equipment for each division, new gloves between tanks and always working with the control group a day before experimental procedures on the exposure group and two days before maintenance.

3.5.4 Treatment with itraconazole

There was a three month acclimatization period established from the entry of the animals to laboratory conditions. As a precautionary step for guaranteeing the absence of any *Bd* element on the animals, every individual was submitted to treatment with Itraconazole (2% Itraskin® Drag Pharma, Oral Solution, Chile) diluted in Amphibian Ringer's Solution. We medicated an initial group at 0.005% but ended up suspending treatment during the fourth day because of behavioral changes in some animals and the death of one out of 18 treated individuals. For the second group of 24 animals, we reduced the concentration to 0.0025%. This was the lowest reported effective concentration and still, it had to be suspended because of the death of five animals on the third day and behavioral changes in others. The six animals of *L. vibicarius* and the individuals corresponding to the other species, that resulted to be more sensitive to treatment, have not been considered for the present study and will be addressed in a parallel manuscript focused on acute toxicity of itraconazol. The concentrations and protocols used are based strictly on previous reports [73] with the only modifications being the size of the containers used in treatment and the volume of Itraconazole solution added (9l x 9w x 7h cm, 350 mL respectively). All containers and tanks were disinfected before and after placing the animals in with a 10% solution of commercial chloride for a minimum of five minutes. Following the submersion, the recipients were rinsed twice with abundant water and set to dry a minimum of 24 h before contact with another animal. Swabs were used on every animal for posterior diagnosis of *Bd* via real-time polymerase chain reaction (PCR) before the first treatment and a day after the last day of treatment. There was a four week period established before initiating inoculation of the animals with *Bd*.

3.5.5 Inoculation process and experimental exposure

Seven days before inoculation, we performed another swab for the detection of *Bd* using real-time PCR. It was considered negative if the animal presented a

negative result a day after the treatment of itraconazole together with the previous sample and was considered apt for initiation of the experiment. The frogs were submitted to the strain *Bd* JGA01; isolated from the species *Lithobates taylori* from the Central Volcanic Mountain Range of Costa Rica, in San Rafael de Heredia. This strain was selected for being isolated from a site of reported amphibian decline in species including *L. vibicarius,* for which there is only one observation from 2013 (Alvarado et al., data unpublished). The preparation of the inoculum with approximately 250,000 zoospores, was carried out following the protocol established [73] for the infected animals and the control group. The exposure took place in containers that were used exclusively for each animal and were of a size that only allowed them to sit in the normal position on a substrate with their pelvic patch, thighs and plantar and palmar surfaces in constant contact with the inoculum. After 24 h of direct contact with the inoculum, we proceeded to re-locate the animals to their respective tanks with closed draining systems. The container used for inoculation was inverted and placed in the upper left quadrant of the platform, where the shelter usually remains so as to drench the most frequented region in the tank with the inoculum. After 24 h, the container was removed. The same procedure was conducted with the control group, with the absence of *Bd* in the inoculum 24 h before. We did not open the drain for the tanks that contained infected animals for two weeks; we also did not change the paper towel in that time in order to favor the growth of *Bd* in the skin of the amphibians but also in the substrate and water inside the tanks.

3.5.6 Experimental Design

Frogs of each sex and provenience were randomly assigned to three treatment groups with 12 animals each. After assignment we tested that all groups had similar size (snout vent length: SVL) and weight using an ANOVA model, to ensure homogeneity of physical conditions across animals between the treatment groups. We established two groups of infected animals and one control group. Every animal was assigned randomly to their respective tanks, whether it is infected or non-infected. We established five times set at 0 days (before inoculation), seven, 14, 28 and 56 days (post-exposure), for the collection of skin swabs and blood samples.

One of the infected groups would end treatment on day 14; the other infected and control groups will end on day 56.

3.5.7 Monitoring techniques and necropsy

One day before inoculation, every animal was clinically evaluated, weighed and measured by a trained veterinarian. After the inoculation, the frogs were monitored daily for any behavioral changes or physical signs of disease. On day eight, the animals were evaluated and classified based on the extension, localization and intensity of the cutaneous erythema into four categories: absent (0), mild (1), moderate (2) and severe (3).

For swabbing, we used sterile fine-tipped swabs (MW113; Medical Wire & Equipment, Wiltshire, England). Swabs were passed 10 times along the dorsum, venter, each side of the body, and length of each femur. We swabbed 5 times across each hand and foot [8]. Swabs were extracted in 50 mL of Prepman Ultra (Applied Biosystems, Foster City, CA, USA) and analyzed for the presence of *Bd* DNA by a TaqMan real-time PCR assay in QuantStudio® 3 Real-Time PCR System in Cell and Molecular Biology Center, University of Costa Rica. In summary, we used *Bd* primers and probes [74] with 2X TaqMan Master Mix and internal positive controls in 20 µL reactions containing 4 µL of DNA template. We ran all reactions, with at least one negative control and two positive controls. The reactions with undetermined results were analyzed once more.

Once the swab was taken, we collect blood sample by puncture in the maxillary vein [75] and collected in one, or at times two, heparinized capillary tubes (Fisherbrand®, USA). The total collected volume would depend on the facility experienced at the moment and could vary between 50-150 µL (from an incomplete capillary to two full capillaries). Two blood smears were done immediately after. A fixed amount of blood was transferred to a 20 µL graduated micropipette (Bryopette® 20 µL Sterile, Bioanalytic) for the Natt- Herrick solution with a mean volume of 1.98 µL and gently inverted several times; the mixture was refrigerated (4° C). When we had enough volume, the capillary tube would be spun down for 5 min at 10 000g in order to determine the packed cell volume (PCV). All these procedures for blood collection are based on the protocols established [76]. For white blood cell count (WBC), differential counts were conducted on blood smears that were air dried in a

few hours and dyed with Diff-Quick stain; every slide was dipped ten times, lasting one second in each container. Excess fluid was drained onto a paper towel after every step. The slides were rinsed under tap water until the water ran clear. The numbers of red blood cells (RBC), WBC, and thrombocytes were calculated by hemocytometry using a light microscope and following a suggested methodology [76].

Before being euthanized on day 14 (n=12) and day 56 (n=12), every frog was weighed and measured again. The euthanasia was preceded by immersion in 0.3% (3X doses for anesthesia) ethyl 3-aminobenzoate methane sulfonic acid solution (tricaine methanesulfonate, Sigma-Aldrich Inc., USA) buffered with sodium bicarbonate solution in neutral pH. Lastly, we conducted cardiac exsanguination.

Each frog was necropsied according to a standard methodology [77] and gross pathologic findings as well as the presence of fat bodies and ovogenesis in females were recorded. The carcasses and internal organs were placed in 10% neutral buffered formalin. For histopathologic examination, we made special emphasis on the skin and recorded detailed information on a previously designed template (Supplementary Table S4)**.** For each frog, we evaluated two sections of skin of approximately 0.5 cm x 2 cm of the pelvic patch parallel to the mid-line, and the medial aspect of the thigh; we obtained at least four cuts of each section. Samples of femoral skin also included the underlying appendicular skeletal muscle. We evaluated systematically the heart, lungs, spleen, liver, stomach, small and large intestines, pancreas, kidney, adrenal glands and gonads. Microscopic findings were documented for every animal. These tissues were trimmed, embedded in paraffin-wax, sectioned at 5µm-thick, and stained with hematoxylin and eosin for microscopic examination. Selected tissue sections were stained with Gram/Twort for bacteria. Periodic-acid Schiff (PAS) and Ziehl-Neelsen (ZN) techniques when deemed necessary.

3.5.8 Statistical Analyses

Statistical analyses were performed in R (Version 3.4.2, R Development Core Team 2019). To test the effect of *Bd* infection between experimental groups and across time on the weight of *L. vibicarius*, we performed Generalized Linear Models (GLMs) and Generalized Linear Mixed Models (GLMMs), respectively. Weight measures were log-transformed prior to analysis to improve normality. On GLMMs,

we used frog id as random effect to account for repeated measures in each adult. Tukey's range test was used for pos-hoc tests between *Bd*-exposed groups and the control group, and between time of each experimental group ($p < 0.05$). When performing pos-hoc tests, Dunnett adjustment of the obtained *p*-values was carried out to control for the family wise error rate. We used odds ratio (OR) and 95% confidence intervals (95% CI)[78], to quantify the deviation between each pairwise comparison. To evaluate the effect of *Bd* infection and across time on the hematological parameters of *L. vibicarius* we conducted GLMMs. As above, we used frog id as random factor. We performed Tukey's range test for pos-hoc tests and used false discovery rate (FDR) to control for alpha-inflation under multiple tests. Results are expressed as means with their ± standard error(SE), unless otherwise stated.

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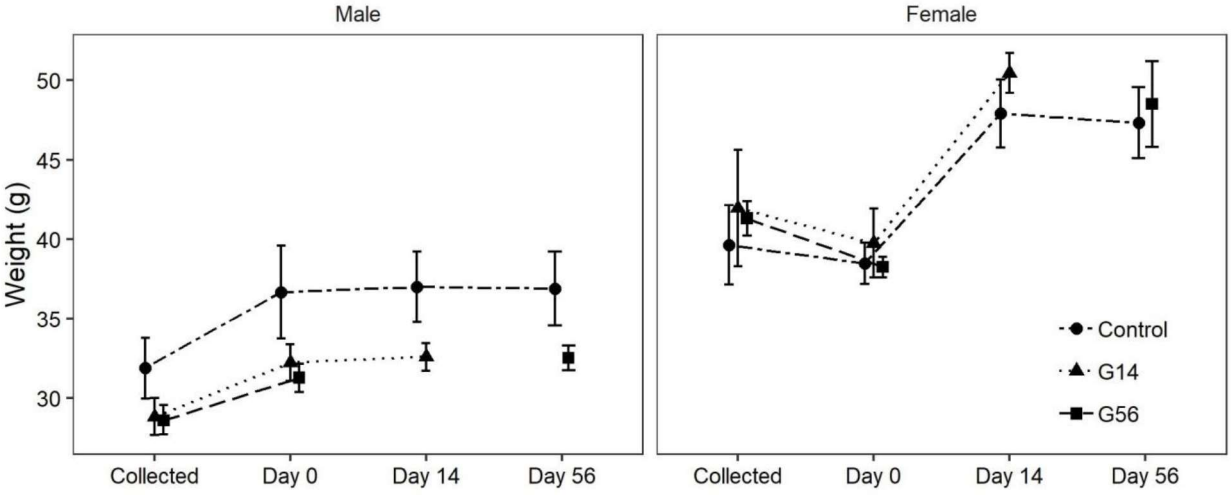

Fig. 1. Mass ($\bar{x}$ ± SE) grams of the animals at the time of the individual was collected and at the different sampling points of the exposure process to *Batrachochytrium dendrobatidis* for each experimental group (Control Group: GC, Day 14 Group: G14 and Day 56 Group: G56).

Table 1. Comparison of the weight in male and female frogs between experimental groups and control group for each experimental day. Asterisk indicates marginally significant difference of means between pairwise comparison and small effect sizes (odds ratio) for males. Control group is the reference for contrasts. Values in parenthesis are 95% CIs of odds ratio.

		MALES			FEMALES		
Contrast	Day	z-ratio	p-value	odds ratio	z-ratio	p-value	odds ratio
G14	Collected	-1.56	0.21	1.11 (0.95 - 1.29)	0.62	0.74	0.94 (0.76 - 1.17)
G56	Collected	-1.67	0.17	1.11 (0.86 - 1.18)	0.44	0.85	0.95 (0.77 - 1.19)
G14	Day 0	-1.72	0.15	1.13 (0.95 - 1.35)	0.63	0.74	0.96 (0.86 - 1.09)
G56	Day 0	-2.09	0.06*	1.17 (0.98 - 1.40)*	-0.11	0.98	1.00 (0.88 - 1.14)
G14	Day 14	-1.93	0.05*	1.13 (0.99 - 1.29)*	0.87	0.38	0.94 (0.84 - 1.06)
G14	Day 56	-1.77	0.07*	1.13 (0.98 - 1.30)*	0.33	0.74	0.97 (0.84 - 1.12)

Table 2. Comparison of the weight in male and female frogs between days of the experiment for each treatment. Asterisk indicates significant difference of means ($p < 0.05$) between pairwise comparisons. Males with small effect sizes (odds ratio) and females with large effect sizes (odds ratio). Day 0 is the reference for contrasts. Values in parenthesis are 95% CIs of odds ratio.

