EL ROL DE LAS SEÑALES ACÚSTICAS EN LAS INTERACCIONES SEXUALES Y LA ESTRUCTURA SOCIAL DE LA RANITA DE DARWIN (*RHINODERMA DARWINII*)

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A mi mama y a mi papá
A Leonardo Varas

Decía que desovan las iguanas… Es el proceso. Ellas
(o las ranas) en el silencioso carbonífero
        emitieron el primer sonido
    la primera canción de amor sobre la tierra,
    la primera canción de amor bajo la luna
        es el proceso
El proceso viene desde los astros.
    Nuevas relaciones de producción: eso
también es el proceso. Opresión. Tras la opresión, la liberación.
La Revolución empezó en las estrellas, a millones
    de años luz. El huevo de la vida
        es uno. Desde
el primer huevo de gas, al huevo de iguana, al hombre nuevo.
Sandino se gloriaba de haber nacido del “vientre de los oprimidos”
        (El de una indita de Niquinohomo).
Del vientre de los oprimidos nacerá la Revolución
Es el proceso.

Fragmento de “Canto nacional” de Ernesto Cardenal

Nací en la ciudad de México, donde parte de mi infancia la viví al lado del Parque Nacional Los Dinamos, en una orilla de ese parque mi abuelita construyó una casa donde quería vivir sus últimos días. Nunca lo pudo hacer, en cambio yo viví dos años y ahí fue que supe que quería ser biólogo y trabajar en un bosque. Después de haber vivido siete años en el puerto de Mazatlán, estudié Biología en la Universidad Autónoma Metropolitana - Xochimilco (UAM-X), aunque había sido aceptado en la Universidad Nacional Autónoma de México (UNAM) para estudiar la misma carrera, me interesó mucho el enfoque del programa de estudios de la UAM-X. Ahí tomé clases con la Dra. Virginia Graue Wilchiers en los meses en que organizó un curso internacional de conservación de anfibios en 2003. Ahí supe que quería dedicarme al estudio de los anfibios. Después, hice dos veranos científicos en el laboratorio de Reproducción animal de la UNAM y me entusiasmé por estudiar la reproducción en anfibios, aunque fue difícil encontrar un lugar donde poder hacerlo. Volví a Mazatlán para hacer mi tesis en Mazatlán e hice una tesis de escritorio analizando por medio de un SIG la distribución de anfibios en el Estado de Sinaloa. Ahí supe que quería estudiar al género *Eleutherodactylus*, por su forma de reproducción terrestre y su vida independiente al agua. En 2006, entré a la Maestría en el Instituto de Ecología A.C., con el apoyo del Dr. Gonzalo Halffter iba a estudiar la anidación de una *Eleutherodactylus* pero una serendipia me llevó a descubrir que las hembras de la especie cantaban muy similar a los machos. Ahí comencé a estudiar los cantos de las ranas. En 2008 fui a dos cursos organizados por la Organización de Estudios Tropicales en Costa Rica, ahí comencé a formarme como ecólogo y herpetólogo, con profesores como Alejandro Farji, Federico Chinchilla, William Eberhardt y Maureen Donnelly. Entre 2010 y 2012 hice clases de Ecología, ecología de la conducta y biología en la UNAM y la UAM-X. En el mismo año de 2010 hice una pasantía estudiando ranas de cristal en el Smithsonian Tropical Research Institute en Panamá con la guía de Rachel Page, Mike Ryan y Karen Warkentin. Ahí conocí al chileno Daniel Zúñiga que me sugirió venir a Chile a hacer el doctorado". Así llegué al laboratorio de Mario Penna y comencé a conocer la bioacústica en su forma más esencial y experimental. En la Universidad de Chile, realizando esta tesis, conocí el valor de la colaboración para la investigación científica y pude coordinar el curso de Tópicos de bioacústica durante varios semestres junto a mis colegas del laboratorio de Mario Penna.

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RESUMEN

El presente estudio se propuso describir el sistema de comunicación en la ranita de Darwin (*Rhinoderma darwinii*), una especie en que los machos realizan cuidado parental incubando las larvas en el interior de su saco vocal. Observaciones naturalistas habían sugerido que la vocalización de los machos adultos consiste en cantos tonales de múltiples notas, que son emitidos para atraer la atención de las hembras, como en la mayoría de los anuros. En cautiverio las hembras han sido observadas emitiendo cantos de una sola nota durante el apareamiento. Frente a este fenómeno surge la interrogante de si los cantos de machos y hembras de *R. darwinii* son dimórficos en sus características bioacústicas y de qué manera son usados como señales de reconocimiento sexual en la interacción social de esta especie. Para este propósito se caracterizó la variabilidad de los cantos de machos de la especie usando grabaciones realizadas en distintas poblaciones. Posteriormente, comparamos las propiedades acústicas y la ocurrencia de cantos de anuncios de hembras y machos preñados y no preñados desde noviembre de 2015 hasta febrero de 2016 en la isla de Chiloé, Chile, además registramos duetos naturales entre los sexos y evaluamos su respuesta vocal evocada a estímulos de playback que consistían en cantos de cada estatus sexual. La tasa de cantos, los ángulos de fase, el nivel de presión de sonido, el número de cantos superpuestos y el retraso de los cantos superpuestos se midieron para determinar las respuestas diferenciales entre los duetos naturales y en los experimentos de playback. Finalmente, empleamos el análisis de redes sociales para evaluar la influencia de la similitud de cantos

y la superposición del ámbito de hogar de *R. darwinii*. Encontramos que, la variación del canto de los machos entre poblaciones está relacionado con diferencias en el tamaño de longitud de los emisores y que los cantos son altamente variables a nivel individual. Además de los machos no reproductivos, las hembras y los machos que están preñados (incubando larvas) producen vocalizaciones en contexto de anuncio sexual (en solitario o en alternancia con conespecíficos) emitiendo un repertorio similar de una a seis notas, siendo más comúnmente emitido el canto de cuatro notas por los tres estatus sexuales. Además, los cantos de machos con distinto estatus reproductivo no difirieron en sus características acústicas, pero los cantos de las hembras difirieron por tener notas y cantos de mayor duración y una frecuencia dominante menor que la de los machos. En registros de duetos naturales, encontramos que los machos interactúan más frecuentemente con otros machos y las hembras pueden vocalizar en interacciones con machos, pero en ninguna ocasión fueron observadas interacciones vocales entre hembras. En los experimentos de respuesta evocada a playback encontramos que machos y hembras mantienen una tasa de canto similar ante los estímulos de los tres estatus sexuales. Sin embargo, encontramos que las hembras responden con menor presión de sonido frente a los estímulos de cantos de hembras y con mayor ángulo de fase frente al estímulo de machos preñados, en tanto que los machos preñados emitieron menor número de cantos superpuestos frente a estímulos de cantos de hembras. Además, encontramos que el solapamiento de cantos es una forma de interacción intrasexual que realizan machos preñados y hembras probablemente como un mecanismo de competencia vocal no agresiva. En la población estudiada en Chiloé, la rana de Darwin utiliza ámbitos de hogar estrechamente asociados con parches de musgo. Evaluando posibles factores que expliquen la estructura social, encontramos que ésta no está relacionada con la similitud de los cantos y el solapamiento de los ámbitos de hogar, sin embargo, utilizando datos empíricos sobre la probabilidad de movimiento entre parches de musgo de machos preñados, machos no preñados y hembras, encontramos mediante un modelo de redes que la estructura social es ligeramente afectada por estos movimientos en grupos de ~30 individuos. Las evidencias mostradas en este estudio respaldan la hipótesis de que ambos sexos pueden reconocer al otro sexo pese a las mínimas diferencias que existen entre los cantos de machos y hembras. Incluso, aunque nuestros análisis no mostraron diferencias entre las características de los cantos de machos preñados y no preñados, las hembras respondieron diferencialmente hacia los machos preñados. Esto sugiere que hay características en el canto de los machos con distinto estatus reproductivo que nuestro estudio no alcanzó a detectar. En conjunto, nuestros datos sugieren que los cantos permiten regular las interacciones sociales de la especie.

