Monitoreo a largo plazo de poblaciones de Ranita de Darwin (*Rhinoderma darwinii*) en el Monumento Natural Contulmo y Reserva Forestal Contulmo

Reporte periodo 2014-2018

.ORG
RANITA DE DARWIN
CONSERVACIÓN DE ANFIBIOS DE CHILE

Universidad Andrés Bello

Centro de Investigación para la Sustentabilidad

ONG Ranita de Darwin es una asociación chilena sin fines de lucro vigente e inscrita bajo el N°213586.

Este reporte es parte del proyecto "Moninotoreo a largo plazo de poblaciones de ranita de Darwin (*Rhinoderma darwinii*)". Para conocer más, visita: www.ranitadedarwin.org/monitoreo

Cita recomendada: Andrés Valenzuela-Sánchez, Soledad Delgado, Jaime Beltrand, Francisco Costas, David Uribe & Claudio Soto-Azat. 2018. Monitoreo a largo plazo de poblaciones de Ranita de Darwin (*Rhinoderma darwini*) en el Monumento Natural Contulmo y Reserva Forestal Contulmo. Reporte periodo 2014-2018. ONG Ranita de Darwin, Chile.

La rana rosácea de hojarasca (*Eupsophus roseus*) es, junto a la ranita de Darwin, la especie de anfibio más abundante en el Monumento Natural Contulmo y Reserva Forestal Contulmo. Esta forma local de la especie era antes conocida como *E. contulmoensis*.

Introducción

La ranita de Darwin del Sur (*Rhinoderma darwinii*) es una especie endémica del bosque templado austral de Chile y Argentina. Esta especie está categorizada como En Peligro de extinción tanto por el Reglamento de Clasificación de Especies (Ministerio del Medioambiente, Chile) como por la Lista Roja de la Unión Internacional para la Conservación de la Naturaleza.

Desde el año 2014, en un esfuerzo colaborativo entre la Asociación Ranita de Darwin y el Centro de Investigación para la Sustentabilidad de la Universidad Andrés Bello, hemos monitoreado dos poblaciones locales de la especie (distanciadas por ~3 km) ubicadas en el Monumento Natural Contulmo (MNC) y la Reserva Forestal Contulmo (RFC), áreas que son administradas por la Corporación Nacional Forestal (CONAF) de Chile. El objetivo general de este estudio es comprender de mejor manera las dinámicas poblacionales de esta especie, de forma de proporcionar información clave para su monitoreo, manejo y conservación.

En concordancia con este objetivo, en este reporte presentamos los principales hallazgos del proyecto obtenidos para el periodo comprendido entre 2014-2018. El apoyo de CONAF ha sido crucial durante este periodo, motivo por el cual esperamos que este documento pueda ser de gran utilidad para esta institución y sus funcionarios.

Breve revisión de la biología de la especie

Rhinoderma darwinii es una especie de anfibio completamente terrestre, esto significa que no requiere de cuerpos de agua (como pozas o ríos) para completar su ciclo de vida. Esta especie vive asociada al bosque templado austral y la abundancia de la especie es mayor en bosques que presentan una alta complejidad estructural, como es el caso de los bosques maduros (A. Valenzuela-Sánchez, datos no publicados). El macho de esta especie cría a los renacuajos en su saco vocal durante todo el desarrollo larvario, siendo la única especie de anfibio en el mundo con este particular modo reproductivo. Los adultos de la especie tienen una alta fidelidad de sitio (Crump 2002; Valenzuela-Sánchez et al. 2014), con desplazamientos interanuales que no superan los 3.64m en promedio (Valenzuela-Sánchez 2017). Los juveniles de la especie son los que realizan los eventos de dispersión, teniendo una capacidad de dispersión muy limitada (la máxima distancia de desplazamiento anual se estima en alrededor de 150m; Valenzuela-Sánchez 2017), lo que podría limitar la capacidad de esta especie para ajustar su distribución geográfica frente al escenario de cambio climático presente y futuro (Uribe-Rivera et al. 2017).

Monitoreo a largo plazo de poblaciones de ranita de Darwin en MNC y RFC

Descripción del estudio

En cada sitio seleccionamos una parcela rectangular dentro del bosque (área de la parcela, MNC = 0.14ha; RFC = 0.25ha) conteniendo una población local de *R. darwinii*. En cada parcela, hemos realizado la captura-recaptura de los individuos durante nueve ocasiones primarias de captura, distribuidas entre marzo de 2014 y diciembre de 2017 (Fig. 1A). Una ocasión primaria de captura es definida como una visita a terreno, dentro de la cual cada parcela es recorrida por dos investigadores durante 1 hora diaria, durante cuatro días consecutivos (cada día es definido como una ocasión secundaria), en busca de individuos de *R. darwinii*. En otras palabras, una ocasión primaria de captura en nuestro estudio es el conjunto de cuatro días consecutivos de búsqueda. El mes de las ocasiones primarias de captura ha sido aproximado por conveniencia para facilitar la interpretación de los resultados; por ejemplo, las visitas indicadas para el mes de diciembre pueden haber sido realizadas durante diciembre o enero, dependiendo de variables logísticas. Detalles sobre el manejo de los individuos, incluyendo el método de identificación de estos y las medidas de bioseguridad utilizadas, se pueden encontrar en el Anexo 1.

En este periodo hemos capturado 80 y

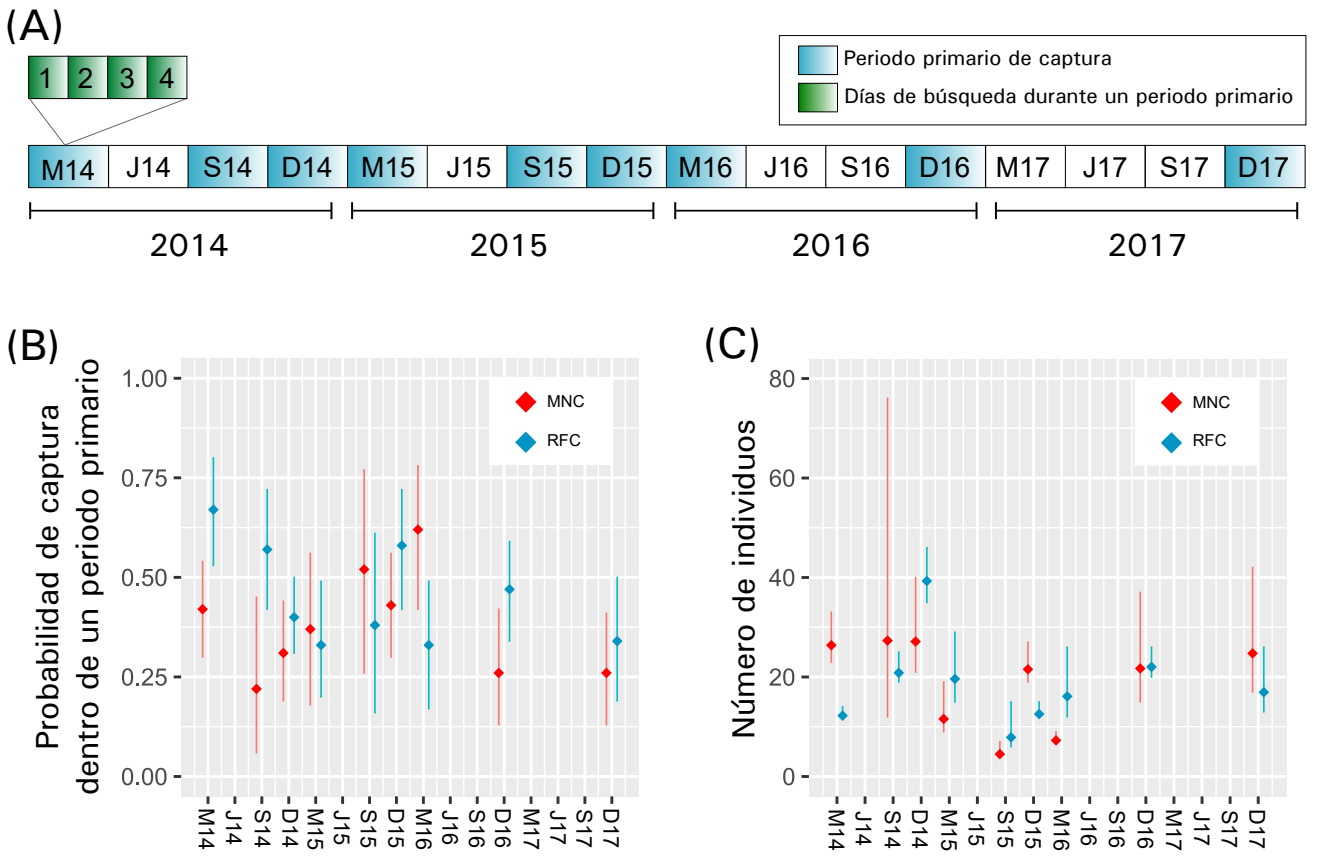

Figura 1. (A) Probabilidad de captura y (B) número de individuos dentro de un periodo primario en dos poblaciones locales de *Rhinoderma darwinii* en el Monumento Natural Contulmo (MNC) y Reserva Forestal Contulmo (RFC), Chile. Las barras representan el intervalo de confianza bayesiano del 95%. Los estimados fueron obtenidos utilizando un modelo de captura-recaptura cerrado (modelo M_0) y estadística bayesiana.

92 individuos diferentes en MNC y RFC, respectivamente. Solo 11% y 12% de los individuos capturados en MNC y RFC, respectivamente, han sido recapturados durante una nueva ocasión primaria de captura.

Número de individuos activos durante cada periodo primario de captura

Utilizando el historial de captura-recaptura de los individuos dentro de una ocasión primaria (esto es, durante los 4 días consecutivos de búsqueda) podemos estimar la probabilidad de capturar los individuos activos (esto es, que están disponibles para ser capturados y que no se encuentran, por ejemplo, en hibernación o estivación) durante cada visita a terreno (Fig. 1B). Esto es muy importante para poder estimar correctamente el número de individuos activos presentes en cada sitio, ya que, aunque estos se encuentren activos, no todos son detectados durante nuestra búsqueda. La probabilidad de captura dentro de cada ocasión primaria varió ampliamente entre los sitios y a lo largo del tiempo, sin un patrón claro. Por ejemplo, durante marzo de 2014 la probabilidad de captura fue mayor en RFC que en MNC, mientras que en marzo de 2016 ocurrió lo opuesto. Esto sugiere que la probabilidad de encontrar un individuo que se encuentra activo depende de factores sitio-específicos que no serían fáciles de predecir *a priori*. Por este motivo, no es recomendable utilizar simples conteos de ranas (esto es, no corregidos por probabilidad de captura) como un indicador de la abundancia de la especie, ya que esto induciría a conclusiones erróneas.