		MALES			FEMALES		
Contrast	Treatment	z.ratio	p.value	Odds Ratio	z.ratio	p.value	Odds Ratio
Collected	Control	-7.12	< 0.0001*	1.16 (1.10 - 1.23)*	1.16	0.50	0.96 (0.90 - 1.04)
Day 14	Control	0.00	1.00	0.99 (0.95 - 1.05)	8.89	< 0.0001*	0.80 (0.75 - 0.85)*
Day 56	Control	-0.07	0.99	1.00 (0.95 - 1.05)	8.38	< 0.0001*	0.81 (0.76 - 0.86)*
Collected	G14	-2.33	0.03*	1.11 (1.00 - 1.25)*	0.86	0.59	0.94 (0.81 - 1.09)
Day 14	G14	0.24	0.94	0.98 (0.89 - 1.10)	4.12	< 0.001*	0.78 (0.68 - 0.90)*
Collected	G56	-2.28	0.04*	1.09 (1.00 - 1.19)*	4.08	< 0.001*	0.92 (0.88 - 0.96)*
Day 56	G56	1.04	0.47	0.96 (0.88 - 1.04)	13.04	<0.0001*	0.78 (0.75 - 0.82)*

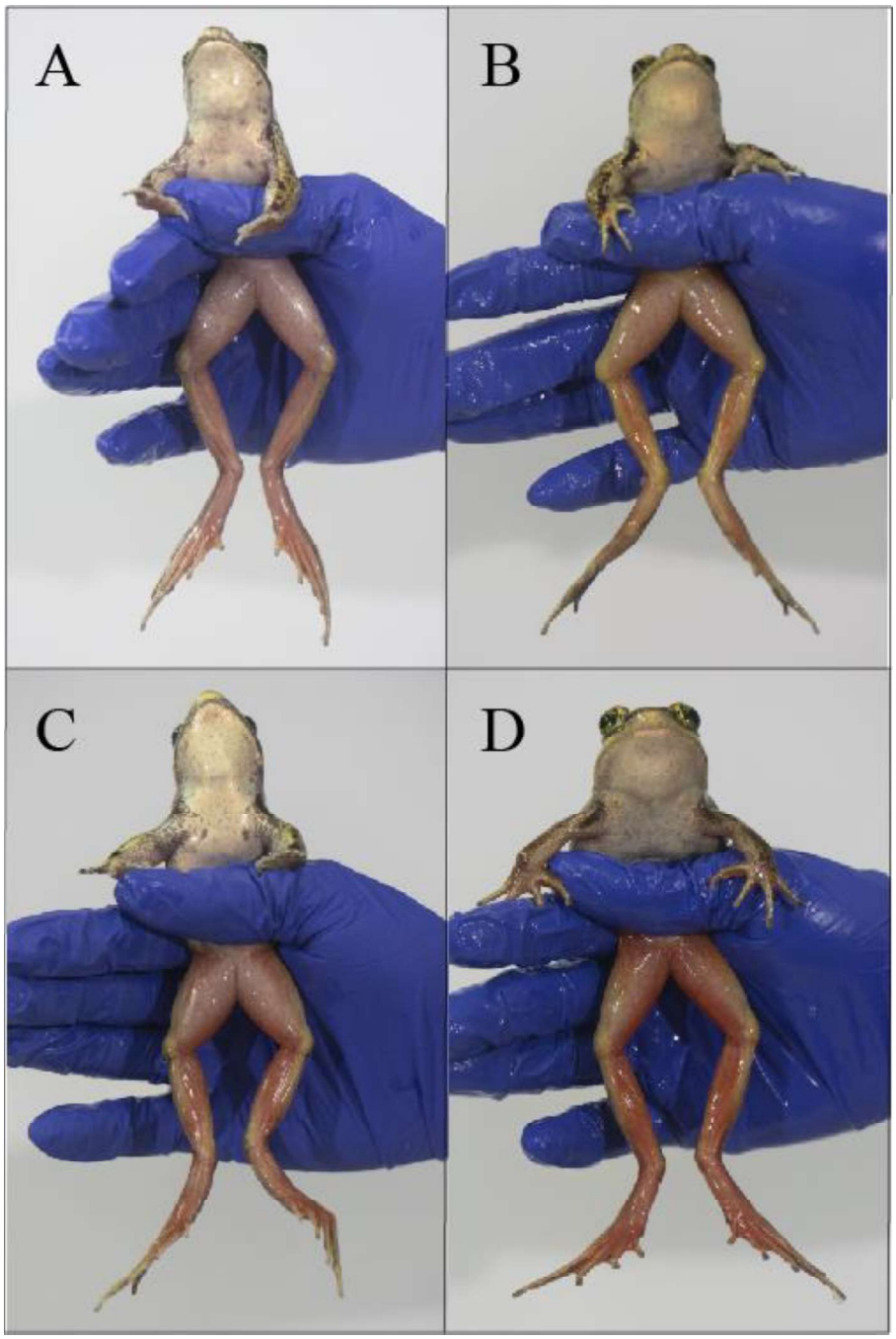

Fig. 2. Visual assessment of cutaneous erythema on the ventral surfaces, primarily inguinal region and medial aspect of caudal limbs of *Lithobates vibicarius* 8 days after exposure to *Batrachochytrium dendrobatidis.* A: Grade 0, no evident erythema (baseline cutaneous appearance). B: Grade 1, mild erythema. C: Grade 2, moderate erythema. D: Grade 3, marked erythema.

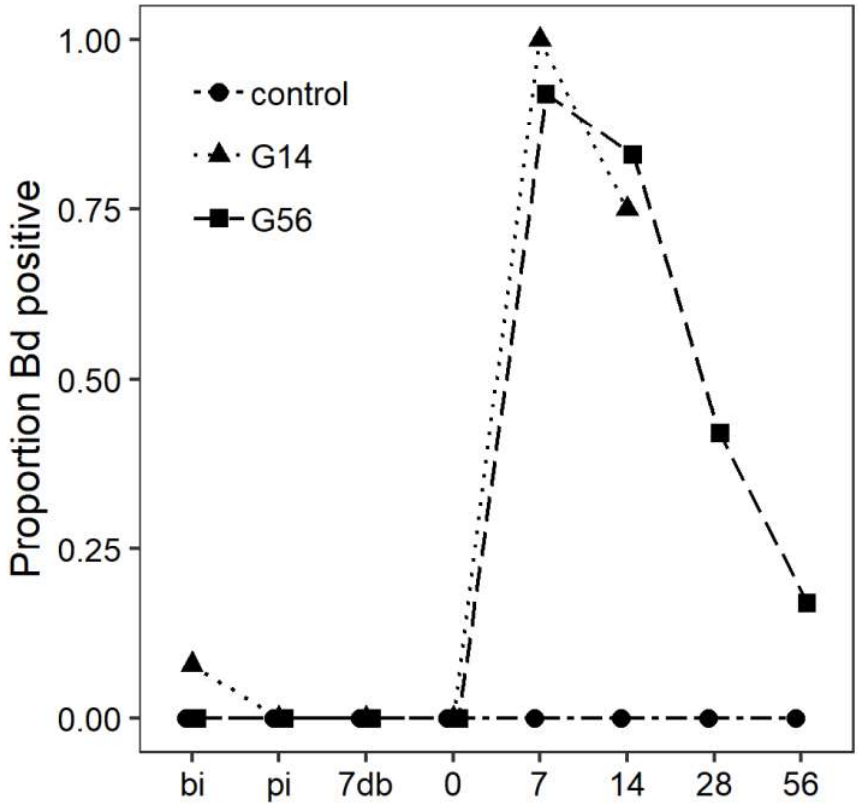

Fig. 3. Proportion of positive individuals to *Batrachochytrium dendrobatidis* (*Bd*) at different sampling points during the experiment. bi: before itraconazole treatment; pi: post itraconazole treatment; 7 db: seven day before exposure to *Bd*; numbers: days post-exposure to *Bd*.

Table 3. Hematology variables and total proteins of adult *Lithobates vibicarius* infected with *Batrachochytrium dendrobatidis* and euthanized 14 (G14, n=12) and 56 (Control Group, n = 12 and G56, n = 11) days post infection (dpi). One individual (G56) died one day before euthanasia. The reference values are indicated as $\bar{x}$ (95% CI). The number of frogs in each group with values outside the proposed RI is indicated if > 0 (0-RI). Asterisk in dpi indicates significant difference of means ($p < 0.05$) between experimental groups and control group for each experimental day. Same letter in means indicates non difference ($p > 0.05$) between days of the experiment for each treatment.

Variable	dpi	Control Group							Infected Groups (G14 + G56)						
Reference Values		n	Mean	SD	Median	Min	Max	0-RI	n	Mean	SD	Median	Min	Max	0-RI
PCV (%)	0*	10	29.95[a]	7.18	32.81	17.68	37.97	1	18	34.82[a,b,c]	6.05	34.05	26.44	44.50	0
33.08 (19.81-46.35)	7	9	31.87[a]	6.47	32.50	23.72	39.88	0	24	33.94[a]	6.11	33.17	22.09	45.68	0
	14*	7	29.64[a]	7.70	32.92	16.66	38.72	1	24	39.12[b]	5.69	39.17	25.55	47.57	1
	28*	10	32.67[a]	7.31	35.57	17.39	39.03	1	12	39.72[b,c]	5.64	41.42	30.10	46.08	0
	56	10	33.25[a]	6.15	34.23	20.87	39.29	0	10	37.79[a,b,c]	4.40	36.56	30.67	45.28	0
RBC (10^12/L)	0	10	0.27[a,b]	0.09	0.29	0.10	0.41	1	18	0.21[a]	0.07	0.22	0.09	0.36	0
0.23 (0.08-0.39)	7	8	0.32[b]	0.14	0.30	0.16	0.55	2	20	0.26[b]	0.06	0.25	0.15	0.42	1
	14*	7	0.20[a]	0.05	0.19	0.14	0.30	0	21	0.27[b,c]	0.07	0.26	0.14	0.43	0
	28	9	0.31[b,c]	0.11	0.28	0.19	0.55	1	11	0.33[d]	0.08	0.33	0.22	0.49	2
	56	11	0.31[b,c]	0.07	0.29	0.23	0.40	2	11	0.29[b,c,d]	0.05	0.30	0.19	0.36	0
Thrombocyte (10^9/L)	0	10	15.40[a]	5.80	14.50	9.00	27.00	1	18	11.83[a,b]	5.80	11.50	3.00	27.00	0
13.11 (1.44-24.77)	7	8	14.50[a]	6.30	15.50	7.00	25.00	1	20	10.50[a]	3.63	10.00	6.00	21.00	0
	14	7	14.86[a]	9.26	13.00	5.00	32.00	1	21	13.95[a,c]	6.87	13.00	4.00	35.00	2
	28	9	18.56[a]	11.45	15.00	6.00	35.00	2	11	15.73[a,d]	4.00	15.00	10.00	21.00	0
	56	11	15.91[a]	6.44	17.00	8.00	25.00	1	11	17.00[b,c,d]	3.69	17.00	11.00	23.00	0
WBC (10^9/L)	0*	10	1.76[b]	1.79	1.39	0.33	6.66	1	18	0.74[a]	0.55	0.56	0.11	2.11	1
1.11 (0.19-3.59)	7	7	0.89[a]	0.32	1.00	0.33	1.22	0	20	0.82[a]	0.51	0.67	0.22	1.89	0
	14	7	0.52[a]	0.31	0.44	0.22	1.11	0	21	0.66[a]	0.57	0.56	0.11	2.89	2
	28	9	1.20[a,b]	0.59	1.11	0.11	2.11	1	11	1.01[a]	0.35	1.00	0.33	1.55	0
	56	11	0.87[a]	0.45	0.78	0.11	1.55	1	11	1.37[a]	0.57	1.44	0.67	2.33	0
Lymphocyte (%)	0	10	36.80[a]	20.60	39.50	6.00	67.00	1	22	30.86[a]	12.49	33.00	8.00	49.00	0

32.72 (2.57-62.87)	7	8	43.63[a]	6.59	44.50	31.00	50.00	0	13	36.69[a]	12.70	39.00	16.00	56.00	0
	14	7	36.71[a]	16.77	40.00	9.00	55.00	0	22	34.18[a]	10.44	38.00	19.00	48.00	0
	28	10	40.90[a]	7.34	44.50	27.00	47.00	0	12	38.50[a]	11.22	39.50	21.00	52.00	0
	56	10	36.20[a]	13.23	42.00	14.00	50.00	0	10	33.70[a]	8.11	33.50	21.00	48.00	0
Lymphocyte (10^9/L)	0*	10	0.48[b,c]	0.32	0.42	0.09	1.10	1	18	0.21[a]	0.14	0.18	0.03	0.55	1
0.30 (0.04-0.90)	7	5	0.43[a,c]	0.14	0.38	0.29	0.60	0	12	0.28[a]	0.17	0.22	0.08	0.61	0
	14	7	0.17[a]	0.08	0.18	0.06	0.28	0	19	0.23[a]	0.27	0.15	0.05	1.30	1
	28	9	0.50[b,c]	0.26	0.47	0.03	0.97	1	11	0.39[a]	0.15	0.36	0.14	0.59	0
	56	10	0.32[a,b]	0.21	0.21	0.05	0.64	0	10	0.44[a]	0.23	0.35	0.25	0.96	0
Neutrophil (%)	0	10	25.60[a]	26.81	14.50	5.00	78.00	1	22	18.91[a]	13.15	15.50	3.00	51.00	0
21.00 (3.00-73.35)	7	8	17.25[a,b]	13.06	13.00	4.00	47.00	0	13	24.38[a]	16.77	18.00	3.00	57.00	0
	14	7	25.14[a,c]	19.70	17.00	6.00	58.00	0	22	22.32[a]	15.06	17.50	6.00	62.00	0
	28	10	19.80[b,c]	12.29	16.00	6.00	46.00	0	12	20.08[a]	14.86	16.50	5.00	61.00	0
	56	10	22.50[a,b]	12.89	20.00	9.00	46.00	0	10	30.10[a]	16.36	24.00	12.00	62.00	0
Neutrophil (10^9/L)	0*	10	0.71[a]	1.47	0.21	0.03	4.80	1	18	0.12[a]	0.10	0.12	0.00	0.37	2
0.33 (0.01-2.32)	7	5	0.21[a]	0.15	0.16	0.08	0.47	0	12	0.21[a]	0.22	0.11	0.03	0.70	0
	14	7	0.16[a]	0.22	0.08	0.02	0.64	0	19	0.13[a]	0.14	0.07	0.01	0.48	0
	28	9	0.21[a]	0.11	0.25	0.05	0.36	0	11	0.21[a]	0.25	0.14	0.06	0.95	0
	56	10	0.21[a]	0.18	0.15	0.02	0.59	0	10	0.42[a]	0.32	0.25	0.12	0.91	0
Monocyte (%)	0	10	22.50[a]	9.96	22.00	8.00	42.00	1	22	20.50[a]	10.34	18.50	7.00	41.00	0
21.13 (1.33-40.92)	7	8	24.50[a]	6.61	25.50	14.00	34.00	0	13	21.85[a]	7.99	25.00	6.00	30.00	0
	14	7	24.00[a]	8.77	21.00	13.00	36.00	0	22	22.55[a]	7.87	25.00	7.00	34.00	0
	28	10	24.60[a]	6.48	25.50	13.00	34.00	0	12	24.58[a]	6.97	23.50	12.00	35.00	0
	56	10	23.90[a]	6.92	24.50	12.00	32.00	0	10	23.00[a]	7.39	24.00	11.00	31.00	0
Monocyte (10^9/L)	0*	10	0.35[a]	0.28	0.26	0.09	0.93	1	18	0.14[a]	0.11	0.12	0.02	0.45	2
0.22 (0.03-0.74)	7	5	0.24[a,b]	0.07	0.23	0.18	0.35	0	12	0.17[a,b]	0.11	0.13	0.03	0.37	0
	14	7	0.11[b,c]	0.04	0.12	0.04	0.16	0	19	0.14[a]	0.15	0.10	0.02	0.72	1
	28	9	0.30[a,c]	0.19	0.30	0.02	0.72	1	11	0.25[a,c]	0.11	0.22	0.07	0.39	0
	56	10	0.23[a,c]	0.15	0.23	0.03	0.44	1	10	0.30[b,c]	0.16	0.27	0.13	0.58	0
Eosinophil (%)	0*	10	12.50[a]	12.17	8.50	4.00	44.00	0	22	29.45[a]	20.51	24.00	6.00	78.00	1