ABSTRACT

The present study aimed to describe the communication system in the Darwin frog (*Rhinoderma darwinii*), a species in which the males perform parental care incubating the larvae inside their vocal sac. Naturalistic observations had suggested that vocalization of adult males consists of tonal calls of multiple notes, which are emitted to attract the attention of females, as in most anurans. In captivity, females have been observed emitting calls of a single note during mating. An unresolved question around this phenomenon is whether male and female calls of *R. darwinii* are dimorphic in their bioacoustic characteristics and in the extent to which they are used as signals for sexual recognition in social interactions. To contribute to clarify these issues, the variability of male calls of the species was characterized using recordings conducted in different populations. Subsequently, we compared the acoustic properties and the occurrence of vocalization of pregnant and non-pregnant males and females from November 2015 to February 2016 on the island of Chiloé, Chile. We also recorded natural vocal duets and evaluated individual evoked vocal responses to playback stimuli that consisted of calls of each sexual status. Call rate, phase angles, sound pressure level, number of overlapping calls and overlapping delay of calls were measured to determine the differential responses in natural duets and in the playback experiments. Finally, we used social network analysis to evaluate the relationship among call similarity of individuals and the home range overlap of *R. darwinii*. We found that call variation of non-pregnant male calls between populations is related to differences in body size of call emitters and that their calls are highly variable at the

individual level. In addition to non-pregnant males, females and pregnant males (males incubating larvae) produce vocalizations in the context of sexual advertisement alone or in alternation with conspecifics, having a similar repertoire of one to six notes, four-note calls being more commonly issued the by the three sexual status. In addition, calls of males with different reproductive status did not differ in their acoustic characteristics, but calls of females differed by having longer notes and calls duration, and a lower dominant frequency than that of both types of males. In recordings of natural duets, we found that males interact more frequently with other males than with females, but we did not observe vocal interactions among females. In the experiments of evoked responses to playbacks we found that males and females maintain a similar call rate in response to stimuli of the three sexual status. However, females respond with lower sound pressure level to the female stimuli and with a greater phase angle as compared to pregnant male stimuli, while pregnant males emitted a smaller number of overlapping calls in response to female stimuli. In addition, we propose that call overlap is a form of intra-sexual interaction performed by males and females in response to stimuli of their own sex probably as a non-aggressive vocal competition mechanism. In the population studied in Chiloé, we found that call similarity of the Darwin's frogs was not related to home range overlap. Using empirical data on the probability of movement of individuals between moss patches, we find through a network model that the social structure is slightly affected by larger individual's movements between groups. Overall, our evidence supports the hypothesis that both sexes can recognize the other sex despite the minimal differences between male and female calls. Even though our analyses showed no differences between call characteristics of pregnant and non-pregnant males, females responded differentially to pregnant male stimuli. This suggests that there are characteristics in male calls that probably signalize the different sexual status of males that remained undetected in our study. Altogether, our data suggest that the calls allow to regulate social interactions in this frog species.

INTRODUCCIÓN GENERAL

En el reino animal, las señales de comunicación producidas por machos y hembras son por lo general sexualmente dimórficas pero también pueden tener un carácter monomórfico. Desde la perspectiva de la teoría de la selección sexual, los caracteres sexuales secundarios se desarrollan como ornamentos o señales elaborados en los machos que atraen parejas e incrementan su competitividad; en tanto que las hembras, que normalmente hacen mayor inversión de cuidado de las crías, carecen de adornos o no producen señales sexuales y generalmente son el sexo limitante que elige a la pareja basado en los caracteres de los machos (Anderson 1994; Clutton-Brock 2004; Snowdon 2004).

La predominancia del dimorfismo sexual como condición para que se manifieste la selección sexual se basa en modelos teóricos generados durante el siglo XX (Kokko y Jennions 2008; Roughgarden 2015). Distintos autores examinaron cómo la selección sexual ha promovido el dimorfismo sexual que caracterizan los sistemas de reproducción poligínicos, suscitando una gran cantidad de estudios empíricos en una amplia variedad de organismos (véase Anderson 1994). Sin embargo, menor atención ha recibido las evidencias tanto empíricas (e.g. Trail 1990) como teóricas (Kokko y Johnstone 2002) de que la selección sexual no requiere necesariamente estar asociada a dimorfismo sexual, pese a que las señales monomórficas constituyen un fenómeno relevante dado el número de especies que las presenta y por las consecuencias de esta uniformidad en el ámbito evolutivo (Wallace 1889).

Uno de los modelos teóricos que respaldan la prevalencia del dimorfismo sexual en varios ámbitos, incluyendo la inversión de cuidado parental que es realizado predominantemente por las hembras, ha sido explicado durante décadas a partir del paradigma de Bateman (Kokko y Jennions 2008; Roughgarden 2015). Este paradigma sostiene que hembras y machos son más y menos selectivos, respectivamente, gracias a que sus probabilidades de éxito reproductivo difieren debido al tipo de gametos que producen con costos energéticos contrastantes. Así, los machos que producen gametos con bajo costo maximizan su éxito reproductivo a través de la competencia intra sexual diseminando gametos pequeños entre el mayor número de hembras posible, en tanto que las hembras maximizan su éxito reproductivo permitiendo que machos competitivos las fecunden y realizando el cuidado de las crías para asegurar el éxito de su inversión en gametos y en su elección (Bateman 1948). Desde esta perspectiva, el cuidado proporcionado exclusivamente por los machos sería un fenómeno improbable en todas las especies con gametos sexualmente dimórficos. Sin embargo, Emlen y Oring (1977) propusieron que alternativamente el cuidado paternal puede presentarse cuando los machos son el sexo limitante a nivel poblacional (proporción sexual operativa sesgada hacia los machos) y estos eligen a las hembras que compiten por ser elegidas y recibir el cuidado paterno. A esta reversión del rol en el cuidado de las crías se le conoce como reversión del rol sexual y se observa claramente en los peces de la familia Syngnathidae (e.g. Miranda et al. 2017).

De acuerdo con un análisis realizado entre diferentes taxones, para revisar la ocurrencia del paradigma de Bateman, que establece que la varianza en el éxito reproductivo es mayor en los machos que en las hembras, se encontró que el rol sexual revertido constituye una excepción (Janicke et al. 2016). A diferencia de la teoría Darwinista, la hipótesis del conflicto entre los sexos ha sido postulada a partir de la modelación matemática de las consecuencias de la anisogamia (diferencia en el tamaño de gametos), desarrollándose a partir de supuestos teóricos y no de observaciones sobre la historia natural de las especies (Roughgarden 2015). En este escenario, la teoría de la selección sexual clásica no proporciona explicaciones claras respecto a la evolución de ornamentos masculinos y femeninos que son usados como señales de comunicación en distintos tipos de interacciones sociales, como los conflictos entre hembras (en especies con rol sexual revertido), conflictos entre crías, la protección de recursos por parte de sujetos reproductivos y no reproductivos y las interacciones interespecíficas no depredatorias (West-Eberhard 1979, 1983; Clutton-Brock 2009; Lyon y Montgomerie 2012; Tobias et al. 2012; Clutton-Brock et al. 2013). Consecuentemente, ante la insuficiencia de la teoría de la selección sexual para dar cuenta de la falta de dimorfismo sexual en las señales producidas por ambos sexos, la selección social ofrecería una explicación teórica y empírica para asociar al monomorfismo con conflictos de grupos sociales de distinta índole (West-Eberhard 1983, 2014; Lyon y Montgomerie 2012; Tobias et al. 2012).

Sin embargo, la selección sexual mutua implica otras alternativas para comprender el monomorfismo sexual de señales (Trail 1990). Desarrollando un modelo matemático, Kokko y Johnstone (2002) sugirieron que la ocurrencia del cuidado parental depende de las necesidades de las crías y por lo tanto, la inversión de cuidado parental es el primer determinante del rol sexual en lugar de la proporción sexual propuesta por Emlen y Oring (1977). Además, el modelo demostró que los factores que promueven la selectividad son especie-específicos e incluyen la tasa de encuentro de parejas, el costo de crianza, el costo de búsqueda de pareja y la calidad de la pareja del sexo opuesto. Además, la aproximación del modelo de Kokko y Johnstone, indica que la elección mutua es improbable en especies que no realizan cuidado parental y sugiere que la coevolución del cuidado parental y la estrategia del apareamiento oculta la elección mutua dado que el aumento de la selectividad de un sexo reducirá la tasa de apareamiento del sexo opuesto, volviendo menos selectivo y más competitivo al sexo elegido. Distintas evidencias empíricas en aves han mostrado que el surgimiento de señales monomórficas es frecuente y se mantiene aún si la selección mutua ocurre de manera desigual entre sexos (Trail 1990; Irwin 1994; Odom et al. 2014; Cain y Langmore 2015).