En la Figura 1C mostramos el número estimado de individuos de *R. darwinii* activos en cada sitio

durante cada ocasión primaria de captura, los cuales fueron obtenidos tomando en cuenta la probabilidad de capturar los animales. En general, se puede apreciar que el número de individuos activos en cada sitio se ha mantenido relativamente constante a lo largo del estudio. Sin embargo, y preocupantemente, ambas poblaciones de *R. darwinii* son muy pequeñas, rondando alrededor de los 20 individuos activos.

Distribución etaria dentro de cada ocasión primaria de captura

Además de la abundancia, la estructura etaria es una característica muy importante de las poblaciones. *R. darwinii* es una especie con una estrategia de historia de vida lenta, esto significa que normalmente los individuos viven varios años (algunos individuos pueden vivir >10 años) pero tienen una baja fecundidad. Por ejemplo, el tamaño de la ovipostura en esta especie es generalmente menor a 20 huevos (Valenzuela-Sánchez et al. 2014). En comparación con otros anfibios, por ejemplo, con las especies del género *Rhinella* (e.g. sapo de rulo) que tienen oviposturas de cientos o miles de huevos, la fecundidad de *R. darwinii* es extremadamente baja. Esta característica de la especie hace que la sobrevivencia de los adultos sea extremadamente importante para el crecimiento poblacional, ya que mientras más años viva un adulto, más oportunidades tendrá este de reproducirse y mayor será el número de nuevos individuos que pueda proporcionar a la población (Valenzuela-Sánchez 2017). Teóricamente, también debería existir una mayor proporción de individuos adultos que de jóvenes en la población. En concordancia con esto, en otras poblaciones de *R. darwinii* hemos observado que un 70% de los individuos corresponderían a adultos y solo un 30% a juveniles.

Basados en rasgos reproductivos morfológicos (e.g. presencia de saco vocal en machos, hembras grávidas) y de comportamiento (i.e. canto), hemos determinado que los individuos de *R. darwinii* de MNC y RFC alcanzan, en promedio, la adultez a los 24mm de largo hocico-cloaca (LHC). Basados en esta información, nuestros resultados muestran que tanto en MNC como en RFC las poblaciones están compuestas por una muy baja proporción de individuos adultos (Fig. 2&3). En RFC la estructura etaria consta de solo dos componentes bien delimitados: juveniles de 1 año y adultos de gran tamaño (>30mm LHC); en esta población hemos detectado una muy baja proporción de individuos de tamaño intermedio (esto es, adultos de corta edad), lo que sugiere que la sobrevivencia de los juveniles es extremadamente baja y que la mayoría de estos no sobrevive hasta la adultez.

Probabilidad de sobrevivencia de las ranitas de Darwin en MNC y RFC

Nuestros datos de captura-recaptura también permiten la estimación de la probabilidad de sobrevivencia de los individuos entre dos ocasiones primarias de captura. Esta probabilidad de sobrevivencia es estimada en base a la probabilidad de recaptura, la que no debe confundirse con la probabilidad de captura dentro de una ocasión primaria. La probabilidad de recaptura es la probabilidad de volver a encontrar en una ocasión primaria un individuo que fue capturado en la ocasión primaria anterior o en ocasiones primarias previas. De esta forma, esta probabilidad está compuesta por la probabilidad de detectar un individuo dentro de la ocasión primaria y por la probabilidad de que este individuo esté disponible para ser efectivamente capturado (esto es, que no haya emigrado a otro sitio o que no se encuentre inactivo hibernando o estivando).

Valenzuela-Sánchez et al. (2017) describen que la probabilidad de recaptura y sobrevivencia es similar en MNC y RFC. Por otra parte, la probabilidad de sobrevivencia es ligeramente menor en juveniles que en adultos (Valenzuela-Sánchez et al. 2017). En esta sección, presentamos resultados generales para MNC y RFC, sin distinción de edad o población.

El primer aspecto importante es que la probabilidad de recaptura muestra una clara variación temporal (Fig. 4A); esta fue siempre mayor durante diciembre que durante los otros meses del año. Esto sugiere que durante septiembre y marzo una mayor proporción de los individuos de la población no se encuentran disponibles para ser capturados, probablemente porque se encuentran hibernando o estivando. Esta información es muy útil para mejorar la relación costo-eficiencia durante el monitoreo poblacional. Diciembre es la mejor fecha del año para realizar

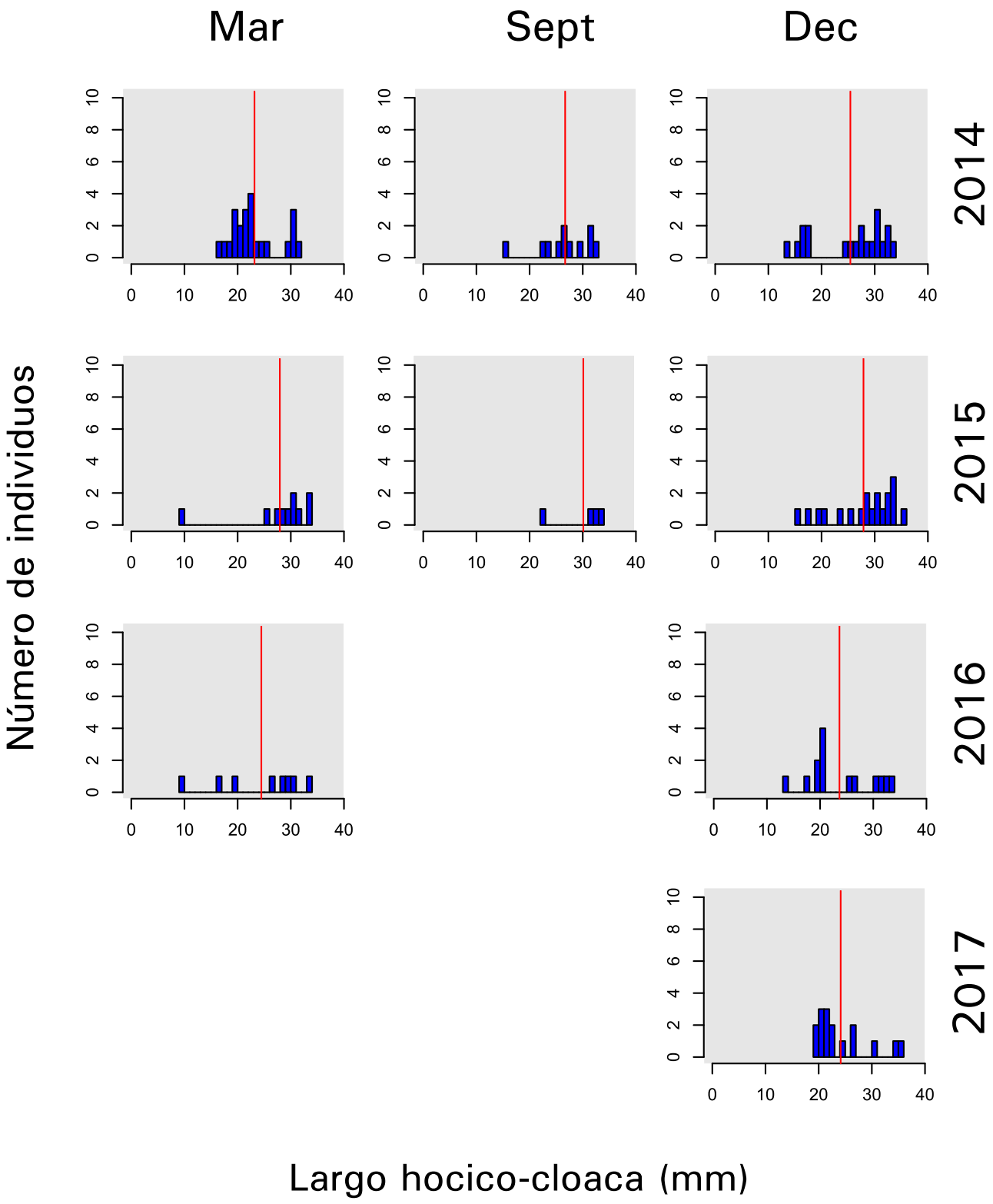

Figura 2. Distribución del largo corporal de individuos de *Rhinoderma darwinii* capturados en el Monumento Natural Contulmo (MNC) durante diferentes periodos primarios de captura. La línea roja vertical representa la media.