24.16 (4.00-70.25)	7	8	13.25[a]	10.59	8.00	3.00	30.00	1	12	18.33[b]	15.19	12.50	9.00	63.00	0
	14	7	13.00[a]	19.62	4.00	4.00	57.00	0	22	20.14[b]	16.46	14.50	0.00	60.00	1
	28	10	12.90[a]	9.47	9.50	3.00	33.00	1	12	16.75[b]	12.86	14.50	1.00	44.00	0
	56	10	16.20[a]	15.15	10.00	4.00	52.00	0	10	13.20[b]	8.13	12.50	2.00	28.00	0
Eosinophil (10^9/L)	0	10	0.19[a]	0.18	0.16	0.03	0.59	0	18	0.27[a]	0.41	0.13	0.01	1.65	2
0.24 (0.03-1.16)	7	5	0.09[a]	0.08	0.06	0.03	0.23	0	11	0.20[a]	0.28	0.10	0.02	0.98	0
	14	7	0.08[a]	0.16	0.03	0.01	0.44	3	19	0.13[a]	0.13	0.05	0.01	0.40	2
	28	9	0.16[a]	0.18	0.12	0.01	0.59	1	11	0.16[a]	0.12	0.09	0.01	0.34	1
	56	10	0.13[a]	0.12	0.07	0.01	0.40	1	10	0.15[a]	0.08	0.16	0.03	0.30	0
Basophil (%)	0*	10	2.60[a]	2.37	2.50	0.00	6.00	0	22	0.23[a]	0.43	0.00	0.00	1.00	0
0.97 (0.00-6.00)	7*	8	1.38[b]	1.19	1.00	0.00	3.00	0	13	0.15[a]	0.38	0.00	0.00	1.00	0
	14	7	1.14[b]	1.07	1.00	0.00	3.00	0	22	0.82[a]	0.91	1.00	0.00	3.00	0
	28*	10	1.80[a,b]	1.14	2.00	0.00	3.00	0	12	0.08[a]	0.29	0.00	0.00	1.00	0
	56*	10	1.20[b]	1.62	0.50	0.00	4.00	0	10	0.00[a]	0.00	0.00	0.00	0.00	0
Basophil (10^9/L)	0*	10	0.03[a]	0.03	0.02	0.00	0.08	1	18	0.00[a]	0.01	0.00	0.00	0.02	0
0.01 (0.00-0.07)	7	5	0.01[b]	0.01	0.01	0.00	0.02	0	12	0.00[a]	0.00	0.00	0.00	0.01	0
	14	7	0.00[b,c]	0.00	0.00	0.00	0.01	0	19	0.01[a]	0.01	0.00	0.00	0.06	0
	28*	9	0.02[a,b]	0.02	0.02	0.00	0.05	0	12	0.00[a]	0.00	0.00	0.00	0.01	0
	56	10	0.01[b,c]	0.02	0.00	0.00	0.05	0	10	0.00[a]	0.00	0.00	0.00	0.00	0
Total Proteins (g/L)	0	10	4.70[a]	0.61	4.80	3.40	5.60	1	18	4.94[a]	0.74	5.00	3.80	6.40	1
4.85 (3.72-5.99)	7	9	4.87[a]	0.50	4.80	4.00	5.60	0	23	4.57[b]	0.51	4.60	3.80	5.40	0
	14	7	4.86[a]	0.75	5.00	3.60	5.80	1	24	5.14[a]	0.64	5.20	3.80	6.20	1
	28	10	4.78[a]	0.55	4.90	3.80	5.60	0	12	5.00[a]	0.46	5.00	4.20	5.60	0
	56	11	4.85[a]	0.63	5.00	3.60	5.60	1	10	5.10[a]	0.44	5.10	4.20	5.60	0

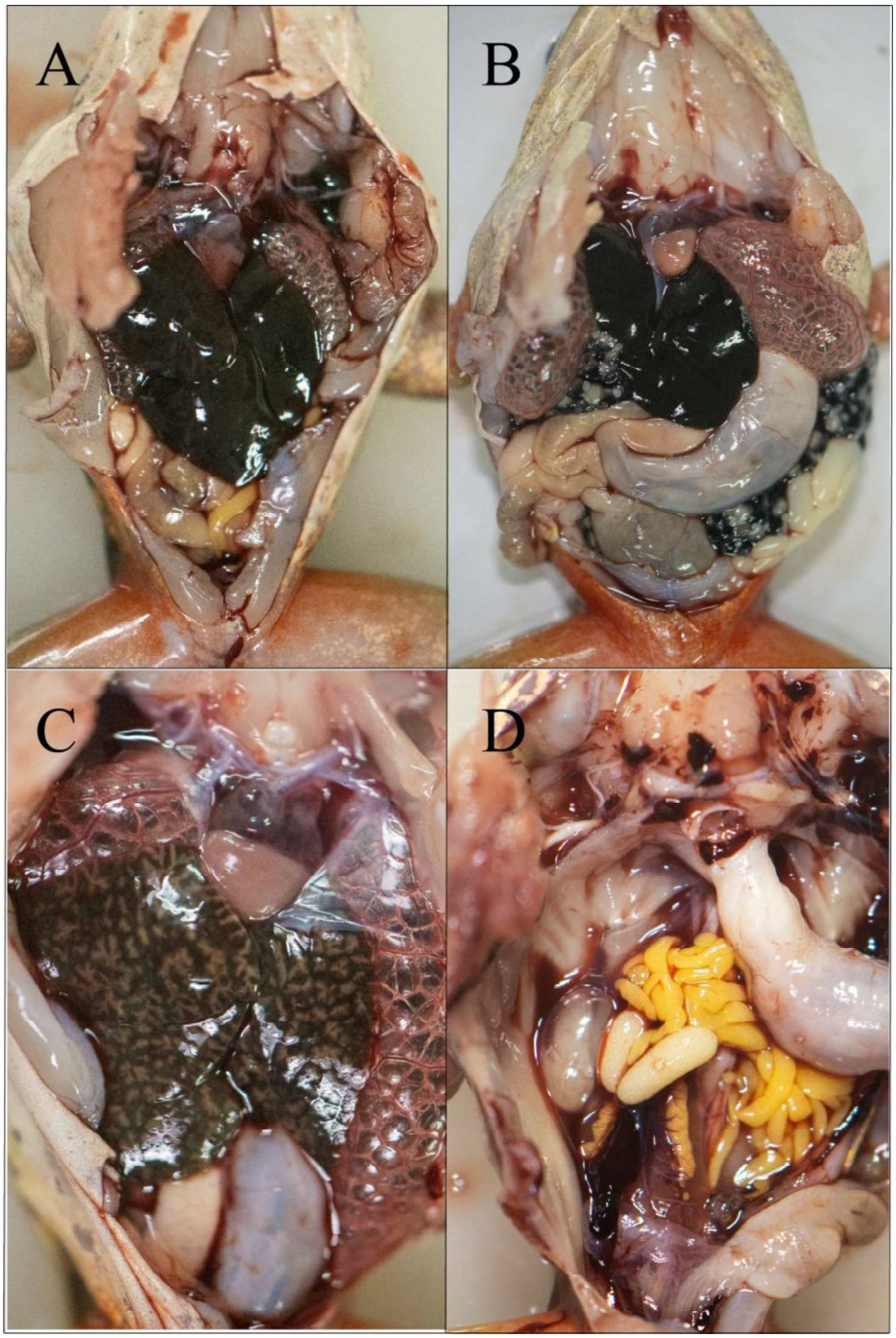

Fig. 4. Gross findings in *Lithobates vibicarius*. The male (A) has a liver proportionally larger to that of the female (B). The female has numerous oocytes expanding the coelomic cavity (B). The liver is diffusely enlarged (hepatomegaly) and has an enhanced reticular pattern (C). Numerous bright yellow fat bodies are in the coelomic cavity (D).

Table 4. Microscopic findings in the skin of adult green-eyed frogs (*Lithobates vibicarius*) experimentally infected with *Batrachochytrium dendrobatidis* and control groups (dpi: days post exposure to *B. dendrobatidis*; af: affected individual; ev: evaluated individuals).

Organ		Microscopic finding	14 dpi (af/ev)	56 dpi (af/ev)	Control (af/ev)
Skin	Epidermis	Apoptosis/Necrosis	4/12	6/12	3/12
		Epidermal exocytosis	7/12	9/12	9/12
		Epidermal hyperplasia (acanthosis)	6/12	9/12	5/12
		Epidermal microvesicles	1/12	1/12	0/12
		Fungal elements compatible with *Batrachochytrium dendrobatidis*	1/12	1/12	0/12
		Hypergranulosis	0/12	0/12	1/12
		Hyperkeratosis	1/12	2/12	0/12
		Intracellular and/or intercellular edema	4/12	3/12	1/12
		Micropustules	0/12	0/12	1/12
		Presumptive intracytoplasmic viral inclusion bodies*	0/12	1/12	0/12
	Dermis	Adenitis	2/12	4/12	0/12
		Congestion	6/12	6/12	1/12
		Edema	9/12	7/12	4/12
		Gland hyperplasia	5/12	5/12	0/12
		Granulocytic to lymphocytic perivascular dermal and/or interface infiltrates	4/12	12/12	7/12
		Granuloma	1/12	0/12	0/12
		Intraglandular nematode larva	0/12	0/12	1/12
		Leukocytosis	4/12	2/12	1/12
		Lymphoid nodule hyperplasia	8/12	7/12	5/12
		Melanophore cell hyperplasia	4/12	2/12	0/12
		Periglandular fibrosis and gland atrophy	2/12	5/12	4/12
		Pigmentary incontinence	1/12	0/12	0/12

* Finding highly suggestive of viral infection.

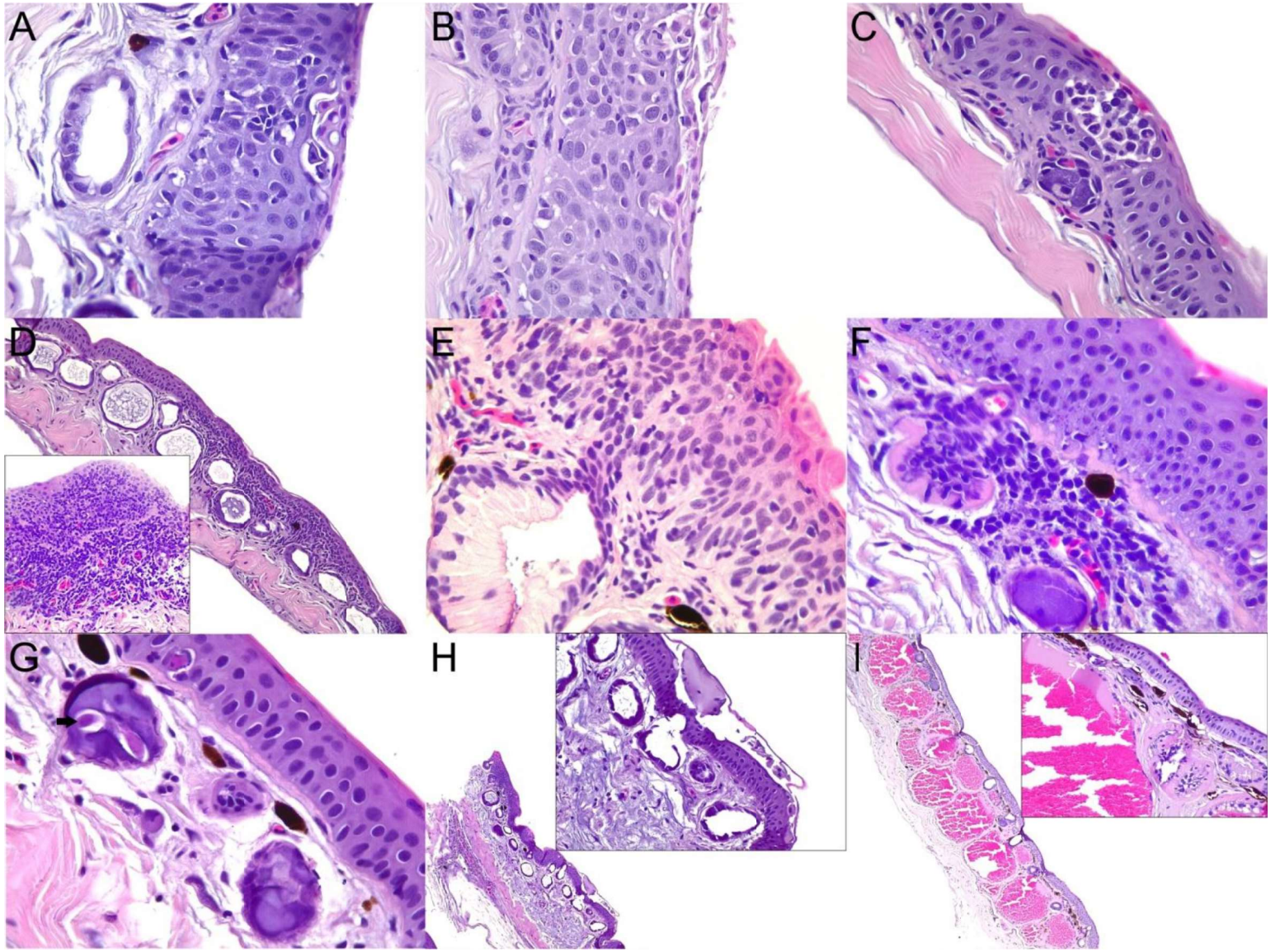
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Fig. 5. Microscopic findings in the skin of adult green-eyed frogs (*Lithobates vibicarius*) experimentally infected with *Batrachochytrium dendrobatidis*. A. Animal 1375. Epidermal hyperplasia (acanthosis) with lymphocytic exocytosis, intercellular edema and rare fungal elements compatible with empty zoosporangia (arrows). Hematoxylin and eosin (H&E). 400x. B. Animal 1375. Epidermal hyperplasia, parakeratotic hyperkeratosis and exocytosis. H&E. 400x C. Animal 1295. Mild epidermal hyperplasia and intra-epidermal micropustule (arrow). H&E. 400x. D. Animal 1708. Cutaneous lymphonodular lymphocytic inflammation with exocytosis. H&E. 100x. Inset: Animal 1379. Detail of dermoepidermal lymphonodular hyperplasia and exocytosis. H&E. 200x. E. Animal 1382. Hyperplasia (acanthosis) with parakeratotic hyperkeratosis and superficial dermatitis with periadenitis. H&E. 400x. F. Animal 1380. Dermatitis and adenitis with gland rupture. H&E. 400x. G. Animal 1294. Nematode larva within a gland. H&E. 400x. H. Epidermal superficial microvesicle and cutaneous myxedema. I. Animal 1716. Focal gland atrophy with thickened and hyalinized basement membrane. H&E. 400x.