A diferencia de otros taxa, los anuros han sido aparentemente mantenidos fuera de esta controversia teórica sobre el rol de las señales femeninas, aún cuando las señales sexuales de anuros constituyen un modelo de comunicación recurrente en estudios sobre elección de pareja por parte de las hembras y desarrollo de señales complejas entre machos que compiten (e.g. Ryan 1997). Esta exclusión ha ocurrido quizás porque en menos del 1% de las especies conocidas las hembras vocalizan (Emerson y Boyd 1999; Serrano y Penna 2018). Además de ello, la producción y percepción de señales fuera del ámbito sexual, por ejemplo, en individuos sexualmente inmaduros, ha sido escasamente estudiada (ten Hagen et al. 2016; Lea et al. 2002). Probablemente la primera descripción de vocalización femenina se hizo en el estudio pionero en bioacústica de la rana toro *Lithobates catesbeianus* (Capránica 1965). Sin embargo, la descripción de que hembras de rana toro producen cantos territoriales que difieren en duración y frecuencia con el de los machos, pasó prácticamente desapercibido para los científicos que estudian el comportamiento sexual en anuros.

Pese a ser poco conspicuos, los cantos de anuncio femeninos de tres especies de sapos parteros *Alytes* spp. han sido intensamente estudiados en décadas recientes (e.g. Bush 1997; Márquez y Bosch 2001). Los sapos parteros reciben este nombre debido a que los machos realizan un cuidado prolongado de los huevos llevándolos en su dorso desde la fertilización hasta que las larvas eclosionan y son depositadas en cuerpos de agua (Verrell y Brown 1993). Se ha propuesto que las hembras de *Alytes obstetricans* y *Alytes cisternasii* podrían preferir aparearse con machos que ya se han apareado previamente o con experiencia reproductiva previa, dado que del 60 al 80% de los machos se mantienen cantando en los coros con una o dos puestas de huevo en sus espaldas (Márquez 1990; Raxworthy 1990), sin embargo, no se han encontrado diferencias en los cantos entre machos que ya se han reproducido y se mantienen cuidando huevos y aquellos que cantan y buscan aparearse sin aún cuidar huevos (Márquez 1990), por lo que se descarta que se sea a través de los cantos del macho que las hembras identifiquen la experiencia reproductiva de éstos. Se conocen detalles relevantes sobre el comportamiento sexual mediado por los cantos de las hembras. Por ejemplo, las hembras de sapo partero aumentan su actividad vocal en respuesta al canto de machos grandes (Bosch 2001) aunque la preferencia sexual de las hembras (Dyson et al. 1998) y el éxito de eclosión de larvas, son independientes del tamaño de los machos (Márquez 1993). Además, la proporción sexual sesgada hacia las hembras no está presente en todas las poblaciones (Márquez y Bosch 2001) y no ocurre competencia vocal entre hembras (Bosch 2002), aunque algunas observaciones en cautiverio sostienen que la rivalidad entre hembras puede ocurrir mediante agresiones físicas (Verrell y Brown 1993; Bush 1997). En conjunto, la vocalización de hembras de

Alytes spp. desafía el paradigma de la selección sexual respecto al cuidado parental, la competencia esperada entre las hembras y la elección masculina.

Dado que la conducta de señalización acústica en anuros ha sido objeto de estudio principalmente en especies poligínicas (Wells 1977), pocos casos han explicado cuáles son las ventajas reproductivas que confiere el despliegue de señales sexuales a las hembras de anuros (e.g. Cui et al. 2010; Goyes-Vallejos et al. 2017). En general, se ha sugerido que en especies con señales sexualmente dimórficas, las hembras pueden anunciar mediante estos sonidos su presencia a los machos (Emerson y Boyd 1999; Cui et al. 2010; Goyes-Vallejos et al. 2017) y en especies con señales monomórficas, éstas podrían conformar un mecanismo de evaluación de los machos por parte de las hembras o un comportamiento social fuera del ámbito de la selección sexual (Serrano y Penna 2018). Investigar la diversidad de contextos en los que ocurre la vocalización en hembras de anuros y las consecuencias ecológicas de las interacciones que sostienen es una tarea pendiente para la mayoría de las especies en que ocurre este fenómeno.

Una perspectiva relativamente nueva que permite comprender el sistema de apareamiento, así como la naturaleza de las estructuras sociales de animales de forma cuantificable es el uso de redes sociales (Whitehead 2008). La perspectiva de redes en la comunicación pone énfasis en que emisores y receptores tienen costos y beneficios adicionales a aquellos considerados en la visión de la interacción entre pares, por ejemplo, haciendo frente al problema de la competencia de conespecíficos y a la discriminación que realizan los receptores expuestos a un exceso de señales de conespecíficos y otros ruidos y contextos ambientales (Grafe 2005).

Desde esta perspectiva sistémica es posible plantear hipótesis acerca de las funciones comunicativas de las señales, la transmisión de información y su relación con la complejidad social en que interactúan las poblaciones, es decir, el tamaño de los grupos sociales, su densidad, el número de roles sociales, el tamaño de su ámbito de hogar y la estructura social (Freeberg et al. 2012). La descripción del fenómeno comunicacional adaptado a las redes requiere por lo tanto de una interpretación de las relaciones espacio temporales que establecen los miembros de los grupos sociales y las consecuencias de las interacciones que pueden conferir los participantes a la red social (Croft 2015).

El caso de la ranita de Darwin (*Rhinoderma darwinii*) permite evaluar el rol de la vocalización femenina en el contexto social donde los machos realizan un cuidado parental extremo, incubando 11 larvas en promedio el interior del saco bucal desde el momento de la eclosión hasta por 52 días después de que han completado el proceso de metamorfosis (Jorquera et al. 1981; Goicoechea et al. 1986). Este fenómeno de crianza es único en el mundo por la estructura en la que ocurre y es un caso conocido de rol sexual revertido entre los anuros (Crump 2010).

La identificación de que los machos de R. *darwinii* son quienes incuban en el interior de su cuerpo fue descrita por primera vez por Jímenez de la Espada (1872) y posteriormente ilustrada por Howes (1888). Ambos autores identificaron que los individuos preñados eran aquellos que tenían las gónadas más pequeñas y que los individuos que formaban los ovocitos poseían gónadas de mayor tamaño, por lo que se concluyó que eran los machos quienes realizaban la crianza y las hembras producían los huevos. Howes (1888) describió que los machos poseen dos poros en el paladar debajo de la lengua por la que las larvas podrían ingresar al saco vocal y posteriormente emerger al exterior como ranas diminutas. A simple vista, machos y hembras no poseen características de dimorfismo sexual que permitan distinguirlos. Sin embargo, algunos autores han descrito que las hembras tienen un tamaño corporal mayor que el de los machos (Crump 2002; Valenzuela-Sánchez 2014).

En su ambiente natural, se ha identificado que los adultos mantienen un ámbito de hogar reducido y una fidelidad al menos durante los meses de reproducción, además de que el ámbito de hogar de los machos suele sobreponerse entre sí a diferencia del de las hembras que se mantienen aislados (Valenzuela-Sánchez et al. 2014). Muchos aspectos acerca del contexto social de la reproducción de esta especie son aún desconocidos. Mediante observaciones hechas en cautiverio se ha reportado que el apareamiento y la fertilización de los huevos están mediados por un ritual que incluye intercambio de cantos de múltiples notas por parte de los machos y de una sola nota por parte de las hembras, además de patadas y saltos por parte de ambos sexos antes del amplexo (Busse 2003).