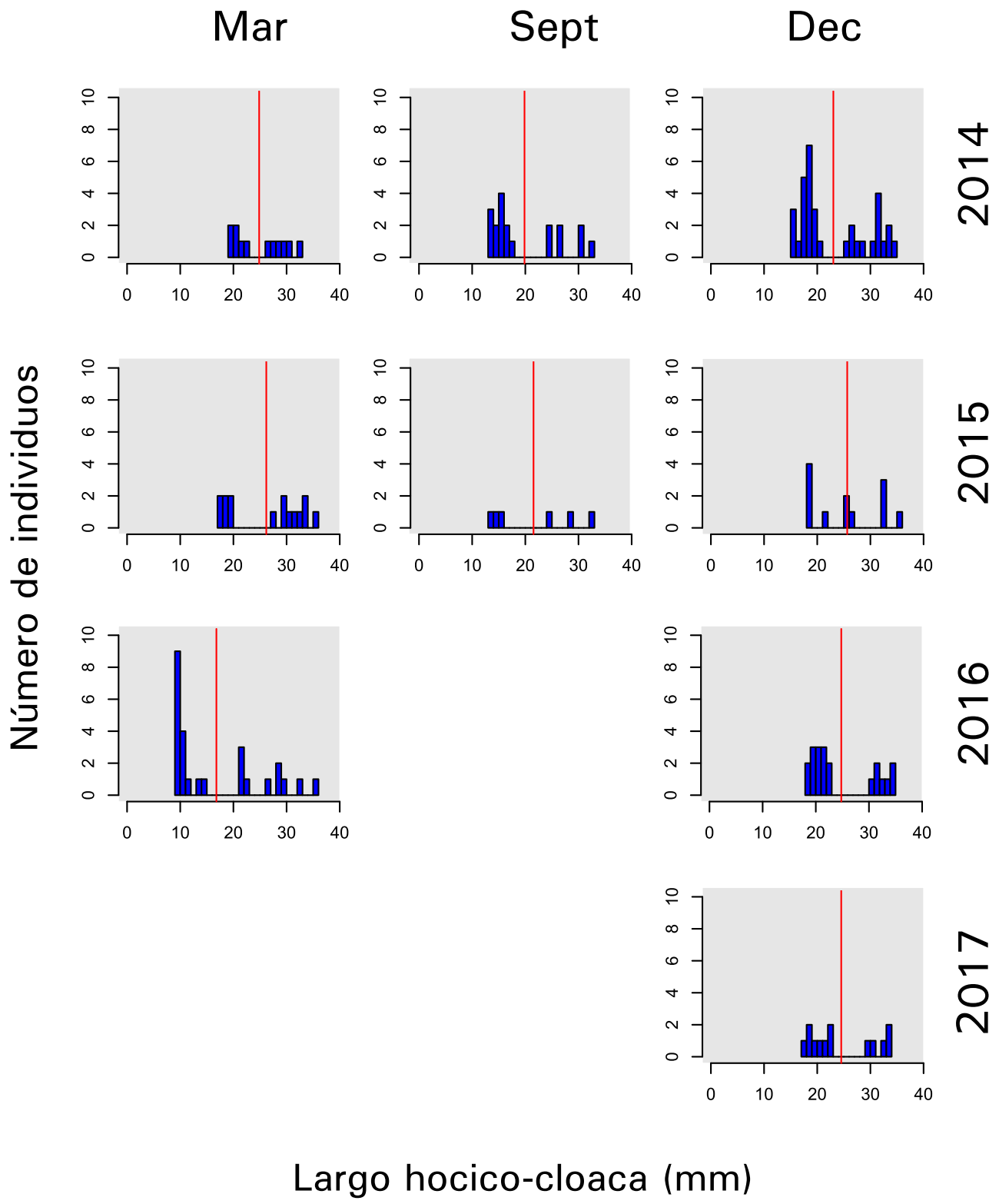

Figura 3. Distribución del largo corporal de individuos de *Rhinoderma darwinii* capturados en la Reserva Forestal Contulmo (RFC) durante diferentes periodos primarios de captura. La línea roja vertical representa la media.

el muestreo de las poblaciones de *R. darwinii* en MNC y RFC, ya que es el momento donde un mayor porcentaje de la población es efectivamente observada. Por este motivo, para hacer un uso eficiente de nuestros recursos, desde 2017 hemos seleccionado solo este periodo del año para continuar con el monitoreo a largo plazo de poblaciones de ranita de Darwin.

La probabilidad de sobrevivencia, expresada aquí en una escala anual, fue relativamente estable a lo largo del periodo de estudio (Fig. 4B), manteniéndose en torno a 0.25. A modo de ejemplo, esta probabilidad de sobrevivencia significa que aproximadamente 1 de cada 20 juveniles alcanza la adultez (~2 años de vida en estas poblaciones) y solo 1 de cada 250 juveniles alcanza los 4 años. Esta baja probabilidad de sobrevivencia, que es varias veces menor a la observada en otras poblaciones de la especie (Valenzuela-Sánchez et al. 2017), explicaría la aparente ausencia de adultos de corta edad en RFC.

Factores que afectan la sobrevivencia de las ranitas de Darwin en MNC y RFC: quitridiomicosis

La quitridiomicosis es una enfermedad fúngica de la piel de los anfibios causada por el hongo *Batrachochytrium dendrobatidis* y *B. salamandrivorans*. En Chile, el hongo *B. dendrobatidis* está presente a lo largo de todo el país. Las cepas o variedades de este hongo que hemos podido aislar desde Chile, indican que en nuestro país se encuentra presente el linaje pandémico global, un grupo del hongo que sería altamente virulento (Valenzuela-Sánchez et al. 2018). Este linaje del hongo surgió durante el siglo XX en el este de Asia y desde esa época, asistido por la actividad humana, se ha dispersado a lo largo de todo el mundo, incluido Chile (O'hanlon et al. 2018).

En base a un análisis de animales de museo, Soto-Azat et al. (2013) proveen evidencia de que *B. dendrobatidis* podría haber sido introducido a nuestro país alrededor de 1970, una sugerencia reafirmada por el análisis genómico de las cepas presentes en Chile (Valenzuela-Sánchez et al. 2018). Esta fecha es coincidente con la desaparición de la ranita de Darwin del Norte (*R. rufum*) y con el inicio de la extirpación de varias poblaciones locales de

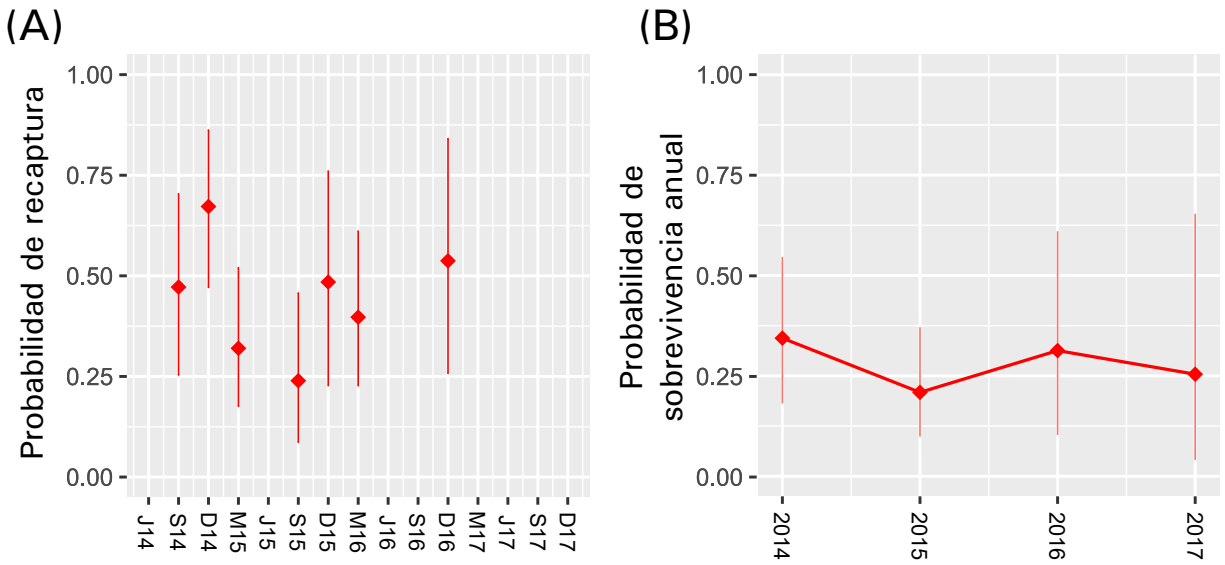

Figura 4. (A) Probabilidad de recaptura y (B) probabilidad de sobrevivencia anual en individuos de *Rhinoderma darwinii* capturados en el Monumento Natural Contulmo (MNC) y Reserva Forestal Contulmo (RFC), Chile. Las barras representan el intervalo de confianza bayesiano del 95%. Los estimados fueron obtenidos utilizando un modelo de captura-recaptura abierto (modelo CJS) y estadística bayesiana.

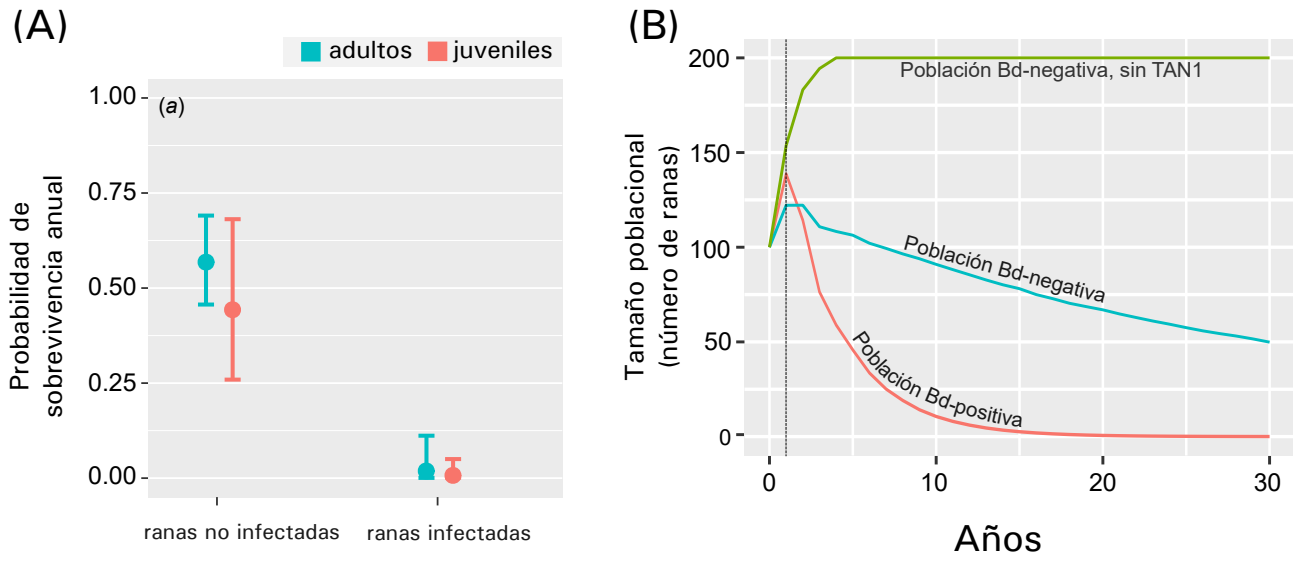

Figura 5. (A) Probabilidad de sobrevivencia anual en individuos de *Rhinoderma darwinii* infectados y no-infectados con el hongo *Batrachochytrium dendrobatidis* (Bd; causante de la quitridiomicosis de los anfibios) capturados en poblaciones silvestres en el sur de Chile. En (B) se muestran predicciones del tamaño de poblaciones con (rojo) y sin el hongo (celeste y verde). Para más detalles ver Valenzuela-Sánchez et al. (2017) en el Anexo 2.

R. darwinii. Los individuos de *R. darwinii* pueden desarrollar la quitridiomicosis, como lo demuestran hallazgos histológicos de animales con signos de la enfermedad capturados en vida silvestre (Soto-Azat et al. 2013).