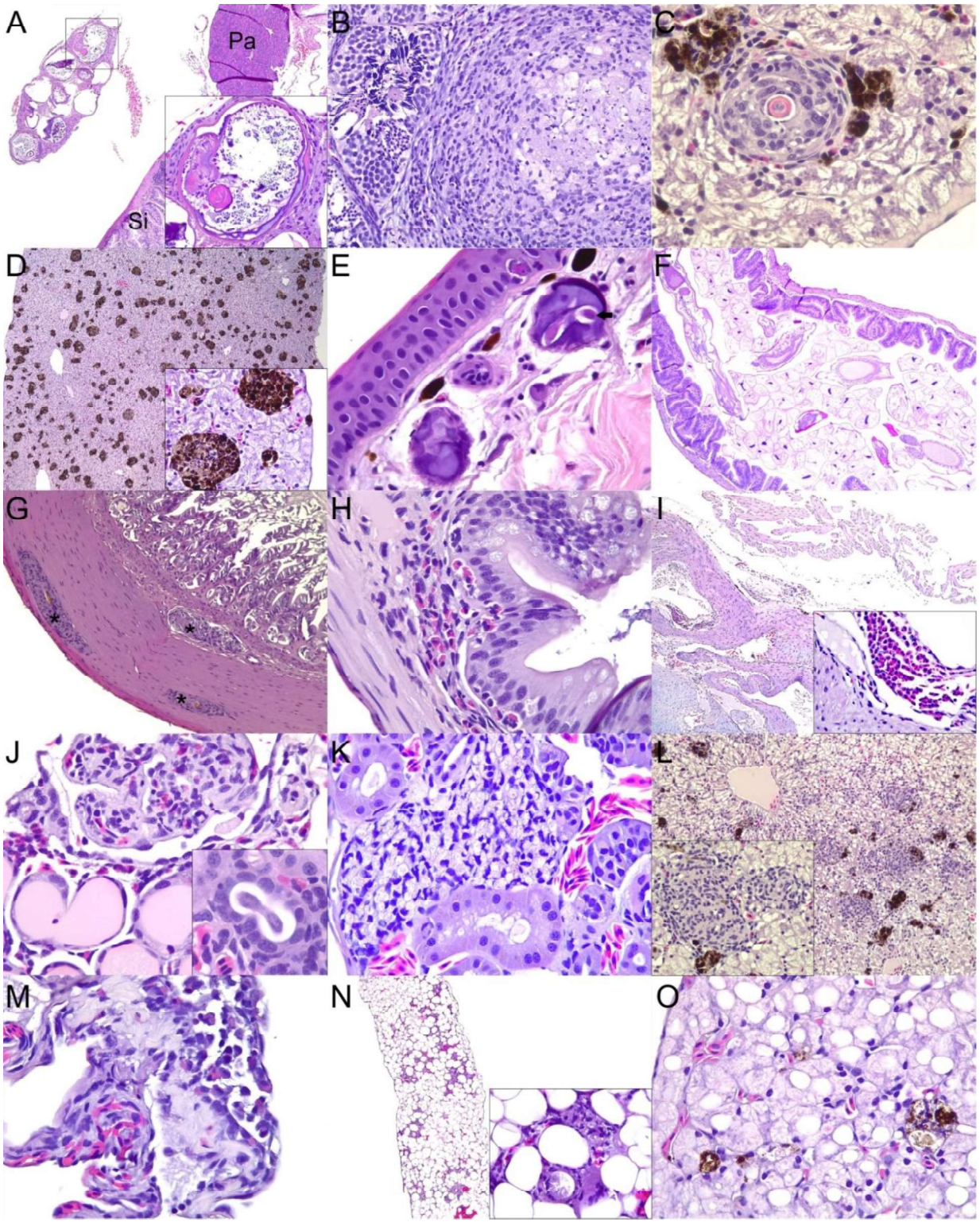
Supplementary Table S1. Hematology variables and total proteins of adult *Lithobates vibicarius* in experimental day 0 before infection with *Batrachochytrium dendrobatidis*. Reference intervals were established as mean ± 1.96 SD (normally distributed data)* or 2.5th– 97.5th interquantile range (nonnormally distributed data)** after outliers were eliminated (outside of Tukey's interquartile fences); distribution normality was established by Shapiro–Wilk test (P > 0.05 = normal distribution).

Variables	n	Mean	SD	Median	Min	Max	p-value	RI (95% CI)
PCV (%)	28	33.08	6.77	34.05	17.68	44.50	0.767	(19.81-46.35)*
RBC (10^12/L)	28	0.23	0.08	0.24	0.09	0.41	0.950	(0.08-0.39)*
Thrombocyte (10^9/L)	28	13.11	5.95	13.00	3.00	27.00	0.159	(1.44-24.77)*
WBC (10^9/L)	28	1.11	1.23	0.72	0.11	6.66	< 0.001	(0.19-3.59)**
Lymphocyte (%)	32	32.72	15.38	33.50	6.00	67.00	0.492	(2.57-62.87)*
Lymphocyte (10^9/L)	28	0.30	0.26	0.23	0.03	1.10	0.001	(0.04-0.90)**
Neutrophil (%)	32	21.00	18.32	15.50	3.00	78.00	< 0.001	(3.00-73.35)**
Neutrophil (10^9/L)	28	0.33	0.90	0.12	0.00	4.80	< 0.001	(0.01-2.32)**
Monocyte (%)	32	21.13	10.10	20.00	7.00	42.00	0.147	(1.33-40.92)*
Monocyte (10^9/L)	28	0.22	0.21	0.16	0.02	0.93	< 0.001	(0.03-0.74)**
Eosinophil (%)	32	24.16	19.79	18.00	4.00	78.00	0.001	(4.00-70.25)**
Eosinophil (10^9/L)	28	0.24	0.34	0.15	0.01	1.65	< 0.001	(0.02-1.16)**
Basophil (%)	32	0.97	1.73	0.00	0.00	6.00	< 0.001	(0.00-6.00)**
Basophil (10^9/L)	28	0.01	0.02	0.00	0.00	0.08	< 0.001	(0.00-0.07)**
Total Proteins (g/L)	28	4.85	0.58	4.90	3.80	6.00	0.536	(3.72-5.99)*

Supplementary Table S2. Microscopic findings (except skin) in adult green-eyed frogs (*Lithobates vibicarius*) experimentally infected with *Batrachochytrium dendrobatidis* and control groups (dpi: days post exposure to *B. dendrobatidis*; af: affected individual; ev: evaluated individuals).

Organ	Microscopic finding	14 dpi (af/ev)	56 dpi (af/ev)	Control (af/ev)
Skeletal muscle	Lymphohistiocytic myositis with necrosis	1	0	0
	Myocyte mineralization	1	0	0
	Myocyte necrosis	0	0	1
	Myocyte regeneration	1	0	0
	Necrotizing vasculitis with thrombosis*	0	1	0
	Perivascular granulocytic infiltrates	0	1	0
	Presumptive intracytoplasmic viral inclusion bodies*	0	1	0
	Vacuolar myocyte degeneration (myofibrillysis)	0	1	0
Kidney	Hematopoiesis (primarily myelopoiesis)	6	8	7
	Interstitial nephritis	2	1	1
	Mesangiocapillary glomerulonephritis with necrosis*	1	0	0
	Tubular proteinosis (with/without hyaline droplets)	3	3	4
	Macrovacuolar tubular degeneration	0	0	1
	Intratubular mineralization	1	0	0
	Acute tubular degeneration	0	1	0
	Tubulitis, cellular casts	0	1	0
	Glomerulopathy/Glomerulonephritis	1	2	4
	Intratubular nematode larva	0	0	1
Spleen	Melanomacrophage hypertrophy/hyperplasia	3	1	3
	Lymphoid nodular hyperplasia	1	0	0
	Necrotizing splenitis (lymphoid depletion)*	0	1	0
	Hemosiderosis	0	1	0
	Mild lymphoid depletion	0	1	0
	Scattered histiocytic aggregates	0	1	0
Liver	Melanomacrophage center hypertrophy/hyperplasia	10	11	11
	Histiocytic nodular aggregates (granulomas)	5	8	8
	Moderate to marked hepatocyte microvacuolar change with glassy cytoplasm	9	7	9
	Hepatocytic macrovacuolar change	1	2	0
	Granulomatous hepatitis with presumptive parasitic structure	1	0	0
	Hematopoiesis	5	5	7
	Necrotizing hepatitis*	0	1	0
Heart	Great vessel/heart base/epicardial granulocytic infiltrates	1	1	3
	Focal heterophilic and histiocytic pericarditis	1	0	0
	Lymphoid nodule associated with serosa of great vessel	0	0	1

	Subendocardial cardiomyocyte vacuolar degeneration	0	0	1
Lung	Focal pleural granuloma	1	0	0
	Scattered interstitial histiocytic infiltrates	1	0	0
	Necrotizing interstitial pneumonia with vasculitis and presumptive ICIBs*	0	1	0
	Focal lymphohistiocytic interstitial pneumonia	0	1	0
	Lymphohistiocytic pleuritis with fungal elements	0	1	0
	Thrombosis*	0	1	0
	Faveolar edema and fibrin*	0	1	0
	Hemorrhage*	0	1	0
	Granulocytosis	0	0	1
Tongue	Myocyte degeneration and necrosis	1	0	0
	One to multiple lymphoid nodules	4	8	5
	Hemosiderophages	1	1	0
	Hemorrhage	1	1	0
	Perivascular lymphocytic infiltrate	1	0	0
	Multifocal glossitis with vasculitis, necrosis and ulcer	0	1	0
	Edema	0	1	0
Digestive tract	Granulomatous gastritis with foreign body (cricket)	2	3	3
	One to multiple lymphoid nodules in stomach and intestines	0	6	1
	Large intestine nematodiasis	4	7	6
	Intraluminal mites	3	0	6
	Small and/or large intestine ciliate protozoa	4	6	6
	Eosinophilic colitis	1	0	2
	Multifocal villi necrosis*	0	1	0
	Mucosa-associated lymphoid tissue lymphocytolysis*	0	1	0
	Serosal granulocytic infiltrate	0	0	1
Pancreas	Multifocal interstitial lymphocytic infiltrate	1	0	0
	Intravascular ciliate protozoa	0	0	1
Gonads	Multifocal ovarian follicular atrophy	0	1	0
	Granulomatous orchitis	0	1	0
Other findings	Granulomatous steatitis	1	0	1
	Granulocytic to granulomatous coelomitis (foreign body, bacteria, nematode, cestodes)	2	3	3
	Heterophilic exocytosis in bile duct	3	0	0
	Pancreatic lymphocytic periductitis	1	0	0
	Multifocal coelomitis with vasculitis*		1	0
	Presumptive ICIBs in various organs*		1	0
	Peripancreatic/perisplenic myelopoiesis		2	3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Supplementary Figure S3. Microscopic findings (except skin) in adult green-eyed frogs (*Lithobates vibicarius*) experimentally infected with *Batrachochytrium dendrobatidis* and control groups experimentally infected with *B. dendrobatidis*. A. Animal 1379. Metacestodal granulomatous coelomitis. Inset. Detail of encysted metacestode. Hematoxylin and eosin (H&E). 400x. B. Animal 1377. Granulomatous orchitis. H&E. 400x. C. Animal 1709. Granulomatous hepatitis with foreign body. H&E. 400x. D. Animal 1716. Melanomacrophage center hypertrophy and hyperplasia (MMCHH). H&E. 40x. Inset: Animal 1716. Detail of MMCHH. H&E. 400x. E. Animal 1382. Hyperplasia (acanthosis) and parakeratotic hyperkeratosis. H&E. 400x. F. Animal 1372. Colonic nematodiasis (asterisks) and protozoosis. H&E. 100x. G. Animal 1705. Mural gastritis with intralesional foreign bodies (cricket fragments; asterisks). H&E. 200x. H. Animal 1296. Eosinophilic and lymphocytic colitis with exocytosis. H&E. 400x. I. Animal 1716. Heterophilic epicarditis. H&E. 100x. Inset: Animal 1716, Detail of heterophilic epicarditis. H&E. 400x. J. Animal 1298. Eosinophilic glomerulonephritis and tubular proteinosis. H&E. 400x. Inset: Animal 1297, intratubular nematode larva. H&E. 400x. K. Animal 1374. Interstitial histiocytic nephritis. H&E. 400x. L. Animal 1710. Nodular histiocytic (granulomatous) hepatitis. H&E. 100x. Animal 1381, detail of nodular histiocytic hepatitis. H&E. 400x. M. Animal 1377. Focal pleuritis. H&E. 400x. N. Animal 1292. Granulomatous steatitis. H&E. 40x. Inset: Animal 1292, detail of granulomatous steatitis. H&E. 400x. O. Animal 1376, detail of hepatocellular macrovacuolar and microvacuolar (lipid) change with glassy cytoplasm appearance. H&E. 400x

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Supplementary Table S4. Template for recording histopathologic findings in the integumentary system of adult green-eyed frogs (*Lithobates vibicarius*) included in this study.