No obstante, se desconoce cuál es el grado de dimorfismo de los cantos y de qué manera estas señales median interacciones entre que hembras y machos durante la época reproductiva. Identificar en qué extremo entre monomorfismo y dimorfismo se ubican las señales sexuales permite plantear cuál es la posibilidad de errores en el reconocimiento (Johnstone 1997), así como plantear las posibles presiones de selección sexual implicadas en el intercambio de señales entre machos y hembras (Trail 1990). Las señales sonoras y el contexto en que se producen en su ambiente natural pueden ser cuantificadas por medio de herramientas bioacústicas, particularmente de especies en peligro de extinción (Laiolo 2010). En el contexto de la Estrategia binacional de conservación de las ranitas de Darwin (UICN Grupo de Especialistas de Anfibios Chile 2018), investigar la relación de la conducta vocal con el sistema de apareamiento de *R. darwinii* es uno de los objetivos prioritarios para desarrollar en los próximos años. Es en este marco investigativo y de estado del conocimiento sobre la especie, que se realiza el presente estudio.

HIPÓTESIS

Antecedentes

En anuros con vocalización de anuncio en hembras, el dimorfismo sexual en los cantos facilita el reconocimiento y la ubicación de pareja a distancia. Los cantos de anuncio de *R. darwinii* son emitidas por machos y hembras sin que hasta ahora se haya evaluado el grado de dimorfismo sexual que presentan. Estudios previos en la especie han mostrado que los individuos de cada sexo presentan relaciones distintas en sus ámbitos de hogar dentro de las poblaciones naturales durante la temporada reproductiva, de manera que los machos mantienen altos solapamientos en sus ámbitos de hogar entre ellos y las hembras suelen mantenerse aisladas entre ellas.

Hipótesis 1

La variación de los cantos de anuncio *R. darwinii* está relacionada con la variación morfológica del sexo del emisor.

Hipótesis 2

Machos y hembras de *R. darwinii* interactúan vocalmente de manera distinta con los cantos de cada sexo.

Hipótesis 3

Las características acústicas de los cantos de los individuos de *R. darwinii* varían a nivel intra-poblacional en relación con la cercanía espacial de su ámbito de hogar.

OBJETIVOS

Objetivo general

Entender el rol de la comunicación acústica en las interacciones sociales que mantienen machos y hembras de la rana de Darwin (*Rhinoderma darwinii*)

Objetivos específicos

1. Caracterizar la variabilidad acústica de los llamados de anuncio en distintas poblaciones de *R. darwinii*.
2. Determinar si las características acústicas de los llamados de anuncio de *R. darwinii* proporcionan información sobre características morfológicas del emisor.
3. Evaluar si las interacciones vocales que machos y hembras de *R. darwinii* realizan con uno y otro sexo son indicativas de reconocimiento sexual.
4. Determinar el grado en que la similitud de los cantos y la proximidad espacial estructuran socialmente a las agregaciones naturales de *R. darwinii*.

CAPÍTULO 1. INDIVIDUAL AND POPULATION VARIATION OF LINEAR AND NON-LINEAR COMPONENTS OF THE ADVERTISEMENT CALL OF DARWIN'S FROG (*RHINODERMA DARWINII*)

Serrano, J.M., M. Penna & C. Soto-Azat

1. Abstract

Non-linear vocal phenomena (NLP) are complex phonation emissions that include complex frequency patterns, which have been reported in an increasing number of taxa. Such acoustic components may play an important role in signalling individual identity and motivation of emitters. Variations in NLP of distant populations within a species have not been explored in any taxa. Here, we evaluate the variability of Darwin´s frogs (*Rhinoderma darwinii*) advertisement calls at an individual level and intra-, inter-population, and the influence of body size in such variation. The occurrence of non-linear acoustic variables was quantified in 606 advertisement calls, of 38 individuals from three distant populations of *R. darwinii* in southern Chile. The results indicate that inter-note intervals, dominant frequency and chaos, differ among populations and that such differences are likely influenced by body size. In addition, discriminant function analysis (DFA) showed that population variations were strongly supported by dominant frequency, while individual distinctiveness was supported by seven acoustic variables including NLP. For the first time, this study demonstrates how NLP features vary among distant populations of a vertebrate species, and contributes with evidence about the influence of body size on individual variation of non-linear vocal components.

2. Introduction

Acoustic signals are relevant for communication in different ecological contexts, such as mate choice, territorial defense and alarm against predators (Wilkins et al. 2012). In conspecific communication, variation in acoustic signals produced by different actors may allow individual recognition under dissimilar behavioral contexts (Bee and Gerhardt 2001; Boughman and Moss 2003; Lynch and Lynch 2017). For example, in territorial animals, owners can recognize familiar neighbors, to whom they respond by calling less aggressively than to strangers, a phenomenon widely known as the dear enemy effect (Temeles 1994). Moreover, inter-population variation of acoustic signals has been used for detecting evolutionary divergence within (e.g. Velásquez 2014) and between (e.g. Irwin 2000) species. Contrasting patterns have been found in different studies, in one case the variation is concomitant with genetic divergence among distant populations (e.g. Päckert et al. 2004; Toews and Irwin 2008; Velásquez et al. 2013), while in another, signal variation does not match with genetic divergence patterns (Prohl et al. 2007; Ruegg et al. 2006).

Acoustic communication signals can have a complex structure (Bradbury and Vehrencamp 2014). In the spectral domain, research has focused on non-linear vocal phenomena (NLP), i.e. signals containing sudden transitions from periodic to chaotic oscillations, period duplication and mode-locking transitions between spectral regimes (e.g. Fee et al. 1998; Bradbury and Vehrencamp 2014; Fig. 2). Unlike harmonic signals such NLP are not described by simple deterministic equations, but by means of square or higher-order terms as mathematical functions (Fitch et al. 2002; Suthers et al., 2006; Titze et al., 2016). The mechanisms of producing NLP are well documented for birds, mammals and anurans (Riede et al. 1997; Fee et al. 1998; Fletcher 2000; Suthers et al. 2006) and include:

subharmonics (integer multiples of the fundamental frequency interspersed between harmonics having double or triple the number of periods between them), chaos (broadband spectra, lacking a banded structure), and frequency jumps (sudden transitions between spectral regimes, accompanied by discontinuous changes in the fundamental frequency).

The communicative significance of NLP has been examined in reptiles (Labra et al. 2013), birds (Nowicki and Capranica 1986; Digby et al. 2013), terrestrial (Fitch et al. 2002; Riede et al. 2004) and marine mammals (Mann et al. 2006; Tyson et al. 2007). In anurans, studies have been restricted to the concave-eared torrent frog (*Odorrana tormota*) from eastern China (Feng et al. 2009a), where NLP occur in a high proportion of advertisement calls in males. Different functions have been assigned to NLP, including prevention of response habituation (Blumstein and Récapet 2009; Karp et al. 2014), recognition of emitters' identity (Fitch et al. 2002; Volodin et al. 2011; Feng et al. 2009b), mimicry of larger individuals (Fitch et al. 2002; Blumstein et al. 2008; Cazau et al. 2016), and quality indicators (Fitch et al. 2002; Riede et al. 2004). However, in some cases it is possible that NLP are physical by-products of vocal systems, not entailing adaptive functions for signal producers (Fitch et al. 2002), or simply being a consequence of vocal disorders (Riede et al. 1997). To our knowledge, no study has reported how NLP vary among geographically distant populations for any taxon within the animal kingdom. Since species with a large geographic distribution vary in their morphometry due to environmental influences (e.g. Valenzuela-Sánchez et al. 2015), and the allometric relationships between body size and vocal apparatus affect the occurrence and strength of non-linearities in acoustic signals (Cazau et al. 2016), it is expected that frequency, duration and NLP characteristics in calls produced by distant populations will relate to the geographic variation in body size.

Identifying the variability of NLP at various levels, will contribute to a better understanding of the role of these complex components in social interactions and evolutionary divergence of sound signals.

Commonly, anurans form social aggregations in which males produce advertisement calls building up choruses that attract females (e.g. Wells and Schwartz 2007). Social recognition occurs in territorial frogs, as neighbours and strangers are discriminated based on acoustic cues (Gerhardt and Bee 2007; Feng et al. 2009b; Gasser et al. 2009; Bee 2016). Such recognition ability varies among anuran species (Bee 2016). However, anuran calls are almost always statistically distinctive at an individual level, even in non-territorial species. While individual distinctiveness of acoustic signals is indeed a prerequisite for neighbour recognition, it is probably not a strong predictor of territorial behaviour (Bee et al. 2016).