Gracias al monitoreo de poblaciones de ranita de Darwin que iniciamos en 2014 en MNC, RFC y otras zonas del país, podemos proveer por primera vez evidencia cuantitativa sobre los efectos de la quitridiomicosis en la probabilidad de sobrevivencia de individuos de *R. darwinii* (Valenzuela-Sánchez et al. 2017). En MNC y RFC hemos detectado diversos individuos infectados con el hongo; nuestros hallazgos muestran que los individuos infectados tienen una probabilidad de sobrevivir que es cercana a cero (Fig. 5A). De esta forma, la presencia de la quitridiomicosis podría explicar la baja probabilidad de sobrevivencia observada en MNC y RFC. Modelos predictivos poblacionales indican que los efectos medidos a nivel individual pueden llevar a la extinción de poblaciones locales de la especie al largo plazo (>10 años luego de la introducción del hongo; Fig. 5B).

Conclusiones

Este proyecto ha permitido generar información clave para el monitoreo, manejo y conservación de *R. darwinii* en MNC, RFC y otras áreas de Chile y Argentina. Nuestros resultados muestran que durante los meses de diciembre-enero es la mejor fecha del año para llevar a cabo el monitoreo de poblaciones locales de *R. darwinii* en MNC y RFC.

De las diferentes poblaciones de *R. darwinii* que hemos monitoreado a lo largo de Chile, las ubicadas en Contulmo presentan el mayor riesgo de extinción. La baja probabilidad de sobrevivencia, los pequeños tamaños poblacionales y la estructura etaria de las poblaciones indican la existencia de altas tasas de mortalidad (probablemente mucho más altas que las naturales) en los individuos de *R. darwinii* en esta zona geográfica. La evidencia existente sugiere que la quitridiomicosis podría ser un factor importante detrás de esta preocupante situación. La prevalencia de esta enfermedad es mayor en poblaciones de *R. darwinii* ubicadas en la cordillera de Nahuelbuta que en otras poblaciones de ranita de Darwin a lo largo de Chile (Soto-Azat et al. 2013; Valenzuela-Sánchez et al. 2017).

La continuidad en el futuro de este proyecto

a largo plazo permitirá determinar el papel que juegan otros factores (e.g. variaciones climáticas) sobre las dinámicas poblacionales de *R. darwinii*. Sin embargo, creemos que es el momento indicado para comenzar el manejo de la quitridiomicosis en poblaciones silvestres de *R. darwinii* en la Cordillera de Nahuelbuta y otras zonas de Chile y Argentina, con la finalidad de asegurar la persistencia de estas poblaciones a largo plazo. Aún existen diversos aspectos claves de la dinámica poblacional de esta especie, y de su interacción con *B. dendrobatidis*, que necesitan ser comprendidos de mejor manera; la utilización de experimentos observacionales y manipulativos en un esquema de manejo adaptativo parecen ser la mejor solución para lidiar con las incertezas existentes y para proporcionar una solución eficiente y oportuna al problema. El proyecto EMERGE (https://www.ranitadedarwin.org/emerge) es un primer paso en esta dirección.

Agradecimientos

Este proyecto fue posible gracias a las autorizaciones N°026/2013, N°11/2015 IX (y su ampliación), N°12/2016 IX y N°04/2018 IX otorgados por CONAF a los investigadores de este proyecto. Estamos particularmente agradecidos con los guardaparques del Monumento Natural Contulmo, liderados por el Sr. Juan Carlos Correa, por su crucial apoyo durante el desarrollo de este proyecto. Estamos muy agradecidos también con el Sr. Marcelo Saavedra por su importante apoyo.

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Como parte de este proyecto, realizamos el primer registro de *Eupsophus vertebralis* en el Monumento Natural Contulmo

PROYECTO EMERGE

ANEXO 1
Protocolo de captura, muestreo y marcaje de anuros vivos

Este protocolo entrega lineamientos para la captura, mantención, manipulación, marcaje y muestreo no-invasivo de anuros vivos en terreno, con el objetivo de reducir el estrés de los animales y la posibilidad de transmisión de patógenos (especialmente del hongo quítrido) entre individuos y entre parcelas de estudio. This protocol provides guidelines for the capture, maintenance, handling, marking and non-invasive sampling of live anurans in the field. The protocol has been designed in order to reduce the stress of animals and the probability of pathogen transmission between individuals and study plots.

Captura

1. Antes del ingreso a cada parcela de estudio, cada investigador debe desinfectar su calzado utilizando una solución de Virkon® (1 g/L de agua) eliminando todo resto de barro, y desinfectar el equipo de trabajo con etanol al 70%, lo que produce la muerte del hongo quítrido luego de 20 s de exposición[1].
2. Una vez que un anuro es avistado, el investigador debe ponerse el guante de nitrilo nuevo (ver kit de captura a la derecha) y capturar al animal con gentileza. El animal debe ser rápidamente depositado en una bolsa plástica nueva, la que debe ser llenada con aire, sellada herméticamente, y mantenida lejos de la luz solar directa hasta el momento del muestreo. No depositar sustrato dentro de la bolsa, ya que esto dificulta el muestreo, aumentando el estrés del animal. Nunca usar guantes de látex, ya que pueden ser más tóxicos para los anfibios que los de nitrilo[2].
3. El lugar exacto de captura debe ser marcado con una bandera con código único idéntico al correspondiente kit de captura.
4. El periodo de búsqueda no debe superar 1 hora continuada. El muestreo debe realizarse inmediatamente transcurrido este tiempo de forma de asegurar que los animales no estarán en ningún caso dentro las bolsas por un periodo superior a 3 horas.
5. Antes del muestreo, todos los individuos capturados deben ser revisados. Los individuos capturados previamente dentro de la misma semana deben ser registrados en la planilla de muestreo y liberados inmediatamente sin ningún muestreo adicional (ver abajo).

Capture

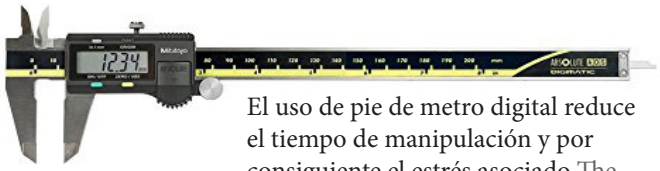
1. Prior to enter the study plots, each researcher should disinfect her/his shoes using a Virkon® solution (1 g/L), removing all the mud, and clean all the equipment with 70% ethanol[1].
2. The researchers should capture each frog wearing a new nitrile glove (see the kit on the right). Each frog should be put in a new ziploc bag filled with air and maintained out of direct sunlight until sampling. Latex gloves should be avoided[2].
3. The exact location of capture should be marked with a flag.
4. The searching period should lasts 1 hour maximum. Sampling should always take place within 3 hours since capture.
5. Individuals captured two times or more during the same week should be sampled only during the first capture.

Kit de captura capture kit:
1 bolsa plástica nueva con código, 1 pinza plástica,1 guante de nitrilo nuevo y una banderita con código

Desinfección disinfection

Bolsa con una rana
Bag containing a frog

Una vez que la rana es depositada en la bolsa, el guante debe ser guardado en la bolsa del kit hasta el muestreo. After capture, the associated glove should be stored in the second bag until sampling



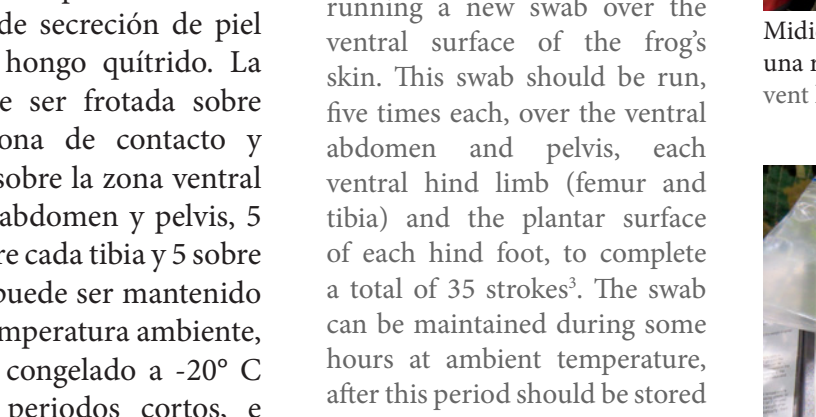
El uso de pie de metro digital reduce el tiempo de manipulación y por consiguiente el estrés asociado The use of a digital caliper reduces the handling time and the related stress

Toma de datos y muestras

1. La toma de datos y muestras debe ser realizada por 2 personas. El investigador 1 debe realizar la manipulación de las ranas y el investigador 2 registrar los datos en la planilla de muestreo.
2. El investigador debe retirar cuidadosamente el aire de la bolsa conteniendo la rana. Con la rana aún en la bolsa, para evitar contaminación cruzada entre los individuos, debe realizar la medición del largo total hocico-cloaca utilizando, idealmente, un pie de metro digital (ver foto en la derecha).
3. El investigador 1 debe retirar la rana de la bolsa usando el guante de nitrilo asociado a esa captura. Utilizando la otra mano debe sostener un hisopo nuevo para realizar el muestreo no-invasivo de secreción de piel para la detección del hongo quítrido. La punta del hisopo debe ser frotada sobre la piel, girando la zona de contacto y realizando 35 pasadas sobre la zona ventral del animal (5 sobre el abdomen y pelvis, 5 sobre cada fémur, 5 sobre cada tibia y 5 sobre cada pata)[3]. El hisopo puede ser mantenido durante unas horas a temperatura ambiente, lejos de la luz solar, y congelado a -20° C para almacenaje por periodos cortos, e idealmente a -80° C para periodos largos.
4. El investigador 2 debe rotular cada hisopo con el código de captura, parcela de estudio, especie, sexo, fecha y hora del muestreo.
5. Utilizando la bolsa de captura como superficie de apoyo para evitar la contaminación cruzada, el investigador 1 debe pesar la rana con una pesa digital (ver foto en la derecha).

Sampling

1. The sampling should be performed by 2 persons. Researcher 1 should handle the frog, and researcher 2 should take notes in the sampling sheet.
2. Researcher 1 should remove the air from the capture bag, and with the frog still inside, measure its snout-to-vent length using a digital caliper.
3. Wearing the nitrile glove used previously to capture that individual, researcher 1 should carefully remove the frog from inside the bag. The sample for chytrid fungus detection should be taken by this person by firmly running a new swab over the ventral surface of the frog's skin. This swab should be run, five times each, over the ventral abdomen and pelvis, each ventral hind limb (femur and tibia) and the plantar surface of each hind foot, to complete a total of 35 strokes[3]. The swab can be maintained during some hours at ambient temperature, after this period should be stored at -20° C, and, ideally, at -80° C for long-term storage.
4. Researcher 2 should label the swab with the required data.
5. Using a digital scale, researcher 1 should weight the frog using the capture bag as surface in order to avoid pathogen transmission between frogs (see photo on the right).