Subcompartment	Finding	
Epidermis	Acantholysis	
	Apoptosis	
	Bacteria	
	Dyskeratosis	
	Intercellular edema (spongiosis)	
	Fungal elements consistent with *Batrachochytrium dendrobatidis*	
	Hypergranulosis	
	Hyperkeratosis	Orthokeratotic
		Parakeratotic
	Hyperplasia (acanthosis)	Up to 5 layers thick
		≥ 6 layers thick
	Inflammation/exocytosis	Granulocytic (G)
		Lymphocytic (L)
		Histiocytic (H)
	Intracellular edema (ballooning degeneration)	
	Keratin pearls	
	Necrosis	
	Vesicles (clefting)	
	Satellitosis	
	Squamous eddies	
Dermis	Chromatophore (melanophores, iridophores)	Hyperplasia
		Incontinence
	Congestion	
	Edema	
	Fibrosis	
	Glands (mucous, serous, mixed, granular)	Hypertrophy
		Hyperplasia
		Atrophy
		Loss
	Inflammation	Perivascular (G/L/H)
		Interface (G/L/H)
		Nodular (G/L/H)
		Diffuse (G/L/H)
		Periglandular (G/L/H)
	Lymphoid nodules	
	Vasculitis	

This paper will be submitted to Animal Behaviour.

4 SPACE USE AND BEHAVIOR OF THE NEOTROPICAL FROG *Lithobates vibicarius* IN THE GENERAL ANIMAL WELFARE ASSESSMENT DURING THE EXPERIMENTAL INFECTION WITH CHYTRID FUNGUS *Batrachochytrium dendrobatidis*

Authors: Gilbert Alvarado, Gabriel Espinoza, Randall Jiménez, Pablo Aragonéz, Kimberly Castro, José Sandoval, Jilma Alemán-Laporte & José Luiz Catão-Dias.

4.1 ABSTRACT

It is an ethical obligation to seek to assure animal welfare in all the experimental infection processes where they are used. Extensive knowledge of the animal's biology and recognition of its clinical signs is essential to achieve this purpose. However, knowledge about the animal's behavior is fundamental to evaluate the general animal welfare and not only aspects related to its health. The aim of this study was to evaluate the effects on the behavior that the infection with the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) infection can produce in frogs of the species *Lithobates vibicarius* in a context of general animal welfare assessment. The clinical aspects of the experimental infectious process were excluded. The enclosure of the animals was designed exclusively for this species and conformed by three levels. A recording methodology was developed and a detailed ethogram was described to register the use of space, body postures, head positions and animal activities. The main space that the animal uses is the left posterior quadrant inside the shelter. The main body posture and head position is that described as B2H2. No significant differences were found between infected and uninfected animals. The creation of baseline information on the behavior of *L. vibicarius* and the absence of clear behavioral alterations due to *Bd* infection is a useful tool for *ex situ* conservation programs.

Keywords: *Batrachochytrium dendrobatidis*, animal welfare, *ex situ* conservation, amphibian, behavior.

4.2 INTRODUCTION

One of the main preoccupations for animal welfare is the emphasis in freedom of injury and diseases in the evaluated individuals (Baumans, 2005). Equally important is the ability the animals will have to live reasonably natural lives with behavior as close as possible to that of individuals raised in the wild (Fraser, 2008). For domestic animals and livestock, basic health and the overall function of the animals have been used as a base for evaluation and improvement of animal welfare. There are clear examples of improvement in cages and housing for birds and pigs by studying their behavior under different conditions or by comparing with the behavior present in the wild (Stolba & Wood-Gush, 1984; Tauson, 1998). However, a great challenge that we still have is the complexity of establishing altered behaviors in wild animals under conditions of *ex situ* conservation (Schaaf, 1984; Wei, Jinghua, Hui, Dan, & Xianfu, 2012). In common marmosets, cage size and cage complexity have deleterious effects on the welfare of the animals and consequently on the reliability of the research (Kitchen & Martin, 1996).

It is essential to accompany any experimental infection study with a stage for determining the effect on the animals' welfare. It is also necessary to evaluate not only the success of the experimental procedure but also the potential implications it may have on the subject. Thus, it is important to create instruments for the evaluation of individual welfare in a veterinary context (Wojciechowska & Hewson, 2005). One of the main mistakes in veterinary medicine is to equate the concepts of welfare and quality of life of animals with their health status. (F D McMillan, 2000; Wojciechowska & Hewson, 2005). In an applied ethological context, the concept of welfare englobes more than just the health of the animals and involves more aspects of the animal's life regarding maintenance (Dawkins, 2008; McMillan, 2014). From this point of view, evaluating animal welfare from a functional stance (level of activity, adaptation, and abilities), could be of importance because of its potential to stand as a base for other studies.

Lithobates vibicarius is a threatened frog thought to have disappeared in the 90s under the context of the mass population declines that this group of vertebrates suffered on a global scale (Hoffmann et al., 2010; Scheele et al., 2019; Stuart, 2004). Currently, we count on two natural, independent protected areas in Costa Rica where this species can be found since its rediscovery in 2002 and 2007 respectively, with

well-established populations (Castro-Cruz & García-Fernández, 2012; Morera Chacón & Sánchez Porras, 2015; Alvarado et al., unpublished data). One of the potential pathogens we attribute many of the population declines for amphibian species around the world is the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). We have a set of seven species that have reappeared in recent years (Whitfield et al., 2017) and we are proposing *Lithobates vibicarius* as a model in conservation for other reappearing species within Costa Rica and the neotropics.

For this reason, we performed experimental infections with the purpose of evaluating the host's response. With this study, we have the objective of evaluating the etiology of *Lithobates vibicarius* in a context of general animal welfare, excluding the clinical aspects of the infection and its potential complications during experimental process. The finality of this is to be able to identify other aspects of the animal's etiology that could be affected by the *Batrachochytrium dendrobatidis* infection.

The characterization of general behavioral aspects related to infection by *Bd*, could represent an important base for the identification and detection of alterations in the welfare of individuals in *ex situ* conservation programs during chytrid infection events. This would allow comparisons amongst similar medical conditions and could help veterinarians make decisions. As a part of this, the development of standardized and valid instruments would be valuable for the evaluations of diseases and treatments (Bateman, Catton, Pennock, & Kruth, 1994; Yazbek & Fantoni, 2005). For this to occur, it is necessary to investigate the influence of individual factors (Wojciechowska & Hewson, 2005; Yazbek & Fantoni, 2005), and particularities of specific diseases (Yazbek & Fantoni, 2005).

4.3 METHODS

4.3.1 Study Species

Lithobates vibicarius is a frog with a historical distribution range that covered the high mountains of Costa Rica in their entirety. The range started decreasing to the point of causing the species to be critically endangered as stated by the IUCN in 2004. However, the species has recovered a great deal since then and began a process of re-evaluation as a vulnerable species with the IUCN in 2013. We collected

36 clinically healthy individuals of the green-eyed frog, *Lithobates vibicarius,* from the Juan Castro Blanco National Park and its periphery. The collected individuals did not present any type of lesion o evident skin alteration. They were captured between May and November of 2017 in six different expeditions and in four different locations chosen according to the monitoring initiated in 2013 (Alvarado et al., unpublished data). The animals were captured manually starting at 19.00 h, each individual manipulated with new nitrile gloves. They were placed and transported in semi-transparent plastic containers (9l x 9w x 7h cm, 0.5 L) with a natural fiber paper towel lining drenched in Amphibian Ringer's Solution from the established protocol (Brannelly, 2014). The animals were always transported to the laboratory between 19.00 and 06.00 h to avoid high temperature during the day.

4.3.2 Animal Husbandry

Maintenance and experimental procedures were performed by the Laboratory of Experimental and Comparative Pathology of the School of Biology of the University of Costa Rica. The laboratory has two structurally independent experiment rooms. These macroenvironments or experiment rooms register a mean temperature of 19.22 ± 1.12 °C and a mean relative humidity of 92.74 ± 6.70 %. Data regarding these two variables was measured and registered every hour after entry with an Arduino microprocessor in conjunction with temperature and humidity sensors. The animals were placed in individual transparent glass tanks (50l x 25w x 35h cm, 37.85 L). Also, they were exposed to a 12 h automated photoperiod, accomplished by combining led hose lights (IP67 Sylvania), approximately 25 cm above the tanks to a timer. Inside each tank, there are three levels: a first level (L1) of eight cm of water; above it a 10 cm high platform (23 x 30 cm) that constitutes the second level (L2) and has coverage of 60% of the water surface. This platform is lined with natural fiber paper towel in place of substrate. In the posterior left quadrant of the platform, there is a plastic flower pot (13 diameter x 13h cm) with an opening cut (10h x 7w cm), that acts as shelter. The pot is inverted and the top is used by the animals as a third level (L3). Each tank is fed water through individual pipes that connect to a filter tank, and a draining system with individual stopcocks. Also, there are water mist nozzles installed over every platform that are regulated by solenoid valves that activate at 15.00, 17.00 and 19.00 h for 30 s to maintain humidity (Fig. 1).

Water used for husbandry and in experimental activities is first passed through two types of filters, a conventional one as a physical method (G0058-02, Purefer) and one with a micro-granular carbon cartridge (UDF- GAC- 2in1, Purefer) in order to reduce the amount of microorganisms present and to eliminate components like chloride in the water. The main water tank is located inside the laboratory so that there are no changes in water temperature. Every week we replaced the paper towel and completely cycle out the old water with any organic material there might be in it. The frogs were fed every 48 hours with adult crickets exclusively (*Acheta domestica*) (one cricket if the individual is < 30g; two crickets if the individual is ≥ 30g). These crickets were raised in the laboratory and maintained on a strict and permanent *ad libitum* diet of chicken feed (Ponedora 18% Golden, Mundi Vet – Dos Pinos), carrots, broccoli, and orange. Any cross-contamination between the infected and control animal modules was prevented by using individualized protection equipment for each division, news nitrile gloves between tanks and always working with the control group a day before experimental procedures on the exposure group and two days before maintenance.

4.3.3 Inoculation process with *Batrachochytrium dendrobatidis*

The frogs were submitted to the strain *Bd* JGA01; isolated from the species *Lithobates taylori* from the Central Volcanic Mountain Range of Costa Rica, in San Rafael de Heredia. The preparation of the inoculum with approximately 250,000 zoospores, was carried out following the protocol established for the infected animals and the control group. The exposure took place in containers that were used exclusively for each animal and were of a size that only allowed them to sit in the normal position on a substrate with their pelvic patch, thighs and plantar and palmar surfaces in constant contact with the inoculum. After 24 hours of direct contact with the inoculum, we proceeded to re-locate the animals to their respective tanks with closed draining systems. The container used for inoculation was inverted and placed in the upper left quadrant of the platform, where the shelter usually remains so as to drench the most frequented region in the tank with the inoculum. After 24 hours, the container was removed. The same procedure was developed for the control group, with the absence of *Bd* in the inoculum, 24 hours before. We did not open the drain for the tanks that contained infected animals for two weeks; we also did not change

the paper towel in that time in order to favor the growth of *Bd* in the skin of the amphibians but also in the substrate and water inside the tanks.

4.3.4 Experimental design & behavioral monitoring

Frogs of each sex and provenience were randomly assigned to three treatment groups with 12 animals each. After assignment we tested that all groups had similar size (SVL) and weight using an ANOVA model, to ensure homogeneity of physical conditions across animals between the treatment groups. We established two groups of infected animals and another control group. Every animal was assigned randomly to their respective tanks, whether it is infected or non- infected. We established five times set at 0 days (before inoculation), 7, 14, 28 and 56 days (post-exposure), for the collection of blood samples and swabs with epidermal samples for analysis in a parallel study. One of the infected groups finished the treatment on day 14; the other infected and control groups finished on day 56. We randomly selected six out of twelve of the infected animals to be monitored up till day 56. We selected six out of twelve control group individuals for the same purpose. We video recorded the animals 24 hours post infection and 12 h before each of the established times (days 7, 14, 28 and 56). We recorded on different days for the infected group and the control group (24 h after) for biosecurity reasons. On the day of recording, we proceeded to set the camera up at a 50 cm distance from the frontal view of the tank. The lens was set to capture all three levels inside the tank. Each recording has a length of 15 minutes (after an initial 5 minute adjustment period), with a resolution of 1280 x 720 pixels and 30 fps. We recorded six videos for each of the animals in the evaluation group between 16.00 and 22.00 h. We divided the animals into groups of three. The first three animals were recorded at 16:00, 17:00, 18:00, 19:00, 20:00 and 21:00 h; the remaining three animals were recorded at 16:30, 17:30, 18:30, 19:30, 20:30 and 21:30 h. At 18:00 h, the permanent light system installed automatically disconnects and the light system with red led lights (P26116 Sylvania) would turn on for the rest of the recordings. These red lights were located in the exact position as the permanent ones. During the recordings, all humans remained outside the experiment rooms. The recording interval was set according to the highest peaks of activity for the species, determined in the field as 18.00 and

20.00 h. We extended the interval two hours before and after the peaks for recording purposes.