Darwin’s frog (*Rhinoderma darwinii*) is an endangered species with a large latitudinal distribution in the temperate rain forests of South America. This species is well known for its unique parental care strategy, as males brood tadpoles in their vocal sacs until metamorphosis is completed (Jorquera et al. 1981). Most studies on behaviour and reproduction of *R. darwinii* have mainly been conducted under captivity (Wilhelm 1927, 1932; Pflaumer 1935; Busse 2003). Reproduction of Darwin’s frog occurs mainly during late spring through summer (November to February), but there has also been breeding activity recorded during winter (Wilhelm 1932; Pflaumer 1935). The fertilization of the eggs by the males occurs minutes to hours after a female has oviposited (Busse 2003). After fertilization, males stay close to the eggs until they hatch, moment when the tadpoles are ingested (Pflaumer 1935). While pregnant males and females produce advertisement calls, these are less frequently observed relative to non-pregnant males (Serrano JM, unpublished data). So, when an individual is heard calling, it is usually assumed to be a reproductively active and non-pregnant male (Crump 2002; Valenzuela-Sánchez et al. 2014). However, vocal exchanges between individuals of different sexes have been observed during mating in captivity (Busse 2003). In the field, males and females of *R. darwinii* call actively at daytime with very similar multi-note calls (Serrano, unpublished data). The advertisement call of *Rhinoderma* consists of a sequence of soft high-pitched notes (Penna and Veloso 1990), and the differences in the vocalizations of *R. darwinii* with its sister species, *R. rufum* (likely extinct), may be due to a difference in size, rather than interspecific variation between two taxa.

A significant geographic variation in body size of *Rhinoderma darwinii* has been documented across its distribution range, larger individuals occur in areas with greater temperature seasonality, where animals probably hibernate (Valenzuela-Sánchez et al. 2015). Such phenotypic variation is relevant for acoustic communication, since call spectral components are inversely related to body size in different taxa, including birds (Ryan and Brenowitz 1985; Podos 2001; Digby et al. 2013), lizards (Labra et al. 2013), mammals (Charlton and Reby 2016; García-Navas and Blumstein 2016) and anurans (Penna and Veloso 1990; McClelland et al. 1996; Castellano et al. 2000; Feng et al. 2009a). However, the influence of body size variation on NLP components has been only inferred from vocal apparatus models (Cazau et al. 2016), but it has never been reported from living animals varying in body size.

Variation in body size across the geographic distribution of *R. darwinii* (Valenzuela-Sánchez et al. 2015), and the potentially complex social interactions of this species (Busse 2003; Valenzuela-Sánchez et al. 2014), provide a model to assess acoustic signal variation at geographical and individual levels. Providing preliminary steps for further explorations of the production and recognition of acoustic signals containing NLP. The aims of this study are to analyse the variation in the advertisement calls of Darwin's frog at an inter- and intra-population and intra-individual levels, and to assess the potential of these acoustic displays for individual distinctiveness. Preliminary observations allow us to propose that the calls of *R. darwinii* differ among populations. Call of larger frogs, from the continent, are expected to have less NLP and lower dominant frequencies, relative to their smaller insular counterparts from the island Chiloé (Valenzuela-Sánchez et al. 2015).

3. Materials and methods

3.1 Study area

Three populations of *R. darwinii* were studied during 2011 and 2014. Two populations from continental Chile: Queulat (44°22´ S; 72° 15´ W) and Huilo Huilo (40°01´ S; 71°56´ W), and one from insular Chile: Tantauco, Chiloé (43° 21´ S; 74° 6´ W). All three localities were >150 km apart in a straight line and where geographically separated by sea or an abrupt topography (Fig. 1). Recent studies have characterized the population abundance of Darwin's frogs in these regions, classifying Huilo Huilo and Tantauco as large abundance, and Queulat as medium abundance (Soto-Azat et al. 2013). However, continental populations including Huilo Huilo and Queulat populations have shown a decreasing tendency in their population size (Valenzuela-Sánchez et al. 2017).

3.2 Acoustic recordings

Vocal signals produced by adult males were first described by Penna and Veloso (1990) as two- or three-note calls with an average call duration of 1.1 s and a dominant frequency of 2.6 kHz. These signals were considered as advertisement calls and recorded from individuals forming chorusing aggregations. In the current study, calls of non-pregnant males were recorded from relatively isolated individuals. No aggressive interactions were observed among callers during fieldwork, but frequent duetting interactions between individuals within a range of 0.5 to 3 m were observed.

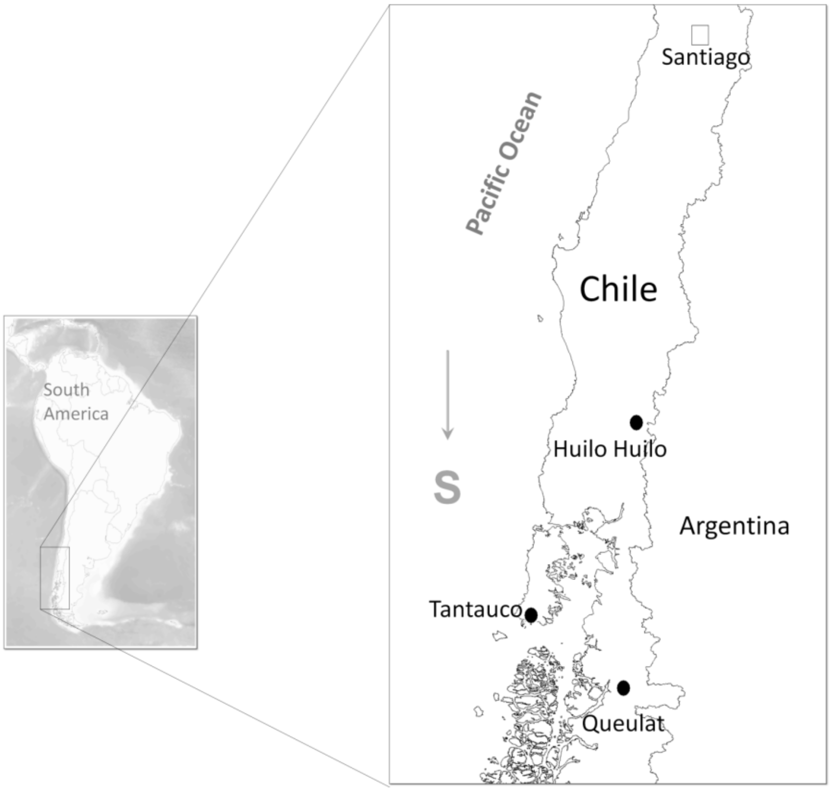

Figure 1. Map of southern Chile showing the sites where advertisement calls of *Rhinoderma darwinii* were studied.

Recordings of vocalizations were carried out with a digital recorder (Tascam DR-100) at a sampling rate of 44.1 kHz and 16 bits resolution, and a directional microphone

(Sennheiser ME66) placed approximately 0.5 m from the focal individual at an angle of ~0° relative to the subject orientation. The sound level meter microphone (Extech 407780) was placed adjacent to the directional microphone to measure the sound pressure level (SPL re 20 µPa, C frequency weighting and fast time weighting) of the calls.

Frogs were sampled in Huilo Huilo and Tantauco, snout-vent length (SVL), weight and sex were determined for each individual. A photograph of the ventral body surface of each frog was taken to record their unique colouring pattern, useful for individual recognition (Soto-Azat et al., 2013). Afterwards, each frog was released at the original point of capture. Morphometric data were not obtained for frogs from Queulat, since they were not captured. Because of this, at this site all recordings were conducted several meters apart, in order to avoid recording individuals repeatedly. Since call characteristics in ectotherms change with abiotic factors (e.g. Penna and Veloso 1990; Márquez and Bosch 1995), air temperature and relative humidity were recorded, after each acoustic recording, by placing a thermohygrometer (Sper Scientific 850070) 5 cm above the position of each of the subjects studied.