Midiendo el largo hocico-cloaca de una rana Measuring a frog's snout-to-vent length

Pesando una rana de Darwin Weighing a Darwin's frog

Hisopo utilizado para el muestreo del hongo quítrido Swab used for chytrid fungus detection
Este es un hisopo estéril, seco, con punta de un material derivado de la celulosa llamado Rayon (MW100, Medical Wire & Equipment, UK).

Muestreo para la detección del hongo quítrido en la superficie de la piel de una rana rosácea de hojarasca Sampling for chytrid fungus detection in a rosy ground frog

Marcaje y liberación

1. Utilizando la bolsa de captura como superficie de apoyo, el investigador 1 debe tomar una fotografía del código de captura y de la zona dorsal y ventral del individuo capturado.
2. En *Rhinoderma darwinii,* la fotografía ventral sirve para la identificación de los individuos[3] (ver foto abajo).
3. Para las otras especies, si la rana no tiene un marcaje previo, el investigador 1 debe sujetar firmemente al individuo usando el guante, dejando expuesta la zona ventral posterior del cuerpo. Con una nueva jeringa con aguja estéril de 29 G, el investigador 1 debe inyectar subcutáneamente un implante de elastómero visible (seguir indiciones del fabricante) sobre los muslos, en combinaciones de 1-3 colores de acuerdo a un esquema de marcaje[5]. Ver recuadro a la derecha.
4. Devolver el individuo a la bolsa de captura, llenarla de aire y dejarla a la sombra hasta la liberación. La manipulación de cada individuo no debe durar más de 5 minutos.
5. Terminado el muestreo de los animales, el investigador 1 y 2 deben liberar lo antes posible los individuos capturados en los lugares exactos

Marking and release

1. Using the capture bag as surface, researcher 1 should take a photo of the capture code, and dorsal and ventral surfaces of the frog.
2. In Rhinoderma darwinii, the ventral patterns are used for individual identification (see photo below).
3. For other species, if not marked previously, the frog should be firmly hold by researcher 1 wearing the nitrile glove. A visible implant elastomer should be injected subcutaneously into the inner thigh by researcher 1 using a 29-gauge insulin needle, in a combination of 1-3 colours following a marking scheme[5]. See box on the left.
4. Researcher 1 should put the frog back into the capture bag, which should filled with air and kept out of direct sunlight until release. The handling of each individual should last less than 5 minutes.
5. Once all frogs have been sampled, researcher 1 and 2 should release, as soon as possible, all the frogs in the exact points of capture. Disinfect equipment and shoes as described before after

Marcaje Marking

Tres métodos son ampliamente utilizados para el marcaje de anuros: (1) corte de dedos, (2) implantes de elastómeros visibles, y (3) transpondedores pasivos (microchips). El (1) es el más confiable, pero el más controversial éticamente porque puede producir dolor e infección[4]. El (2) es el que produce menos dolor y reacciones adversas, pero no es efectivo en todas las especies debido a migración y pérdida de las marcas[4,5]. El (3) requiere de una incisión para la instalación del microchip en cavidad celómica o tejido subcutáneo; un gran porcentaje de los microchips pueden ser expulsados, incluso con el uso de sellante[4]. Antes de ser usado en un estudio de largo plazo, es preferible evaluar la efectividad de cada método a nivel de especie[4]. En ausencia de tal evidencia, debería escogerse el que produce menos dolor y reacciones adversas (i.e. (2)).

Three methods are widely used for marking anurans: (1) toe clipping, (2) visible implant elastomers, and (3) passive transponders. (1) is the most reliable, but controversial from an ethical point of view because it may cause pain and infections[4]. (2) is the least painful and detrimental, but the marks may be lost in some species[4,5]. (3) requires of an incision to implant the transponder, which may be commonly expelled from the frog's body even using glue[4]. The efficacy of each method should be evaluated species by species before its use in a long term study[4]. In the absence of such evidence, the less painful and detrimental method should be preferred (i.e. (2)).

Recapturas de una rana de Darwin macho Recaptures of a male Darwin's frog

Enero 2015
SVL (largo): 15.62 mm

Enero 2016
SVL: 27.65 mm

Enero 2017
SVL: 31.09 mm

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Como citar How to cite

Proyecto Emerge (2018) Protocolo para la captura, muestreo y marcaje de anuros vivos en el campo. ONG Ranita de Darwin & Universidad Austral de Chile.

Una versión anterior de este protocolo fue utilizada exitosamente durante un estudio de largo plazo publicado por los autores[6].

Centro de Investigación para la Sustentabilidad

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Cover image
A Darwin's frog (*Rhinoderma darwinii*) from Contulmo, Nahuelbuta Range, Chile. This species is threatened by an emerging infectious disease known as amphibian chytridiomycosis. Photo credit: Andrés Valenzuela Sánchez. See the paper 'Cryptic disease-induced mortality may cause host extinction in an apparently stable host–parasite system' by Valenzuela-Sánchez *et al*, published in *Proceedings B* (http://dx.doi.org/10.1098/rspb.2017.1176)

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PROCEEDINGS OF THE ROYAL SOCIETY B

BIOLOGICAL SCIENCES

In this issue: Barn owls have ageless ears

Quantitative study of developmental biology confirms Dickinsonia as a metazoan

Earless toads sense low frequencies but miss the high notes

THE ROYAL SOCIETY PUBLISHING

ANEXO 2

Portada de la revista científica Proceedings B, resaltando una ranita de Darwin del Monumento Natural Contulmo parte de este proyecto

PROCEEDINGS B

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Research

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THE ROYAL SOCIETY PUBLISHING

Cryptic disease-induced mortality may cause host extinction in an apparently stable host–parasite system

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The decline of wildlife populations due to emerging infectious disease often shows a common pattern: the parasite invades a naive host population, producing epidemic disease and a population decline, sometimes with extirpation. Some susceptible host populations can survive the epidemic phase and persist with endemic parasitic infection. Understanding host–parasite dynamics leading to persistence of the system is imperative to adequately inform conservation practice. Here we combine field data, statistical and mathematical modelling to explore the dynamics of the apparently stable *Rhinoderma darwinii*–*Batrachochytrium dendrobatidis* (Bd) system. Our results indicate that Bd-induced population extirpation may occur even in the absence of epidemics and where parasite prevalence is relatively low. These empirical findings are consistent with previous theoretical predictions showing that highly pathogenic parasites are able to regulate host populations even at extremely low prevalence, highlighting that disease threats should be investigated as a cause of population declines even in the absence of an overt increase in mortality.

1. Introduction

In his pioneering work, Anderson [1] used epidemiological models to show that highly pathogenic parasites are likely to induce their own extinction before that of their host. Subsequent theoretical and empirical work showed that, under certain circumstances, parasites can drive host populations to extinction [2–5]. For instance, the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter Bd) [6] has been associated with mass mortality events, the extirpation of local amphibian populations and the extinction of amphibian species on multiple continents [7,8]. As in other host–parasite systems [5], the capability of Bd to have these devastating effects on host populations is largely attributed to the presence of multiple reservoirs, the existence of a free-living infective stage and the introduction of the parasite into naive host populations [3,7,9,10].

Diverse emerging host–parasite systems (e.g. morbillivirus disease in mammals, white nose syndrome in bats, Ebola in primates), including the amphibian–Bd system, show a common pattern of disease-induced host population decline: the parasite invades a naive host population producing a disease outbreak or epidemic, leading to mass mortality, population decline and, eventually, extirpation [4,11–17]. For the amphibian–Bd system, theory predicts and empirical evidence confirms, that populations of highly susceptible hosts (i.e. hosts that develop the fatal, Bd-induced disease chytridiomycosis) can survive the epidemic state and persist in relative stability with endemic Bd infection

dynamics [18–23]. The persistence of a population of susceptible hosts with endemic Bd infection could arise as a consequence of several processes that include (but are not restricted to) an increase in recruitment that compensates for the Bd-induced mortality [19,24,25], changes in biotic or abiotic factors that reduce average infection intensity and increase parasite aggregation [21,26], and density-dependent transmission dynamics [24]. As these processes are general, they are not restricted to the amphibian–Bd interaction but should play a key role in the dynamics of most host–parasite systems [27–29].

As emerging infectious diseases have become a significant threat to biodiversity [3,30], it is urgent to gain a thorough understanding of host–parasite systems that can transit from an epidemic to an endemic state, and therefore where a population of susceptible hosts is able to persist in the face of recurrent parasite infection and high probability of disease-induced mortality if infection occurs [29]. In this study we combined field data, statistical and mathematical modelling to explore the dynamics of an apparently stable amphibian–Bd system. To this end, we focused on the southern Darwin's frog (*Rhinoderma darwinii*), an amphibian species that inhabits the austral temperate forest of southern South America [31]. The *R. darwinii*–Bd system is a suitable model for the study of endemic Bd infection dynamics in a susceptible host species because: (i) Bd infection can produce mortality in *R. darwinii* individuals [32]; (ii) retrospective and cross-sectional data are consistent with the chytridiomycosis-driven extirpation of local populations of *R. darwinii* and its sister species, the northern Darwin's frog, *R. rufum* [32]; and (iii) prior to the beginning of this study, neither epidemics nor mass die-offs have been observed in Bd-positive *R. darwinii* populations over 5 years of monitoring [32,33] (A. Valenzuela-Sánchez 2016, unpublished data).

Here, we used data from a 24-month capture–recapture (CR) study covering multiple seasons (i.e. spring, early summer and early autumn) in eight wild populations of *R. darwinii* to estimate demographic and epidemiological parameters. We incorporated these parameters into matrix population models in a way that is analogous to classical compartment disease models (e.g. SIR model) [2,34,35], to predict the long-term dynamics of this apparently stable system. We provide evidence suggesting that disease-induced population extinction is possible in the absence of epidemic dynamics and at relatively low infection probabilities in our study system. In fact, our population model predicted that *R. darwinii* populations are probably in slow decline due to chytridiomycosis and that Bd-infected populations will eventually become extinct. Our findings provide rare empirical support for previous theoretical predictions showing that highly pathogenic parasites can regulate a host population even at extremely low prevalence [2,4].