For the video analysis, we used BORIS (Behavioural Observation Research Software, available at http://www.boris.unito.it/;Friard & Gamba, 2016), and configured the ethogram detailed in Table 1. The data of the videos was analyzed without their information source and randomly according to the behaviors previously established (use of space, postures and activities). Both level one (L1) and level two (L2) were divided into quadrants (left anterior quadrant: LAQ, left posterior quadrant: LPQ, right anterior quadrant: RAQ and right posterior quadrant: RPQ). In the case of L1, we established a special variable RAQt that corresponded to the moments when the animal was supported on the drain tube. For L2 we established LPQi for when the animal had more than 50% of its body inside the shelter. The data for each observation period for each individual were combined. The percentages of time in each level are based on the total duration (seconds) of the video analysis for each sampling time. The use of space for each level is based on the average time of use of that space by observation. The time body postures and head positions were held, were combined for each individual within each observation period. The percentages of time for these two categories are based on the total duration (seconds) of the video analysis for each sampling time. Small sample size for many of the postures, head positions, use of space or activities (n = 1 or 2) made impossible the application of conventional statistical analyzes.

4.3.5 *Batrachochytrium dendrobatidis* molecular diagnosis.

We used a swabbing technique that involves the passing of a swab (MW113; Medical Wire & Equipment, Wiltshire, England) 10 times along the dorsum, venter, each side of the body, and length of each femur. We swabbed 5 times across each hand and foot (Whitfield et al., 2017). Swabs were extracted in 50 mL of Prepman Ultra (Applied Biosystems, Foster City, CA, USA) and analyzed for the presence of *Bd* DNA by a TaqMan real-time polymerase chain reaction assay in QuantStudio® 3 Real-Time PCR System in Cell and Molecular Biology Center of the University of Costa Rica. In summary, we used *Bd* primers and probes (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004) with 2X TaqMan Master Mix and internal positive controls in

20 µL reactions containing 4 µL of DNA template. We always ran all reactions, with at least one negative control and two positive controls.

4.3.6 Ethical note

This study has been developed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Research Council of the National Academies of Science. The protocols used in the investigation were approved by Institutional Animal Care & Use Committee of University of Costa Rica (CICUA 028-15) and Ethics Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil (N°2657080715). The project was approved by the Biodiversity Commission of the Vice-Rectory for Research of the University of Costa Rica (VI 6457-2016 Resolution N°69). All field locations and activities, including collection of frogs from the wild, were authorized and all the animals were collected under the permission of the Arenal Huetar Norte Conservation Area (ACAHN) of the National System of Conservation Areas of Costa Rica (SINAC-ACAHN-PI-R-001-2017) of the Ministry of Environment and Energy of the Government of Costa Rica.

4.4 RESULTS

The animals were analyzed according to the experimental group to which they were assigned regardless of their molecular diagnosis for *Bd*. All the animals were negative to *Bd* on day 0 before performing the experimental infection. The six infected animals filmed were positive for the presence of Bd on day 7 and day 14 of the experiment, so it is assumed that 24 hours after the infection, the animals were also positive. For day 28 and day 56, two and one of the animals were diagnosed for the infection respectively.

All animals showed a clear preference for L2, that corresponds to the platform or terrestrial part of the primary enclosure, regardless of whether they were infected or not. At the sampling point of 28 and 56 days there was a slight increase in the use of L1 (n = 2) and L3 (n = 2) respectively. However, these increases were related to animals that had already resolved their infection and were negative. L3 was a space

that animals used more than L1 (Fig. 2). Of the two animals that remained infected with molecular diagnosis from day 28, only one of them made use of L1 on the day 56 and corresponded to less than 1% of the use of space by the group analyzed for that treatment. The rest of the time the animals remained in the terrestrial part of the enclosure.

When animals used L1, there was a greater use of the posterior quadrants. However, in the anterior region, the animal makes a greater investment of time supported on the drain tube (RAQt) or around it (RAQ) (Fig. 3). Almost all the time that the animal remained in L1 was supported with one or several of its extremities by the bottom of the enclosure, some of the walls or by the drain tube RAQt. Only one animal was recorded floating in two observations on day 7 for mean time period of 10.5 ± 2.76 s. In all experiment only 4 (one infected) animals swam for short periods of time between 6.15 s and 27.95 s with mean of 15.41 ± 2.92 s. The posture of floating and swimming activity was for both infected and uninfected animals.

When animals used L2, they spent more time on the LPQ on average, especially in the LPQi, using the shelter placed in the enclosures (Fig. 4). Throughout the experimental process six animals (two infected) were recorded walking for short periods of time between 1.90 s and 9.49 s with an average of 4.60 ± 0.81 s.

In relation to body posture, positions B2 or B3 are the most used by animals. Body posture B1 is a position used in a smaller percentage of time (Fig. 5). Regarding the position of the head, the normal position H2 is independent if the animals were infected or not. Head position H1 or H3 occurred in punctual and brief situations (Fig. 6).

Prolonged movements were observed in three animals (two infected) in L1 for a period of time between 11.51 s and 81.80 s with mean of 49.61 ± 11.10 s. In L2 they were presented by five animals (two infected) for a period of time between 8.07 s and 61.83 s with mean of 21.64 ± 7.03 s. Brief movements, rotation and jumps appear to be more numerous in the recording times (24h, 7d and 14d) where all the animals were positive to *Bd* (Table 2). Only one infected animal in two different recording times (24 hrs and 14 d) vocalized. All their vocalizations were brief (37 total) and one was prolonged with a duration of 137.63 ± 28.23 s.

4.5 DISCUSSION

One of the biggest challenges of the experiment was the construction of a space that would provide the best housing conditions for this particular species. *L. vibicarius* belongs to a gender of frogs associated with wetlands and small lakes in the highlands of Costa Rica. In Panama this species is possibly extinct (Savage, 2002). Considering this, we decided to create an environment that would give the animals the possibility of being in an aquatic (L1) or terrestrial space (L2). The third level (L3) arose from the preference of some animals towards a higher perch, giving use to the top view of the shelter; the preference of some species for higher perches is known with the intention of improving the characteristics of their calls (Santos & Rossa-Feres, 2007). In Costa Rica we have ranids (including *L. vibicarius)* that, despite having a dependence on water for reproduction, show a certain preference towards terrestrial environments as adults (Savage, 2002). This preference manifested itself in the same way in the laboratory when making a greater use of the terrestrial space of the primary enclosure.

There is information on the importance of the design of enclosures in reptiles and birds (Alberts, 1994; Mallapur, Qureshi, & Chellam, 2002; Tan et al., 2013), but data available for amphibians is very scarce. The design of the enclosures and the knowledge of natural history are critical aspects in assuring animal welfare during the development of *ex situ* programs of any animal species by offering the indicated space quality (Estevez & Christman, 2006; Kohn, 1994; National Research Council, 2011).

There are few reports of behavioral associations with *Bd* infection; however, there are reports of Australian rainforest stream frogs with interspecific variations in the probability of transmission where the frequency of contact with other frogs and with water, as well as the use of substrate could define the infection and susceptibility of these species (Rowley & Alford, 2007). Also in Australia, it is suggested that higher thermal preferences selected naturally or artificially by some frogs could reduce susceptibility to this potential pathogen. On the other hand, there are suggestions of an influence of the pathogen improving the properties of the call in order to achieve an earlier reproduction in the host and increase its probability of transmission (An & Waldman, 2016). This could not be confirmed in another Australian species where there were no effects of the infection status of the fungus or intensity of the infection on the properties of the call (Greenspan, Roznik, Schwarzkopf, Alford, & Pike, 2016).

This type of reports made us think about the possibility of some behavioral alteration that we could identify in the experimental infection process.

We were motivated to establish a systematic monitoring for the experimental infection because of an acute toxicity event on the third and fourth days of treatment with itraconazole in *L. vibicarius* and *Lithobates taylori* where behavioral alterations were observed (Alvarado et al., data unpublished). Behavioral evaluation methodologies are well established especially for the evaluation of ecotoxicological aspects (Cohn & MacPhail, 1996), but behavioral studies in infection processes are less common. From an animal welfare perspective, we are concerned about the possible manifestations of distress that animals may experience and the compromise of their health, but possible indicators to establish this distress in these animals are poorly understood. In wild frogs, distress call is an indicator well studied for situations of high emergency in the animals (Hodl & Gollmann, 1986; Santana & Moura, 2011). From a scientific perspective there is a risk that the distress influences the quality of the data generated with animals in the research.

In general, amphibian enclosures are not considered enriched environments; this may be due to the complex and specific requirements of temperature, humidity, housing, water quality and live food assumed as essential for its successful maintenance. (Burghardt, 2013). Regarding the investment of time by quadrants in L2 their preference for the refuge and its surroundings, as well as the RPQ where the water misting nozzle was located, is an interesting phenomenon. Also in L1 there was an association with the presence of the drain tube in RAQt. This suggests that the presence of elements in the enclosure could favor the preference of use of space. We know that the best chance of finding a *L. vibicarius* in our enclosure was inside the shelter in L2. Similarly, the design of the enclosure is a base that can be modified according to the different research needs of the researcher.

Unfortunately, working with amphibians in captivity is based mainly on informal anecdotal reports lacking documentation. This experiment represented a unique opportunity to see possible alterations in the use of space and behavior of frogs during an experimental infectious process. There are reports of abnormal postures caused by chytridiomycosis, but they have never been characterized (Voyles et al., 2012). Our goal was not to associate these body postures or head positions with specific behaviors; we wanted to determine some possible difference between uninfected and infected experimental groups. However, this study can be a first step

for the objective and systematic characterization of a body posture and head position for a frog. The posture and head position B2H2 seems to be the expected form for *L. vibicarius* in our laboratory conditions.

Observations on the increase of brief movements, rotations or jumps should be considered in further detail in future studies as possible indicators of quantifiable behavioral alterations due to *Bd* infection. However, the interpretation of this information must be done carefully. In acute intoxication events due to the treatment of itraconazole, the increase in the frequency of these behaviors was very noticeable (Alvarado et al., data unpublished). The calling was not one of the best indicators due to the fact that the animals were placed in separate enclosures and this did not allow the adequate interaction between individuals.

In the early stages of an applied research field, anecdotal data is often the only available source of other people´s observations or the success of their implemented work. The key value of these reports is to provide precise details about what was done and the behavior observed. In behavioral analysis, only one or a few individual animals are often studied even in experiments (Saudargas & Drummer, 1996), so small-scale studies can be extremely useful.

It is important to evaluate how the animal perceives, interprets and experiences its environment (Burghardt, 2013). This is the basis to be able to guarantee the welfare of the individuals that are part of an experimentation process or an *ex situ* conservation program. All clinical and health aspects have been organized in a parallel study focused on the experimental infection performed (Alvarado, unpublished data). Regarding the evaluation of space use and behavior, they do have significant alterations that generate an important difference between infected and uninfected animals groups. This suggests that the *Bd* infection does not cause a behavioral alteration with a clear pattern in *L. vibicarius* under laboratory conditions and it gives rise to a methodology and ethogram for the evaluation of amphibian behavior indicators under experimental conditions.

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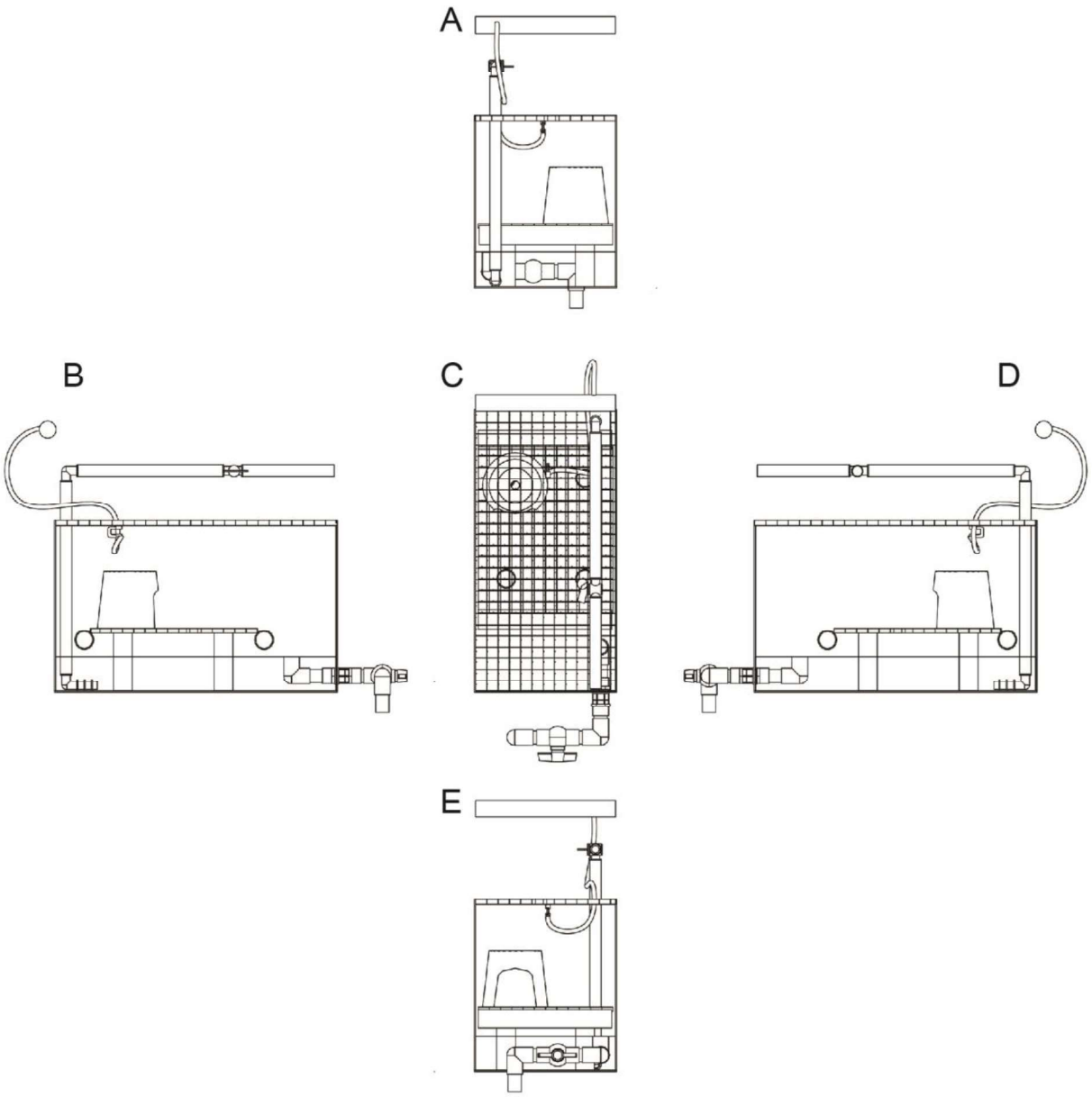

Fig. 1. Views of the microenvironment designed for *Lithobates*. A. Back view. B. Left side view. C. Top view. D. Right side vie E. Frontal view.