4.3 Acoustic delimitations and definitions

After visual inspection of a recording, the boundary of each call was delimited with the Manual finding tool of Sound Ruler 0.9.6.0 (Gridi-Papp 2007) using a 50-point resolution, 30-point smoothing, peak amplitude of 0.1 ± 0.2 V, estimated note duration of 1500 ± 0.4 ms, and expected interval between notes of 700 ± 0.4 ms. Calls recorded consisted of a sequence of high-pitched notes separated by regular silent intervals. The nomenclature of Köhler et al. 2017 was used for call description. Six common acoustic variables were analysed: note duration, call duration, inter-note interval, dominant frequency of the note, delta frequency (the difference between note onset frequency and note end frequency), and call repetition rate (the ratio of the number of calls recorded during the 5 min following the first call emitted). Call duration and inter-note intervals were directly measured in oscillograms. Spectrograms were obtained with a Hamming window and 512-sample fast Fourier transform (FFT).

Note segments containing NLP were identified with PRAAT 5.3.82 (Boersma and Weenick 2008) and selected based on visual inspection of narrowband spectrograms (1024 samples FFT, Hanning window). The NLP analysed were identified according to different authors (Fee et al. 1998; Fitch et al. 2002; Riede et al. 2004; Mann et al. 2006; Feng et al. 2009a) by measuring their duration on notes (subharmonics and chaos) or quantifying their proportion of occurrence among total notes due to their very short duration (frequency jumps). When no such phenomena occurred, zero values were computed for ratio calculations (see Results, Fig. 2 for representative three- and four-note calls produced by non-pregnant males containing the three NLP regimes reported in this study).

4.4 Statistical analyses

Simple Pearson correlations were used to explore the association between acoustic variables of the calls with environmental (relative humidity and air temperature) and morphometric (SVL and weight) variables. An analysis of variance (ANOVA) was performed to evaluate differences in acoustic variables between populations (Huilo Huilo and Tantauco) by stepwise simplification of models. In order to discard whether population differences in acoustic variables were supported by body size differences among populations, datasets were re-analysed correcting the data by SVL (following Platz and Forester 1988) for those variables at which significant relationships were detected (see

Results) and ANOVAs were repeated afterwards. When required, Box Cox transformation was performed to achieve normality assumptions (Osborne 2010).

Additionally, we used the coefficient of variation (CV = [SD/mean] x 100) to compare variation of the acoustic variables at three levels: inter-population (CVp), intra-population (CVip), and intra-individual (CVi). Inter-population CV was calculated for each acoustic variable from the means and SDs obtained for all the individuals within the three populations, CVip was obtained from the means and SDs obtained for individuals in each population, and CVi was obtained from the mean value of the CVip of all the individuals in a given population (Dale et al. 2001; Velásquez et al. 2013). From the results of CVi, we classified the acoustic variables as static or dynamic according to their rank within a continuum between low and high extremes of variability, respectively (Gerhardt 1991). The dispersion of the calls at the population level was assessed with the ratio (CVi/CVip) for all variables analysed. A value larger than one, means that such variable has a high potential to encode individuality, as its intra-individual variability is smaller than its inter-individual variability (Aubin et al. 2007).

Discriminant function analyses (DFA) were performed in order to evaluate inter-population and intra-individual distinctiveness. The data for these analyses included 207 four-note calls from the three populations, corresponding to the type of call most frequently emitted, the mean values of calls emitted by all the 38 individuals for the analysis of inter-population distinctiveness, and the mean value of the 150 calls of a random sampling of the 207 four-note calls for the analysis of individual distinctiveness. Random sampling allowed to balance the number of samples in the analysis of individuals for which recordings contained few calls. In order to reduce the set of variables and evaluate comprehensively their contribution to overall call variability, variables showing collinearity were deleted from the model. To evaluate the contribution of the different variables to the distinctiveness models, we conducted a stepwise forward variable selection using the Wilk’s Lambda criterion. To do this, an overall model was defined first by considering the whole set of variables to establish which one contributed to discriminate the groups. In a step forward, a differential model was then constructed by testing each independent variable putting it into the model and then taking it out. Both models yielded a Wilk's Lambda statistic and a probability value. In this way, the overall model estimates which variables contribute significantly to build the entire model, while the differential model calculates which variables allow to discriminate the groups. Wilk’s Lambda values ranges from 0 to 1, where 1 means no discrimination, and 0 full discrimination. The significance of the change in the lambda statistic was evaluated with a F-test, and if the F-value was greater than the critical value, the variable was kept in the model, indicating which variables supported differentially the significance of lambda values. All analyses were performed using R software v.3.2.3 (http://www.r-project.org). MASS package was used for DFA, Box-Cox transformations and klaR package for Wilk’s Lambda statistical analyses.

4. Results

Overall, we recorded 606 advertisement calls of 38 individuals of *R. darwinii* from three populations from southern Chile: Huilo Huilo (*n* = 12), Queulat (*n* = 12) and Tantauco (*n* = 14). The mean number of calls recorded for each individual was 14.8 (range: 3–40) and the number of calls recorded for Huilo Huilo, Queulat and Tantauco were 196, 181 and 229, respectively. All the individuals recorded showed non-linearities in most of their calls (587 out of 606 calls) in different proportions (Table 1; Fig. 2).

Table 1. Proportions of non-linear vocal phenomena (NLP): chaos, subharmonics and frequency jumps in 587 advertisement calls of *Rhinoderma darwinii* in three populations from Chile. Chaos and subharmonics percentages are calculated from the ratio between the duration of these components and the total duration of the calls; and the percentage of frequency jumps were calculated from the number of calls in which this NLP occurred for each individual.

Population	Individuals	Number of calls	% Chaos	% Subharmonics	% Frequency jumps
Huilo Huilo (Mainland site North)	1	22	6.56	0.04	10.34
	2	14	13.10	0.42	35.42
	3	24	13.40	0	0
	4	7	19.74	5.90	0
	5	20	20.17	0	26.03
	6	5	13.62	0.62	2.08
	7	20	13.34	2.33	0
	8	19	18.64	9.56	35.16
	9	18	14.46	0.29	0
	10	31	10.24	0	11.54
	11	3	7.27	0	0
	12	12	7.34	3.14	0
Queulat (Mainland site South)	1	10	20.11	2.52	0
	2	11	29.47	2.63	13.16
	3	11	21.37	6.21	62.50
	4	17	15.44	4.91	2.56
	5	22	28.10	2.87	18.75
	6	12	18.44	3.14	0
	7	11	27.77	2.12	14.29
	8	10	6.45	0.63	55.56
	9	18	21.56	34.39	0
	10	25	11.53	2.45	60.00
	11	11	9.55	2.19	0
	12	9	11.68	2.81	0
Tantauco (Chiloé Island)	1	4	21.97	17.85	55.88
	2	40	39.95	21.91	5.17
	3	19	14.26	5.40	4.44
	4	17	18.47	3.46	2.56
	5	21	19.87	10.31	68.18
	6	38	17.07	4.86	0
	7	6	26.70	5.05	78.18
	8	9	62.52	28.14	0
	9	7	12.68	2.63	79.01
	10	10	30.74	5.12	5.15
	11	4	18.05	14.18	12.5
	12	18	88.84	4.54	0
	13	23	21.28	1.28	0
	14	9	19.48	0.04	0