2. Methods

(a) Model species and study area

Rhinoderma darwinii is a fully terrestrial, forest specialist frog [36]. Its populations are not homogeneously distributed in native forest but clustered in specific sites, with individuals exhibiting high site fidelity and small home ranges [37]. From 2014 to 2016 we surveyed two sites with known presence of *R. darwinii* [31] in each of four geographical areas of Chile (figure 1): (i) Nahuelbuta range (Monumento Natural Contulmo, 'MNC' and Reserva Forestal Contulmo, 'RFC'); (ii) the Andes (Reserva Biológica Huilo Huilo, 'HUI1' and 'HUI2'); (iii) Chiloé Island (Parque Tantauco, 'TAN1' and 'TAN2'); and (iv) Patagonia (Reserva Natural Melimoyu, 'MER1' and 'MER2'). Within an area, the minimum distance between sites was at least 300 m. Considering the short inter-annual movements observed in *R. darwinii* (mean adult displacement between years = 6.3 m) [38] we considered these sites as independent units and call them 'populations' hereafter. At each site, we defined a rectangular plot of different size (electronic supplementary material, table S1) to demarcate each population and in which to conduct our CR study.

(b) Capture–recapture study

We surveyed northern populations (MNC, RFC, HUI1 and HUI2) on seven occasions. Due to the difficulties reaching southern populations (TAN1, TAN2, MER1 and MER2), which were located in remote areas, we surveyed them only on five occasions. Each captured *R. darwinii* individual was measured (snout–vent length, SVL), photographed for individual recognition [31] and skin-swabbed for Bd detection following Soto-Azat *et al.* [32]. Details on searching and handling methodology can be found in the electronic supplementary material. Syntopic anurans were captured opportunistically when seen (electronic supplementary material, table S2); these animals were held in individual, disposable plastic bags until the survey was completed, sampled for Bd detection and then released at the site of capture without being marked.

We defined three age classes for *R. darwinii*: recently metamorphosed frogs (SVL $<$11 mm), juveniles (SVL $\geq$11 to 24 mm, but SVL $\geq$11 to 19.5 for TAN1 and TAN2) and adults (SVL $>$24 mm, but SVL $>$19.5 for TAN1 and TAN2). The smaller adult size used for frogs on Chiloé Island follows observations that these animals are smaller and reach sexual maturity at approximately 19.5 mm SVL [36]. Recently metamorphosed frogs were rarely captured (4.8% of all captures); since their individual ventral markings were not completely developed, we did not include them in our CR analyses.

(c) *Batrachochytrium dendrobatidis* detection

Extraction of DNA from skin swabs and subsequent detection of Bd using a specific real-time PCR assay (qPCR) was done following the methods described by Boyle *et al.* [39] as amended by Soto-Azat *et al.* [32]. We assumed that a Bd-positive swab indicated Bd infection of the swabbed animal. By including known concentrations of Bd DNA in serial diluted control wells on each PCR plate, we were able to quantify infection intensity, which we defined as the number of zoospore equivalents (ZE) per swab. For this, infection intensity was corrected by multiplying the genomic equivalent value obtained from the qPCR assay by 120 (see Hudson *et al.* [40] for further details).

(d) Capture–recapture models

In order to evaluate differences in individual frog survival between Bd-positive and Bd-negative populations, we used Cormack–Jolly–Seber (CJS) models [41]. The Bd status of each population was defined by the qPCR results: if any animal in a population tested positive for Bd at any time during the course of the study, that population was considered Bd-positive. In the CJS and related non-spatial CR models, mortality cannot be disentangled from emigration, and survival probability is considered to be 'apparent' [41]. Emigration could lead to a sub-estimation of true survival probability [41]; however, our estimates of 'apparent' survival probability (ϕ) are likely to be near to the true survival probability because *R. darwinii*

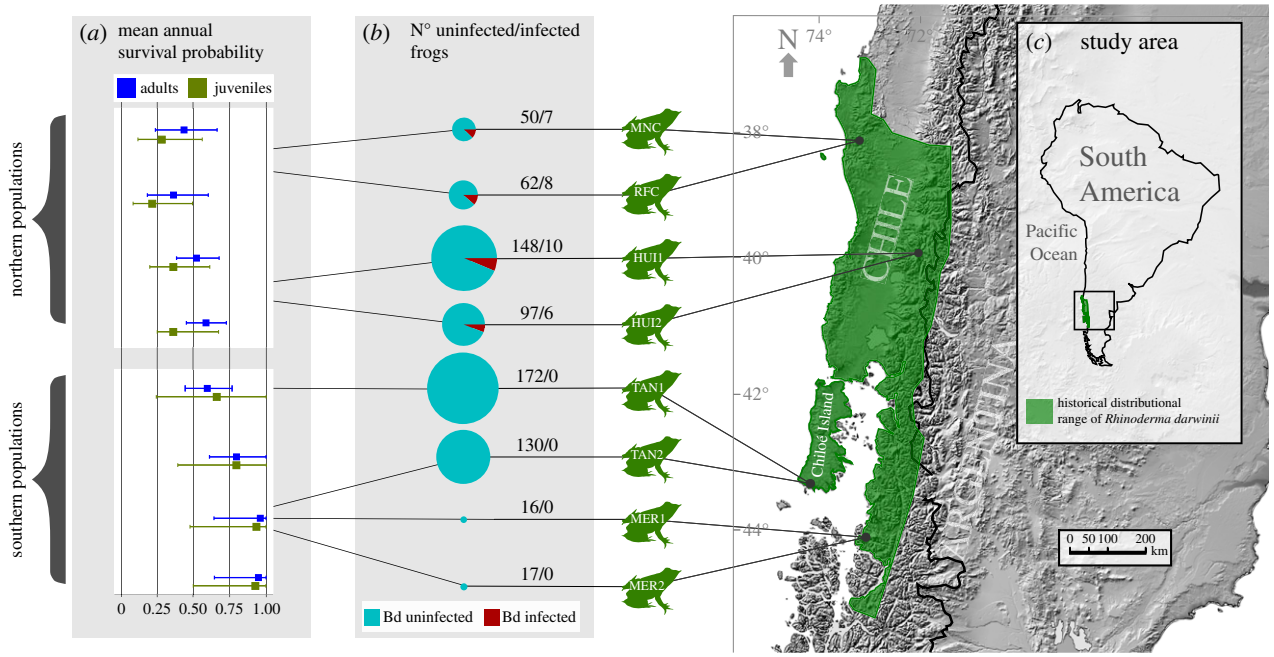

Figure 1. (*a*) Annual apparent survival probability from a Cormack–Jolly–Seber model applied to capture–recapture data from eight wild populations (*c*) of *Rhinoderma darwinii* located in Chile. In (*b*) the proportion of frogs uninfected and infected with the fungus *Batrachochytrium dendrobatidis* is shown. The size of the chart is proportional to the number of frogs captured in each population. Error bars in (*a*) represent the 95% credible interval. (Online version in colour.)

individuals move only short distances between seasons and years, and because each study site was centred on a population that was several times larger than the average home range of individuals [37,38]. The step-by-step process used for CJS model construction and the comparison between Bd-positive and Bd-negative populations are described in the electronic supplementary material. In 'Results' we show a CJS model where ϕ was constrained by age (adult and juveniles) and population.

Subsequently, we used multistate CR (MSMR) models to estimate the effect of Bd infection on ϕ [18,34,40,42,43]. In MSMR modelling, ϕ and recapture probability (p) can be separately estimated for different states [42]. We constructed models with two states according to the observed Bd-infection status of individuals (infected or uninfected). Additionally, individuals may change states between survey periods and therefore transition probability (ψ) can be estimated. We defined the transition from the uninfected to the infected state as 'infection probability' (ψ_{UI}) and the transition from the infected to the uninfected state as 'recovery probability' (ψ_{IU}). The return rate of infected frogs (i.e. percentage of infected frogs in Bd-positive populations that were recaptured at least once during the course of the study) was very low (only two frogs), therefore ψ_{IU} and p of infected frogs (p_I) were unidentifiable parameters with our data [41]. We performed a simulation study to evaluate the likelihood of observing only by chance such a low return rate of infected individuals. To this end, we ran 10 000 simulations where the capture history matrix from Bd-positive populations was held (a total of 388 frogs) but the position of the 31 infections was re-sampled (without replacement) following a flat categorical distribution.

We fitted a first MSMR model to evaluate if the difference in ϕ estimates between Bd-infected (ϕ_I) and uninfected (ϕ_U) frogs was consistent across Bd-positive populations (MSMR model 1). Subsequently, we constructed a model to evaluate any effect of age on ϕ and ψ_{UI} estimates (MSMR model 2; see electronic supplementary material for further details on MSMR model construction). Previous studies on amphibian–Bd systems have found no differences between p of uninfected individuals (p_U) and p_I (*Rana sierra* [21]; *Litoria rheocola* [18]), while for *Leptodactylus fallax* p_U was lower than p_I [40]. Therefore, we constrained the MSMR models in such a way that p_I and p_U were equal. To test the sensitivity of ϕ_I estimates to violations on this assumption, we re-ran the MSMR model 2 several times using a time-constant p_I that ranged from 0.1 to 0.9.

We analysed the CR models in a Bayesian framework, as described by Kéry & Schaub [41], using the package jagsUI in R [44,45], which internally calls and runs the program JAGS [46]. The ϕ and ψ estimates were obtained for the time interval between each survey period for each population, but for simplicity and comparability between populations, all estimates were calculated and are presented as annual probabilities in 'Results' (estimates in the original time intervals are presented in electronic supplementary material, tables S3 and S5). All our estimates are presented as the mean of the posterior distribution of the parameter with a 95% credible interval (CRI). We used vague priors for all parameters [41]. For most models, we ran three chains of 110 000 Markov chain Monte Carlo iterations with a burn-in of 10 000 thinning every 10th observation. Otherwise, MCMC were run as long as was necessary to reach convergence in all parameter estimates, which was evaluated using the Gelman–Rubin $\hat{R}$ statistic (i.e. $\hat{R}$ values <1.1) and by a visual inspection of the chains [41].

(e) Matrix population models

To estimate and compare the asymptotic population growth rate (λ) and the extinction risk in Bd-positive and Bd-negative populations, we developed deterministic state-structured matrix population models [34,35,47]. In our models, individuals change between states and reproduce in discrete 1-year time steps and the population is sampled just after breeding (i.e. post-breeding census) [47]. Six states were defined based on a combination of Bd-status (uninfected and infected frogs) and age class (1-year-old juveniles, 2-year-old juveniles and adults). A detailed explanation on the criteria used to classify animals by age, model parameters and full model structure, is shown in the electronic supplementary material.