Table 1. Ethogram used to code the behavior (use of space, postures and activities) of *Lithobates vibicarius* during the experimental infection with chytrid fungus *Batrachochytrium dendrobatidis*.

Behavior	Category	Level	Description	Type
Posture	Body 1	2, 3	Elbow forms an angle < 80°	State
Posture	Body 2	2, 3	Elbow forms an angle between 80 ° and 100°	State
Posture	Body 3	2, 3	Elbow forms and angle > 100°	State
Posture	Head 1	2, 3	Vocal sac is rested on the supporting surface.	State
Posture	Head 2	2, 3	The angle formed by the corner of the mouth and a line parallel to the supporting surface, is <45°.	State
Posture	Head 3	2, 3	The angle formed by the corner of the mouth and a line parallel to the supporting surface, is ≥ 45°.	State
Supported	Posture N1	1	One or several extremities are in contact with a surface.	State
Floating	Posture N1	1	No extremity is in contact with any surface.	State
Submerged	Posture N1	1	Head is completely submerged in water.	State
Swimming	Locomotion	1	Unsupported movement using posterior extremities, anterior extremities are out-stretched and parallel to the body.	State
Walking	Locomotion	2	Supported movement using extremities, resulting in the displacement of 100% of its body.	State
Prolongued vocalization	Communication	1,2,3	Composed of two or more continuous pulses with less than 10 seconds between them.	State
Brief vocalization	Communication	1,2,3	Composed of a single pulsation.	Punctual event
Prolongued movement	Body adjustment	1,2,3	A slight interruption of inactivity by sporadic body adjustment, ≥ 3 seconds.	State
Brief movement	Body adjustment	1,2,3	A slight interruption of inactivity by sporadic body adjustment, < 3 seconds.	Punctual event
Rotation	Body adjustment	1,2,3	A rotation > 45°, on its own body axis.	Punctual event
Jump	Locomotion	1,2,3	Aerial displacement with a single impulse using the posterior extremities.	Punctual event
Other		1,2,3	Any other behavior not contemplated.	Punctual event

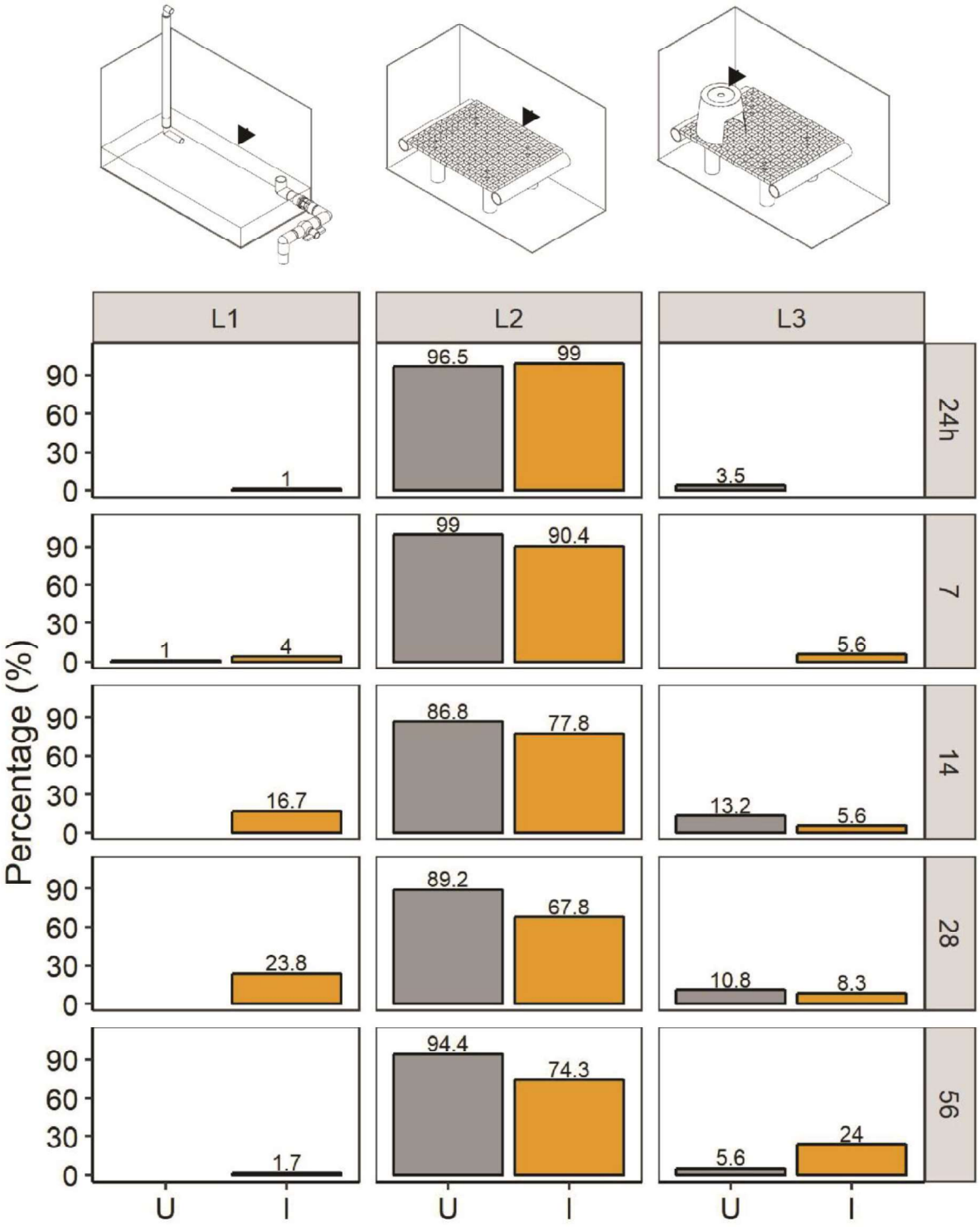

Fig. 2. Percentages of time of use in each level between uninfected (U) and infected (I) frogs per sampling point (24 h, 7, 14, 28 and 56 days). L1: Level 1, L2: Level 2, L3: Level 3.

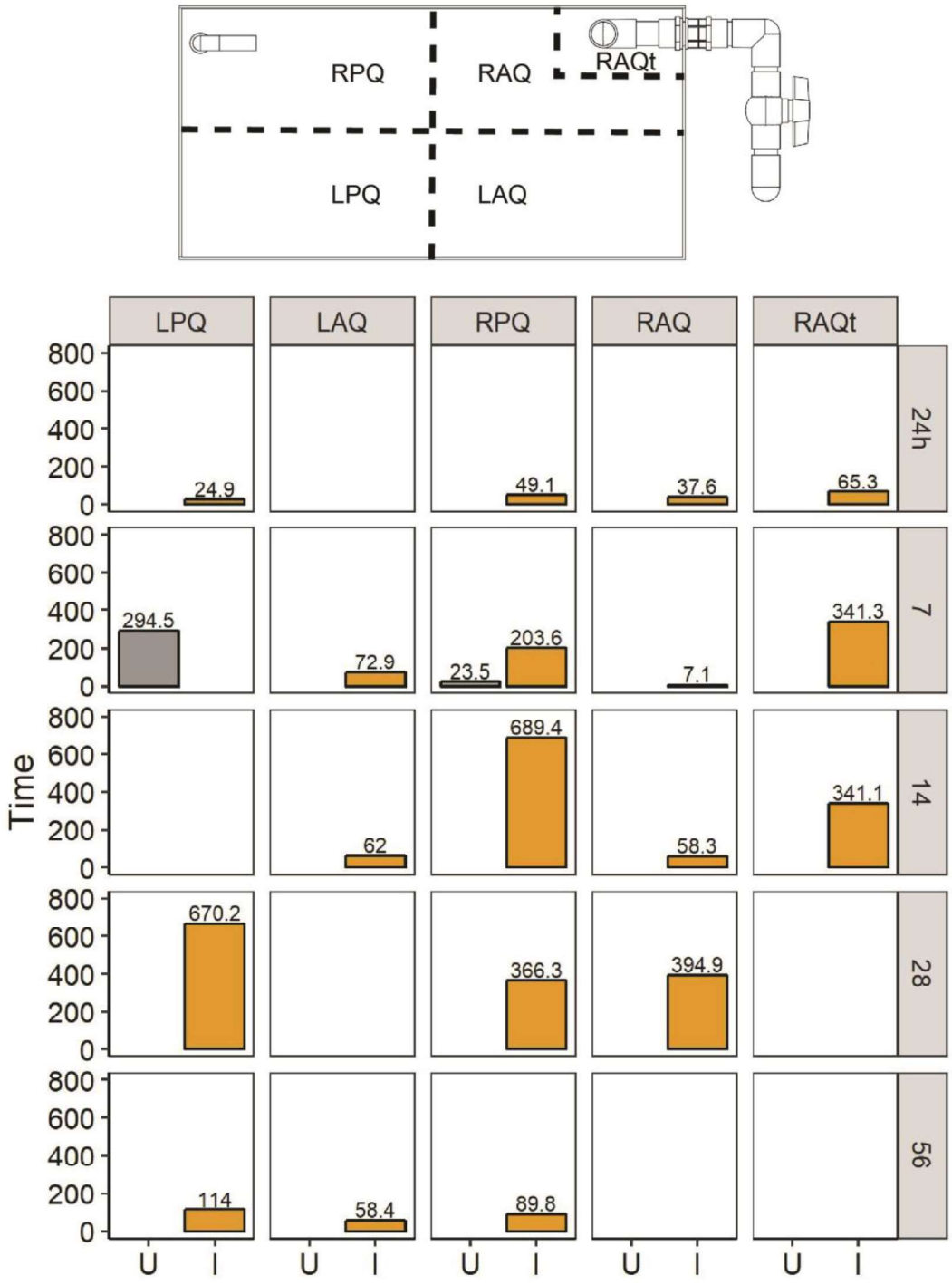

Fig. 3. Time spend (seconds) in the quadrants of the L1 by uninfected (U) and infected (I) frogs per sampling point (24 h, 7, 14, 28 and 56 days). LPQ: left posterior quadrant, LAQ: left anterior quadrant, RPQ: right posterior quadrant, RAQ: right anterior quandrant, RAQt: right anterior quandrant with animal supported in the tube.

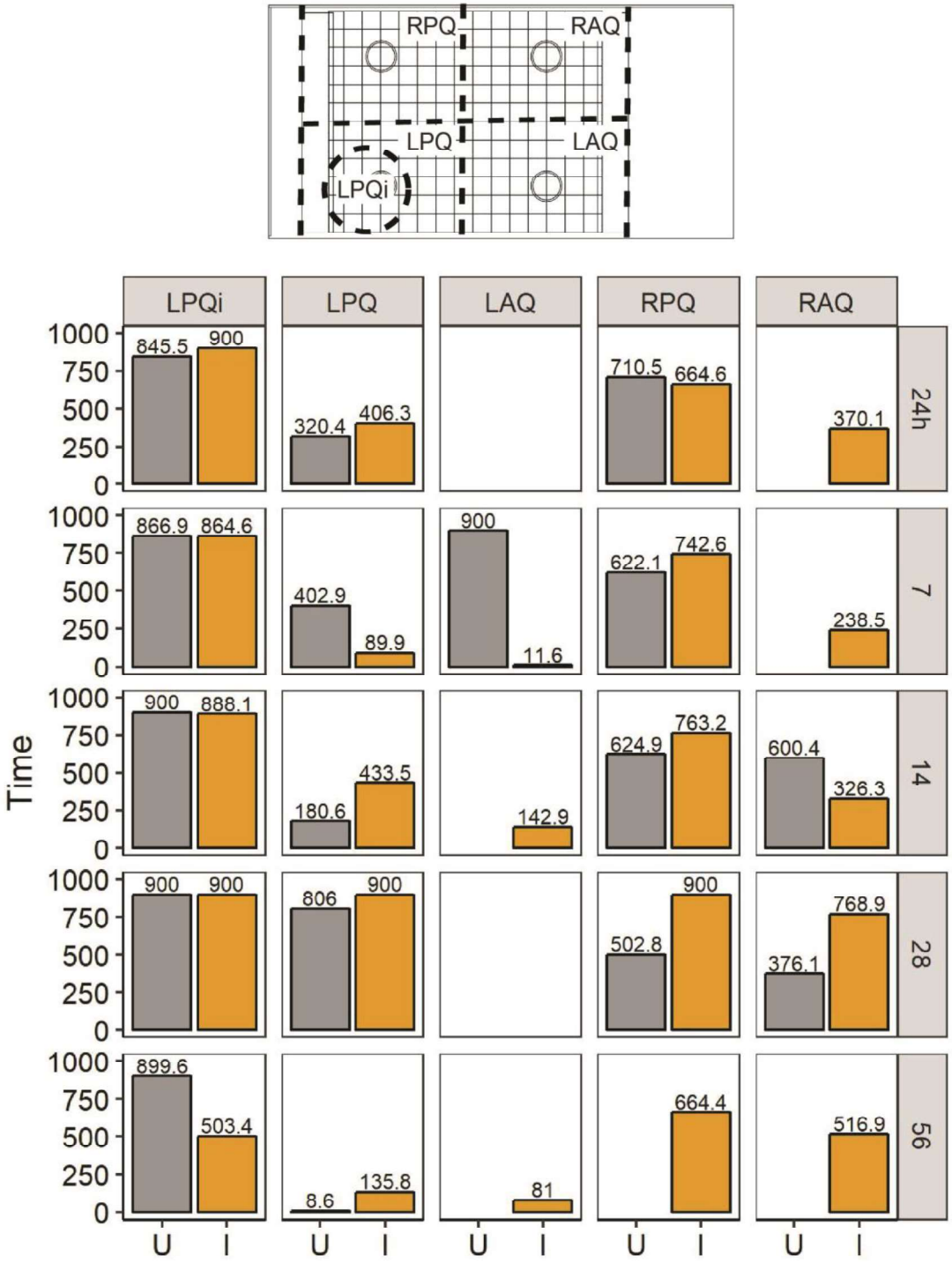

Fig. 4. Time spend (seconds) in the quadrants of the L2 between uninfected (U) and infected (I) frogs per sampling point (24 h, 7, 14, 28 and 56 days). LPQ: left posterior quadrant when animal is inside of shelter, LPQ: left posterior quadrant, LAQ: left anterior quadrant, RPQ: right posterior quadrant, RAQ: right anterior quandrant.