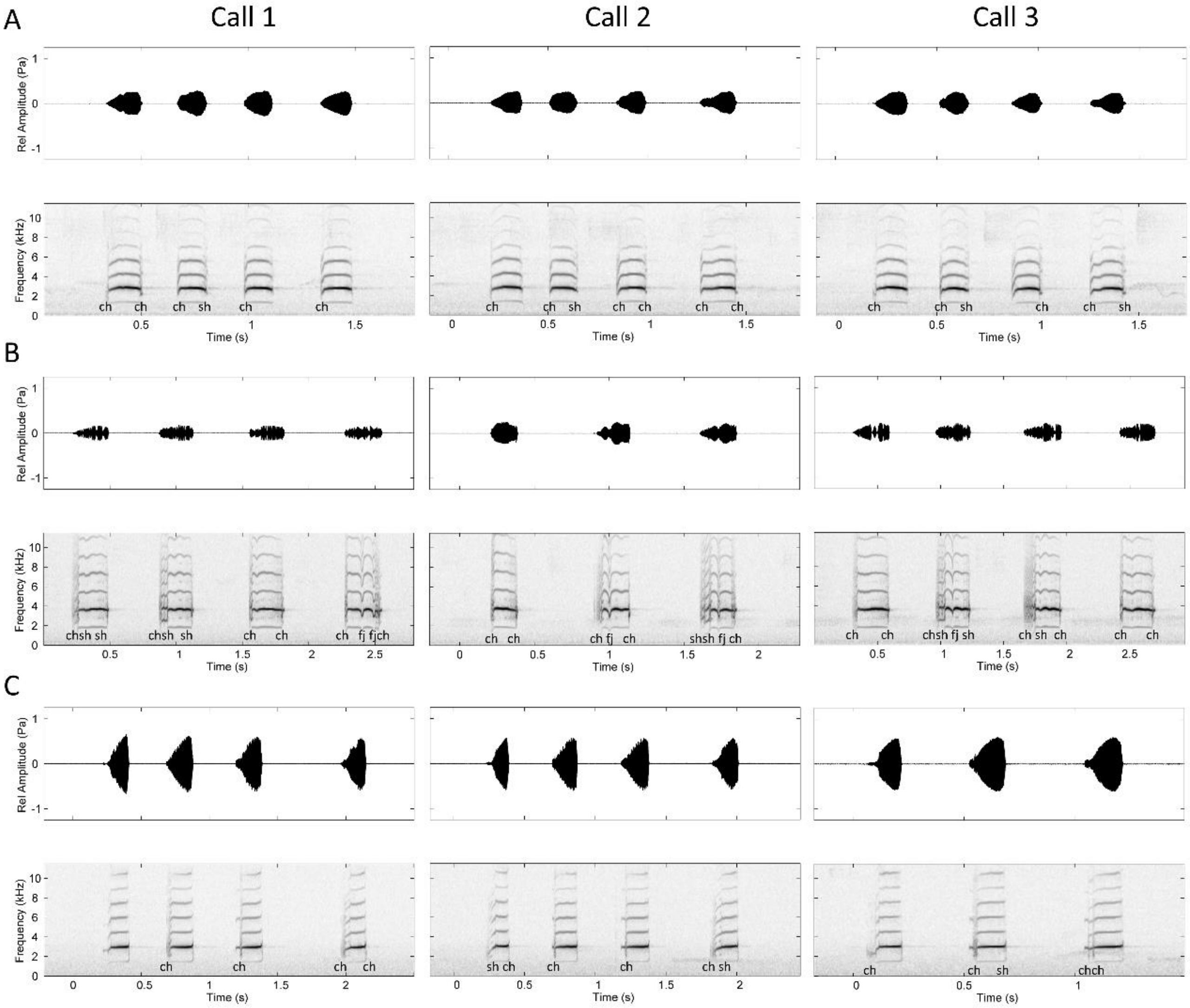

Figure 2. Representative calls from three different non-pregnant males of *Rhinoderma darwinii* from Huilo Huilo (A), Tantauco (B) and Queulat (C). Air temperature: 27.5, 17.0 and 15.7 °C, respectively. Body size of A and B: 27.5 and 21.8 mm, respectively. Sample rate was 44.1 kHz for all the recordings. Oscillograms and spectrograms were obtained using Sound Ruler. Abbreviations: ch: chaos, fj: frequency jumps, sh: subharmonics.

Advertisement calls of *R. darwinii* consisted mostly of three to six notes in Huilo Huilo and Tantauco, and three to four notes in Queulat. Four-note calls were the most frequently produced in the three populations studied (Huilo Huilo: 4.3±1.0, range= 1-7; Tantauco: 3.8±0.6, range= 1-6; Queulat: 4.1±0.9, range= 1-7). Most of the notes had a frequency modulation pattern with a slight increase in frequency at the beginning and a slight decrease at the end, and contained from seven to eight harmonics, the second harmonic always corresponding to the dominant frequency (Fig. 2). The mean values of acoustic variables, including two NLP variables in the calls of the three populations of *R. darwinii* are listed in Table 2. The data of Huilo Huilo and Tantauco populations were analysed pooled together for correlation calculations, inter-note intervals, dominant frequency and chaos, were significantly related to SVL, and inter-note intervals and dominant frequency were significantly related to weight (Table 3).

Table 2. Acoustic variables of the advertisement calls for three populations of *Rhinoderma darwinii* from Chile. Average ± standard error.

		Population	
	Huilo Huilo (Mainland site North) (*n* = 12)	Queulat (Mainland site South) (*n* = 12)	Tantauco (Chiloé Island) (*n* = 14)
Note duration (ms)	166.0 ± 18.65	155.7 ± 18.90	188.1 ± 17.78
Call duration (s)	1.746 ± 0.307	1.397 ± 0.172	1.796 ± 0.363
Inter-note interval (s)	0.315 ± 0.049	0.303 ± 0.042	0.368 ± 0.071
Dominant frequency (Hz)	2806 ± 40.47	3006 ± 45.62	3749 ± 96.58
Delta frequency (Hz)	-17.66 ± 255.07	94.74 ± 217.11	16.01 ± 316.10
Call repetition rate (calls/min)	1.6 ± 0.60	1.3 ± 0.30	1.4 ± 0.50
Chaos (ms)	22.01 ± 12.94	28.12 ± 17.61	125.19 ± 59.59
Subharmonic (ms)	3.61 ± 10.38	6.38 ± 11.57	17.07 ± 23.48
Sound pressure level (dB)	64.10 ± 4.84	64.21 ± 3.41	61.00 ± 3.19

Inter-note intervals, dominant frequency, and chaos differed among populations (ANOVA test, F= 67.35 p <0.001; F= 10.15, p <0.01; F= 4.44, p <0.05, respectively; Fig 3 A-C), but the rest of acoustic variables did not (ANOVA Tests, P >0.05). When datasets were re-analysed correcting the data by SVL, ANOVAs showed that all the acoustic variables that initially showed differences among populations (inter-note intervals, dominant frequency and chaos) were rendered non-significant (ANOVA Test, P > 0.05), indicating that differences among populations in these acoustic variables were strongly influenced by body size (Fig. 3).