We constructed two matrix population models to represent different epidemiological scenarios. Population model 1 was constructed to represent an average Bd-positive population. Therefore, this model had all above described states and was parameterized with averaged demographic and epidemiological parameters estimated with the MSMR model 2 in the Bd-positive

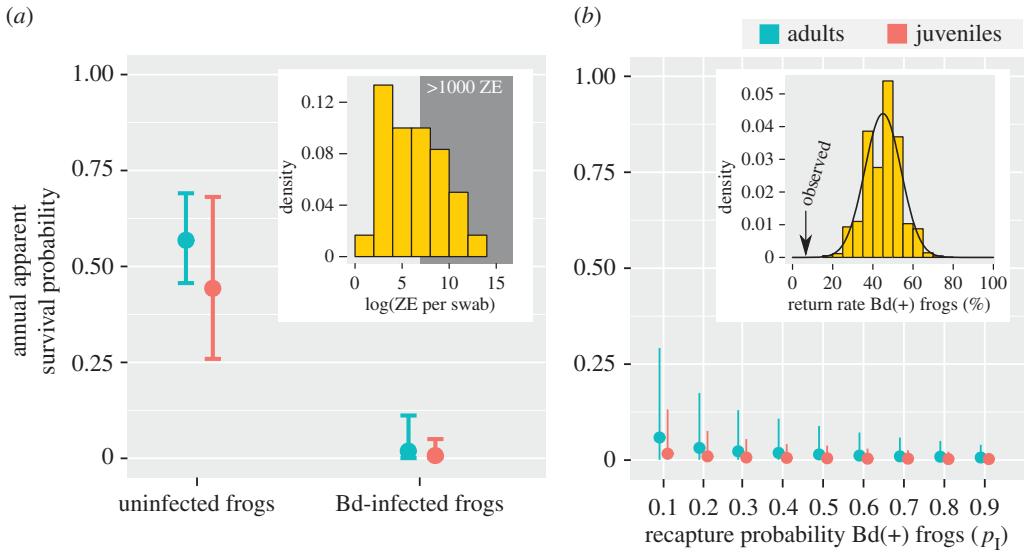

Figure 2. (*a*) Annual apparent survival probability of uninfected and Bd-infected frogs from four wild populations of *Rhinoderma darwinii* and infection intensity (zoospores equivalents per swab) of infected frogs (inset). In (*b*) we show the survival probability of Bd-infected frogs at different recapture probabilities and the distribution of the observed and simulated return rate of the 30 Bd-infected frogs (inset). Error bars represent the 95% credible interval of the posterior distribution of the parameter estimated using Markov chain Monte Carlo in a multi-state capture–recapture model. (Online version in colour.)

populations. Population model 2 was constructed to represent an average Bd-negative population, thus this model had only uninfected states and was parameterized with averaged demographic parameters estimated with the CJS model in the Bd-negative populations (electronic supplementary material, CJS model 3). Additionally, we made an *ad hoc* split of Bd-negative populations in order to illustrate that the λ and the population size projection produced by population model 2 were largely influenced by ϕ estimates coming from a single Bd-negative population (i.e. TAN1). To this end, we constructed population model 3, which also represents a Bd-negative population and had the same structure as population model 2, but was parameterized with averaged demographic parameters from TAN2, MER1 and MER2 only (electronic supplementary material, CJS model 4).

Under our study design, ψ_{UI} and ϕ_U could be underestimated because an unknown proportion of Bd infections are not detected if disease-induced mortality takes place in a period of time shorter than three months (i.e. both infection and mortality occurred between study visits). Assuming that the differences between mean ϕ_U from individuals at Bd-negative and Bd-positive populations (estimated using CJS models) are only due to unobserved disease-induced deaths, we can use our fully parameterized matrix population model (population model 1) to provide corrected ψ_{UI} estimates. With this single purpose we ran population model 4. This model has the same states and parameter values as population model 1, but two modifications were made. First, we used the highest ϕ_U values (i.e. from TAN2-MER1-MER2). Second, we tested different ψ_{UI} values and checked which of them led to the same mean λ that was observed with the population model 1.

To include uncertainty of our parameter estimates in model outputs, we ran 10 000 simulations of each matrix population model. In each simulation the parameter values were randomly sampled from different probabilistic functions fitted with parameter values presented in the electronic supplementary material, table S7. We started each simulation with a Bd-free population and ran the model for a 30-year period. We calculated λ from models 1–4 during each simulation using eigenanalysis, as described by Stevens [48]. Further details on model structure, parameter estimation and simulation setting are provided in the electronic supplementary material.

3. Results

(a) Captures

We made a total of 1182 captures of 723 different frogs (figure 1*b*). Of these, 284 (39.3%) were recaptured at least once across survey periods. Adults presented a slightly, but consistently, higher number of recaptures than juveniles (electronic supplementary material, figures S1 and S2). Despite the large number of individuals captured, we did not observe dead frogs or individuals with abnormal behaviour, nor other possible signs of chytridiomycosis during the course of this study.

(b) Bd-infected frogs and infection intensity

Only the four northern populations were found to be Bd-positive (i.e. at least one positive sample during the study; figure 1*b*). Of the 338 individual frogs captured in these populations, only 8.9% were positive for Bd at least once (30 individuals and one re-infection). The return rate of infected frogs was 6.6%; lower than would be expected by chance (95% CI = 29.0%–64.5%; figure 2*b*). Only two frogs, both having low infection intensities (10 and 13 ZE per swab), were recaptured as uninfected. One of these frogs subsequently gained a heavy infection (27 649 ZE) and was never captured again.

The infection intensity ranged from 3 to 326 786 ZE per swab (mean = 15 498, bootstrapped 95% CI = 2810–38 719; median = 365, bootstrapped 95% CI = 71–1380). Of infected frogs, 36.7% had more than 1000 ZE per swab at least once. The distribution of log-transformed ZE per swab was bell-shaped (figure 2*a*), slightly right skewed (bootstrapped skewness = 0.26) with a variance to mean ratio of 1.45.

Eighty-six individuals of six other amphibian species were captured from seven sites; no syntopic amphibians were found at site HUI1 (electronic supplementary material, table S2). One syntopic anuran species (*Eupsophus contulmoensis*) was positive for Bd. This species was found only in the two most-northern sites of MNC and RFC, where 6 of 59 (10.2%) animals tested positive for Bd, but with a low infection intensity (mean = 287 ZE, bootstrapped 95%

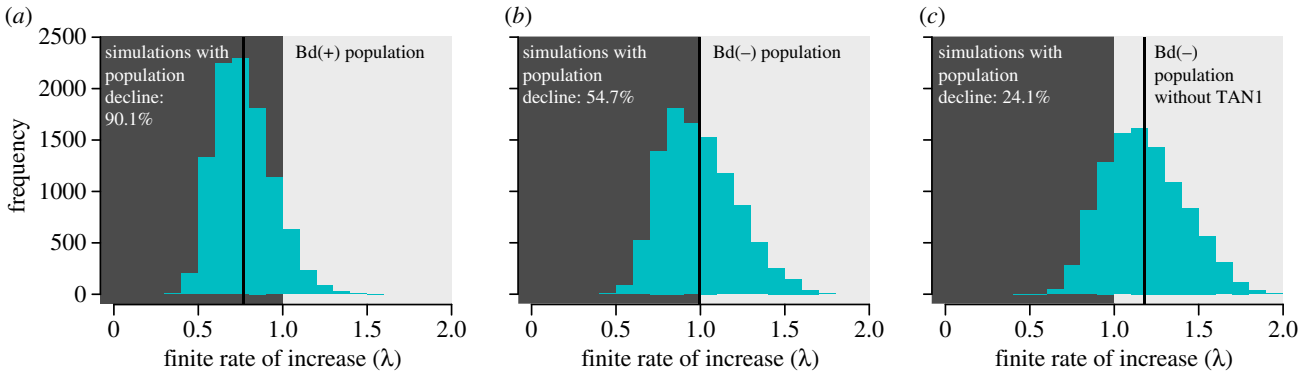

Figure 3. Distribution of the finite rate of increase (λ) for 10 000 simulations of three matrix population models of *Rhinoderma darwinii*. The Bd-positive population model (*a*) uses parameters estimates obtained from a multi-state capture–recapture model of four wild populations, while the Bd-negative models use parameters estimates obtained from Cormack–Jolly–Seber models of another four (*b*) or three (*c*) Bd-negative wild populations. The black lines represent the mean. The darker grey area represents simulation with population decline (i.e. $\lambda < 1$). (Online version in colour.)

CI = 43–624; median = 71 ZE, bootstrapped 95% CI = 21–769; electronic supplementary material, table S2).

(c) Capture–recapture models

The CJS models showed that adult mean annual ϕ was, on average, lower in Bd-positive than in Bd-negative populations (mean difference = −0.354, 95% CRI = −0.506 to −0.173), although it was similar between the Bd-positive populations and TAN1 (figure 1*a*; electronic supplementary material, table S4). The same pattern was observed for juveniles (mean difference = −0.397, 95% CRI = −0.560 to −0.196), but with considerably larger credible intervals for ϕ estimates from Bd-negative populations (figure 1*a*).

The MSMR models showed that ϕ was considerably lower in infected frogs in comparison with uninfected frogs (figure 2*a*). This difference was consistent across all study populations (electronic supplementary material, figure S3*a*) and between age classes (figure 2*a*). Infected adults and juveniles were 55.0% and 43.5% less likely to survive 1 year than uninfected adults and juveniles, respectively (figure 2*a*). The difference between ϕ_U and ϕ_I was consistent across all the tested p_I values (figure 2*b*).

Annual ψ_{UI} was higher in juveniles than in adults (26.8% [95% CRI = 9.1–52.5%] versus 14.7% [95% CRI = 7.2–25.2%]), and was similar across all Bd-positive populations (electronic supplementary material, figure S3*b*).

(d) Matrix population models

Matrix population models suggest that Bd infection has a profound impact at the population level (figure 3): mean λ in the Bd-positive population model (model 1) was 0.77 (95% CI = 0.51–1.13), while in the Bd-negative population model (models 2) it was 0.99 (95% CI = 0.648–1.473). The percentage of simulations with population decline (i.e. $\lambda < 1$) dropped from 90.1% to 54.7% between the Bd-positive and the Bd-negative population models (figure 3). The mean λ in the Bd-negative population model 2 was close to stability, but it was largely influenced by survival rates in the Bd-negative population TAN1. Excluding this population from the analysis (i.e. population model 3) results in a λ of 1.18 (95% CI = 0.78–1.66) and a percentage of simulations with population decline of 24.1%. Median extinction time (i.e. fewer than two frogs) was predicted at year 17 in model 1, while the other populations (model 2 and 3) did not go extinct during the 30-year period (figure 4).