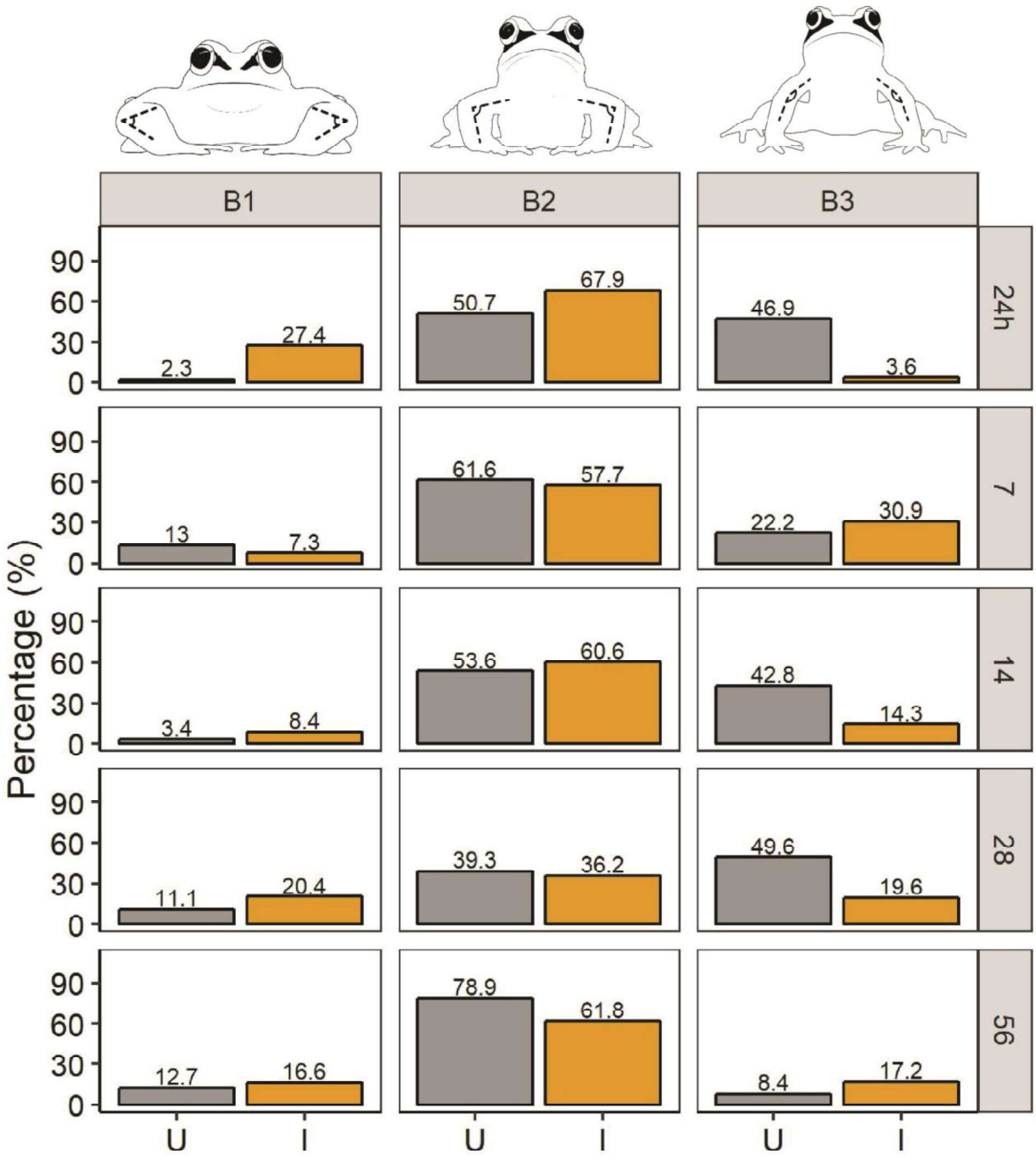

Fig. 5. Percentages of time in each different body posture between uninfected (U) and infected (I) frogs per sampling point (24 h, 7, 14, 28 and 56 days). Body position descriptions are detailed in the ethogram (Table 1).

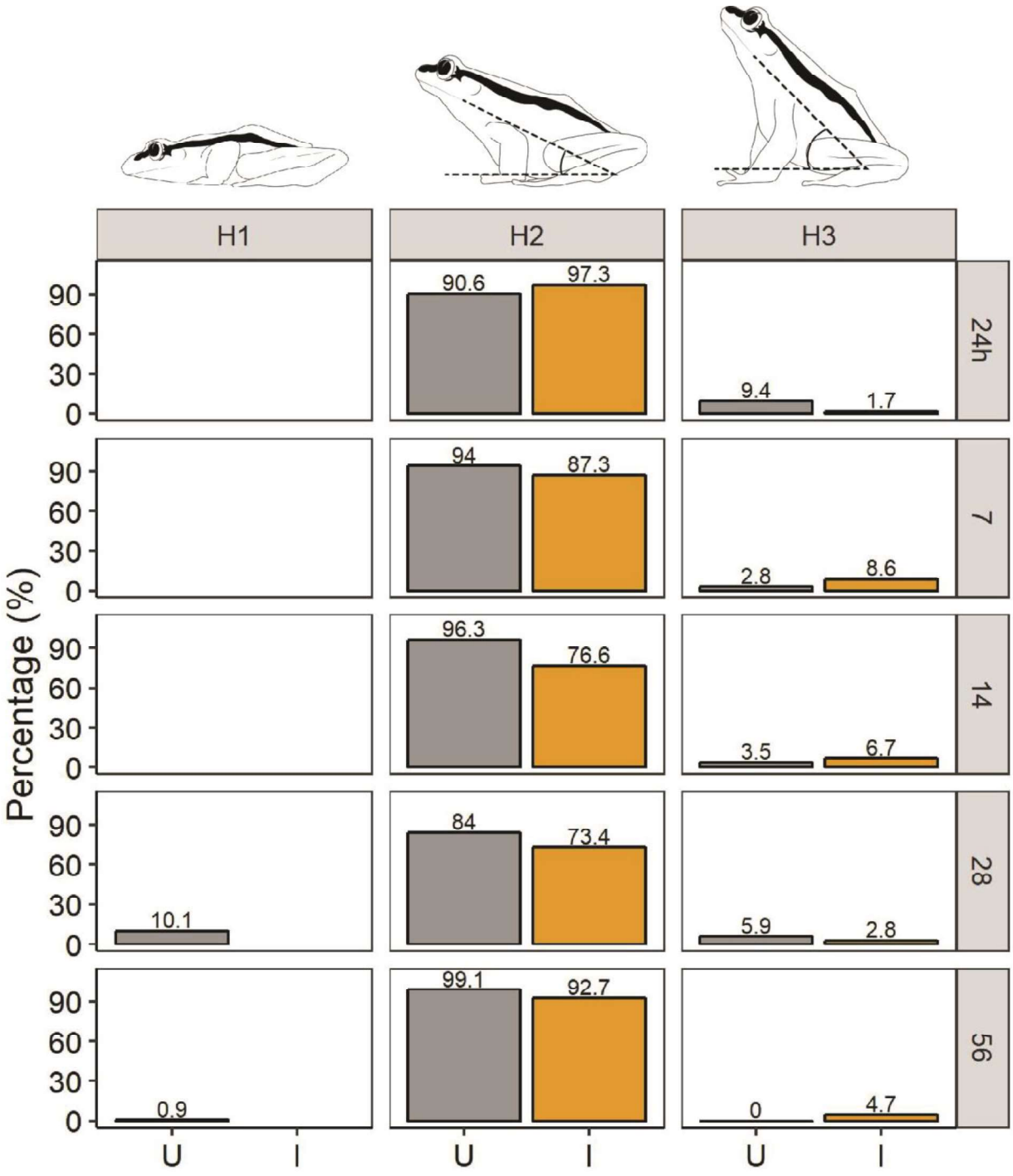

Fig. 6. Percentages of time in each different head position between uninfected (U) and infected (I) frogs per sampling point (24 h, 7, 14, 28 and 56 days). Head positions descriptions are detailed in the ethogram (Table 1).

Table 2. Frequencies (n) in different activities between uninfected (U) and infected (I) frogs per sampling point (24 h, 7, 14, 28 and 56 days). Asterisk is n infected. Activities descriptions are detailed in the ethogram (Table 1).

Recording Time	Brief movement		Rotation		Jump	
	U	I	U	I	U	I
24 h	17 (5)	40 (4)	3 (2)	13 (3)	1 (1)	13 (2)
7 d	5 (4)	37 (4)	3 (1)	2 (1)	14 (2)	13 (1)
14 d	28 (4)	44 (5)	7 (3)	13 (2)	1 (1)	17 (1)
28 d	14 (4)	33 (4, 0*)	5 (2)	0 (0)	4 (2)	4 (3)
56 d	11 (4)	38 (5, 1*)	0 (0)	6 (2, 0*)	0 (0)	18 (2)

5 FINAL CONSIDERATIONS

Costa Rica represents, on a small scale, the global phenomenon of amphibian population declines observed from the end of 80s to the current days. Around 20 species were believed to be extinct in the 90s. These species mainly came from the highlands of the country and fast-moving waters streams; however, the phenomenon also included species like *Lithobates vibicarius*, which is one of the most common ranids from all the mountain ranges of the country. The last specimen that was collected and registered was in 1990, according to the Zoology Museum from the University of Costa Rica.

Since 2002, there have been reported a series of reappearances in the country, starting with *L. vibicarius;* this population was the only one reported, and with it, new registrations were expanded and determined, up to the point of getting two extensive protected areas with well-established populations of this species. Nonetheless, the populations from the northern, middle-eastern and eastern parts of the country have not been reestablished.

One of the main hypotheses that explain the amphibian population declines is the one that states that the presence of the strain *Batrachochytrium dendrobatidis,* ethiological agent responsible for the disease known as Chytridiomycosis in the amphibians, played a major role in the process. However, there is not a robust causality study that confirms this strain to be the agent causing lesions that cause the death of animals at the moment these declines happened. Without a doubt, this has been an event that the scientific community was not expecting; therefore, there is not enough context or any interdisciplinary investigation regarding this kind of phenomena.

The reappearance of amphibian species does not solve the conservation aspects, but rather it reactivates the process by placing a species under human responsibility and very harsh conditions for its survival. The reappearance of species will normally be in small numbers and in restricted locations; therefore, this will be a species that will be on the brink of extinction again.

Before thinking of a reintroduction or translocation program for the *L. vibicarius*, it is important to know its response as a host when exposed to *Batrachochytrium dendrobatidis.* It is important to mention that in the current research the used strain of *Bd* was the one exposed to this species in order to

manage it eventually. Knowing the susceptibility of the species to *Bd* is ideal before assuming the costs of an *ex situ* management and conservation program.

The biggest challenge on an experimental infection process is the establishment of the best husbandry and maintenance conditions for the animals. Defining the most appropriate food, housing, animal welfare and biosecurity measures, the consolidation and correct application of all the standardized operational procedures, are all indispensable elements that guarantee the repetitiveness of the data and quality of this investigation. This is one of the aspects that require the largest investment of time and economic resources. The fact we are dealing with a wild animal, with no previous captivity background, represents an even greater challenge.

L. vibicarius was capable of resolving clinical signs in a period of two weeks, and in a period of eight weeks, where more than 80% of the experimentally infected animals successfully eliminated *Bd*. Additionally, there were no signs of anatomo-histopathological, hematological or behavioral alterations directly associated with the experimental infection process. Based on our results, it is feasible to consider the *Bd* infection in this species is self-limiting with a low morbidity and no death results.

Our study suggests that the *Bd* infection does not cause a behavioral alteration with a clear pattern in *L. vibicarius* under laboratory conditions but it gives rise to a methodology and ethogram for the evaluation of behavior indicators under experimental conditions. The description of the use of space, body posture and head position most likely valuable information. It is important knowledge to be able to guarantee the welfare of the individuals that are part of an experimentation process or an *ex situ* conservation program.

According to the results of our investigation, it is suggest that it is not possible to attribute the decline *of L. vibicarius* solely to chytridiomycosis based only on the diagnoses of the infection, despite the mortality observed in wild in the 90s. Complementary studies are needed to uncover the various aspects related to *in situ* mortalities.

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