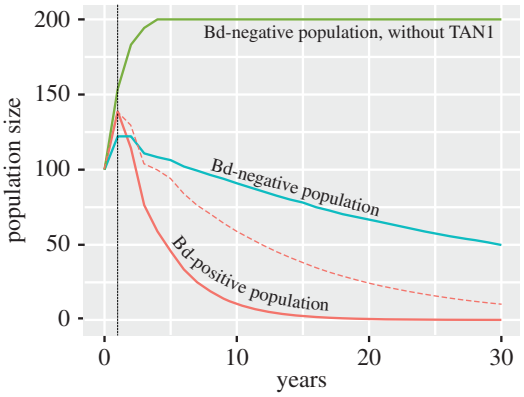

Figure 4. Predicted variation in the size (median from 10 000 simulations) of a *Rhinoderma darwinii* population using matrix population models. The projections are shown for Bd-positive and Bd-negative populations. The dashed black line represents the year of the introduction of Bd (only for the Bd-positive population model). It is worthwhile noting that if Bd is not introduced to the Bd-positive population (i.e. $\psi_{UI} = 0$; dashed red line), this population is still predicted to decrease, but at a much slower rate (mean $\lambda = 0.93$, 95% CI = 0.61–1.36). (Online version in colour.)

Model 4 indicated that a onefold increase in the estimated annual ψ_{UI} led to an equal mean λ as observed in model 1; therefore, the corrected annual ψ_{UI} was 53.6% for juveniles and 29.4% for adults.

4. Discussion

As in other wildlife populations threatened by infectious diseases (e.g. [11–13,43]), well-documented amphibian population declines due to chytridiomycosis have been characterized by the occurrence of disease outbreaks and mass mortalities following pathogen introduction into naive populations [14–17]. With such epidemics, the pathogen obviously threatens population survival. A less evident pattern of disease-induced population decline was predicted by Anderson [2]: if the probability of disease-induced mortality is sufficiently high when infection occurs, a parasite can regulate a host population even at extremely low prevalence. Empirical evidence supporting this important theoretical prediction, however, has remained largely elusive [4]. Our current study provides empirical support to this prediction. Our results suggest that *R. darwinii* populations are unlikely to persist where Bd infection is endemic, even in

the absence of mass mortalities and where infection prevalence is low. At current fecundity, infection and survival probabilities, our matrix population models predicted slow population decrease and eventual extirpation of Bd-positive populations in most of the simulations (figures 3 and 4).

Less obvious than epidemics and rapid local population extirpations, a slow population decline might go unnoticed in short-term studies or might be attributed to other causes (such as a change in climatic conditions) [49]. Low infection probability (i.e. parasite transmission) could be a characteristic of some terrestrial amphibian–Bd systems, preventing the occurrence of epidemic spread and mass mortalities, even if Bd-induced population declines are occurring. For instance, in this study we found a lower Bd infection probability in *R. darwinii* compared with that observed in other amphibian species (e.g. [18,21,40,50]), even when correcting for an assumed underestimation due to our sampling design. This might have implications for current understanding of the biological and environmental factors that predict host susceptibility to Bd, such as the findings that amphibian species with an aquatic life-stage are more likely to suffer Bd-induced population declines than terrestrial species [7,10].

The low mean survival probability observed at Bd-positive populations suggests that Bd-induced mortality is not compensatory to other natural causes of mortality in our model species. Being aware that many extrinsic and intrinsic factors can drive amphibian population dynamics, and that we have not quantified such effects, we propose lines of evidence that support our conclusion that Bd infection is the main reason for the observed difference in mean survival probability between Bd-positive and most Bd-negative populations. First, we detected a strong negative effect of Bd infection on individual survival (figure 2*a*), this effect being consistent across age classes and Bd-positive populations, and at a wide range of p_I values. Second, even though climatic conditions in TAN2, MER1 and MER2 populations differ considerably [36], mean survival estimates for frogs in each of these Bd-negative populations were similar. Third, population size in fully terrestrial amphibians shows a relatively low temporal variance, probably due to a relatively low environmental stochasticity [51], adding support to our suggestion that it is unlikely that the observed differences in survival probabilities were associated with environmental differences across sites. Finally, our results are consistent with retrospective and cross-sectional evidence suggesting chytridiomycosis as an explanation for the documented recent extirpation of several northern populations of *R. darwinii*, particularly those within protected areas where other recognized threats (habitat loss/degradation, pollution, over-extraction) are unlikely to operate [31,32]. The lower mean survival probability estimated for frogs in TAN1 compared to that observed in the other Bd-negative populations (figure 1*a*) could be due to density-dependent mortality and/or dispersal. At the beginning of this study, this population had the largest known population density of *R. darwinii*, which was around 10 times higher than the population density observed in most of the study populations (electronic supplementary material, table S1). Our ongoing spatial capture–recapture work suggests the presence of density-dependent mortality in this species (A. Valenzuela-Sánchez 2016, unpublished data); long-term time-series data might be necessary to confirm this hypothesis.

In other amphibian–Bd systems, the survival of Bd-infected individuals is also lower than the survival of uninfected frogs [19,40], including in a population of *Litoria pearsoniana* that had coexisted with the parasite for approximately 30 years [50]. In some cases, however, individuals from species that have been documented as being highly susceptible to Bd can develop a decreased susceptibility when their populations have been infected with the parasite for a few decades [18,52]. Although Bd was probably introduced into Chile in the 1970s [32,38], our results suggest that *R. darwinii* individuals from all the studied Bd-positive populations are highly susceptibility to chytridiomycosis. The time of Bd introduction into these populations, however, is unknown and we cannot discard the possibility of a gradual, long-term decrease in host susceptibility in our study species.

When parasite-induced mortality occurs in a parasite-load-dependent fashion, parasite aggregation (i.e. a small proportion of hosts holding high parasite burdens while most hosts have low burdens) may allow populations of susceptible hosts to persist in the face of recurrent infection [2,21,26]. It is worth noting, however, that even under the presence of strong parasite aggregation, theory predicts that a parasite can regulate a host population if the turnover of heavily infected hosts occurs at high rates [2]. Our field data do not allow us to discern between individuals that are in an early infective stage from those holding long-lasting, low parasite burdens. This situation limits our capability to draw strong conclusions from the distribution of parasite burdens in the host population. Even though we were not able to incorporate parasite load as a covariate in our modelling (three-month infection probability and survival probability of infected frogs were very close to zero, therefore, the effect of any covariate cannot be modelled reasonably), our results suggest that, regardless of parasite burden, case mortality is extremely high in our model host–parasite system.

An additional mechanism that may allow host populations to persist with endemic parasitic infection, even though the parasite has detrimental effects on host survival, is compensatory recruitment. This population response to disease has been observed in the badger–*Mycobacterium bovis* system [29] as well as in the amphibian–Bd system [24,25]. In *R. darwinii*, paternal care may inhibit the occurrence of compensatory recruitment, as offspring are carried internally by adult males until metamorphosis and, therefore, Bd-induced death in brooding males also leads to the death of developing offspring. The small egg clutch size in *R. darwinii* [37], along with the limited number of larvae that each male is able to brood, probably decrease further the species's ability to offset chytridiomycosis-induced mortality through compensatory recruitment. Additionally, our results indicate that over a half of *R. darwinii* juveniles in Bd-positive populations become infected and die from chytridiomycosis before reaching adulthood.

Parasite transmission is a central component of the host–parasite interaction [28]. The mode of Bd transmission in *R. darwinii* is unknown and should be studied in more detail, but, as this species lives in moist vegetation and substrate, indirect transmission is possible as well as transmission via direct contact with conspecifics and syntopic amphibian species [53,54]. We used a constant infection probability in our modelling, and therefore other models of parasite transmission such as density-dependent or frequency-dependent were not considered here. This choice is

supported by three lines of evidence. First, parasite transmission is low, and *R. darwinii* individuals are highly sedentary and commonly live at low densities [37,38], suggesting that intra-specific transmission is unlikely to be important for Bd transmission and persistence within local populations. Additionally, the low survival rate of infected *R. darwinii* individuals further decreases the chances of intra-specific transmission [1]. Second, we found a similar infection probability across the four Bd-positive populations, which have different population sizes and densities. Third, reservoir hosts may lead to constant infection probability. In our system, syntopic species are probably important for the introduction and maintenance of Bd within *R. darwinii* populations. As with previous reports [32,33], we detected Bd only in northern populations of *R. darwinii*. This is coincident with a spatial pattern of a markedly higher Bd prevalence in sympatric anurans towards the northern distribution of the range of *R. darwinii* [32]. For instance, *E. contulmoensis* could be acting as a Bd reservoir in our study system. As for most anurans syntopic to *R. darwinii*, *E. contulmoensis* individuals have a higher vagility than our model species and use both aquatic (where individuals can easily come into contact with the infective stage of Bd) and terrestrial (where individuals overlap spatially and temporally with *R. darwinii* individuals) environments. The role of syntopic species in the transmission of Bd to *R. darwinii* requires further investigation, as management interventions, such as exclosures, to limit interspecific contact might be a feasible (short-term) mitigation measure for the conservation of *R. darwinii*, especially as discrete local populations of this species exist within small, manageable areas.

5. Conclusion

Epidemics and mass die-offs are tacitly or explicitly assumed as a pre-requisite for the occurrence of disease-induced extirpation, even though theory predicts that a parasite with extremely low prevalence can regulate host populations if case mortality is sufficiently high [2]. We showed, with empirical evidence, that a cryptic pattern of disease-induced host population decline is an alternative route to population extirpation. Our findings challenge the way we conceive pathogen threats to host populations and show that disease should be investigated as a cause of population regulation even in the absence of an overt increase in mortality.

Ethics. This research project was approved by the Animal Welfare Committee at the Universidad Andrés Bello, Chile (no. 13/2015) and by the Zoological Society of London's Ethics Committee (WLE709), and was conducted in accordance with Chilean law under permits no. 5666/2013, no. 230/2015 and no. 212/2016 of the Servicio Agrícola y Ganadero de Chile, and no. 026/2013 and no. 11/2015 IX of the Corporación Nacional Forestal de Chile.

Data accessibility. Complete dataset supporting our results is available at https://doi.org/10.5281/zenodo.583629.

Authors' contributions. A.V.-S., A.A.C. and C.S.-A. conceived the study. A.V.-S., B.R.S., A.A.C. and C.S.-A. formulated the ideas. A.V.-S., D.E.U.-R. and F.C. performed fieldwork. A.V.-S. and B.R.S. analysed samples and data. A.V.-S. wrote the first draft of the manuscript, and all authors contributed to revisions.

Competing interests. We declare we have no competing interests.

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Monitoreo a largo plazo de poblaciones de Ranita de Darwin (*Rhinoderma darwinii*) en el Monumento Natural Contulmo y Reserva Forestal Contulmo

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