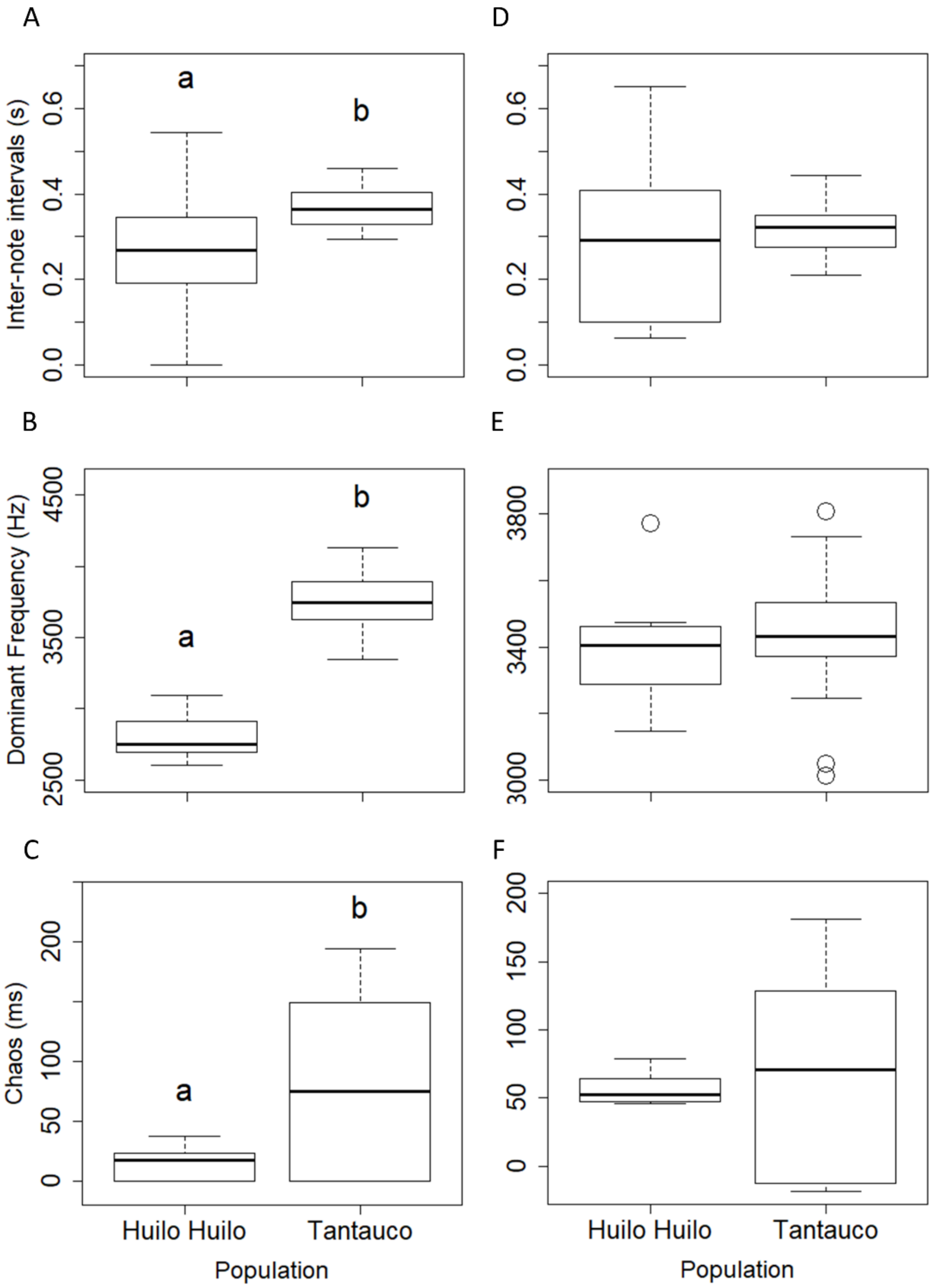

Figure 3. Population comparisons of inter-note intervals, dominant frequency and chaos before (A-C) and after corrections for body size (D-F). Letters (a, b) represent significant differences.

Table 3. Pearson correlations between acoustic and environmental (relative humidity and temperature) and morphometric (SVL and weight) variables of *Rhinoderma darwinii* from Chile (Significance levels= *: p <0.05, ***: p < 0.001). Abbreviations: SVL: snout-vent length.

	Humidity %	Temperature °C	SVL (mm)	Weight (gr)
Call duration	-0.10	-0.46*	0.31	-0.31
Note duration	0.17	-0.26	-0.32	-0.35
Inter-note intervals	-0.31	-0.12	-0.49*	-0.50*
Dominant frequency	-0.04	0.22	-0.90***	-0.92***
Delta frequency	-0.02	0.30	0.09	0.06
Call rate	-0.01	0.39	-0.30	0.11
Chaos	0.13	-0.18	-0.41*	-0.32
Subharmonics	-0.05	0.11	-0.36	-0.35
Sound pressure level	0.18	-0.17	-0.12	-0.23

The analysis of coefficients of variation showed that the largest variation at the intra- and inter-population levels corresponded to delta frequency and the lowest to dominant frequency. We considered that static variables were those having a CV <8, whereas those with a CV >8 were classified as dynamic. As such, SPL at the three levels of comparison, and dominant frequency at the intra-population and intra-individual levels corresponded to static characters, while the remaining variables corresponded to dynamic characters. A high potential for individual distinctiveness was observed for the acoustic variables call duration, note duration, inter-note interval, dominant frequency, delta frequency, chaos and call repetition rate, as indicated by their corresponding CVip/CVi values >1 (Table 4), with the only exception of delta frequency in Huilo Huilo, having a value < 1 for this ratio. Subharmonics had a low potential for individual distinctness in the three populations, with values < 1 for this ratio.

Table 4. Coefficients of variation (CV) for advertisement calls at the inter-population (CVp), intra-population (CVip) and intra-individual (CVi) levels in three populations of *Rhinoderma darwinii* from Chile. Populations: H: Huilo Huilo (Mainland site North), Q: Queulat (Mainland site South), T: Tantauco (Chiloé Island).

Acoustic variables	Inter-population (CVp)	Intra-population (CVip)			Intra-individual (CVi)			CVip / CVi		
		H	Q	T	H	Q	T	H	Q	T
Call duration	33.34	39.59	21.06	29.84	20.70	12.71	23.70	1.91	1.66	1.26
Note duration	24.94	22.90	18.42	25.72	11.31	12.59	9.82	2.03	1.46	2.62
Inter-note interval	30.16	37.27	24.74	23.19	15.76	13.31	19.15	2.37	1.86	1.21
Dominant frequency	13.72	5.44	4.08	5.95	1.44	1.52	2.65	3.78	2.68	2.24
Delta frequency	1027.59	1444.28	229.15	1974.16	2453.74	80.35	378.08	0.59	2.85	5.22
Chaos	97.30	58.84	62.49	47.59	43.54	54.73	37.99	1.35	1.14	1.25
Subharmonics	190.75	289.46	188.05	137.53	256.82	176.41	103.56	0.89	0.94	0.75
Sound pressure level	6.26	7.55	5.31	5.37	2.68	2.19	3.86	2.82	2.43	1.39

The distinctiveness analyses based on DFA showed dissimilar results at population and individual levels. The eight variables considered in the analysis contributed significantly to the overall model explaining population distinctiveness, however, only dominant frequency was essential for discriminating populations in the differential model ($P < 0.001$; Table 5). The first linear discrimination axis explained most of the variation among populations (LD1= 97.85%;) allowing to discriminate two groups: the two continental populations (Huilo Huilo and Queulat) and the insular population (Tantauco). The two continental populations were less discriminable between each other (along LD2= 2.05%). In addition, the discriminant analysis showed that 75.68% of the subjects recorded were correctly classified as belonging to their population of origin, while correct classifications were lower within the continental populations (Huilo Huilo= 66.67% and Queulat= 58.33%), but higher within the insular population (Tantauco) for which all the subjects were correctly classified (Fig. 4A). In addition, the individual distinctiveness model was run with seven variables (dominant frequency, chaos, call duration, note duration, subharmonics, delta frequency and inter-note interval; Table 5).

Table 5. Relative importance of acoustic variables for geographic and individual distinctiveness in *Rhinoderma darwinii* from Chile. Linear discriminant coefficients (LD), eigenvalues, and explained variance percentages of discriminant function analysis. F overall indicates the contribution of the acoustic variable for the overall model. F differential indicates the contrast comparing the model including and excluding the corresponding acoustic variable (Significance levels for F values= *: p <0.001).

Distinctiveness model	Variable	LD1	LD2	LD3	Wilks´ Lambda	F overall	F differential
Populations	Dominant frequency	2.30	-0.118	-	0.140	104.42*	104.42*
	Call rate	0.544	0.388	-	0.120	31.13*	2.75
	Delta frequency	-0.398	-0.484	-	0.101	22.85*	2.96
	Call duration	0.059	0.680	-	0.090	18.02*	1.86
	Subharmonics	0.420	-0.410	-	0.082	15.00*	1.61
	Chaos	1.188	-0.148	-	0.076	12.74*	1.15
	Note duration	-0.641	0.231	-	0.064	11.86*	2.65
	Inter-note interval	0.234	-0.299	-	0.061	10.29*	0.58
	Eigenvalues	14.34	2.07	-			
	Explained %	97.92	2.08	-			
Individual	Dominant frequency	-10.473	4.211	-0.324	7.287^{-03}	937.87*	937.87*
	Chaos	-1.202	-3.422	0.128	4.089^{-04}	331.70*	115.15*
	Call duration	-1.200	-1.440	-1.739	4.091^{-05}	192.65*	61.25*
	Note duration	0.249	-1.143	2.786	5.921^{-06}	133.31*	39.99*
	Subharmonics	-0.152	-0.191	0.261	2.146^{-06}	87.66*	11.84*
	Delta frequency	-0.178	0.250	0.251	9.675^{-07}	63.30*	8.15*
	Inter-note interval	0.105	-0.773	-1.177	7.176^{-07}	45.92*	2.32*
	Eigenvalues	34.50	12.32	7.28			
	Explained %	79.39	12.36	4.10			

Call rate was excluded from this model because there was only one value per individual for this variable. According to the stepwise procedure, all seven variables contribute to discriminate individuals in the overall and differential models (P <0.001; Table 5). The first two linear discriminant axis explain most of the variation among individuals (LD1= 82.09%; LD2= 10.47%). In this analysis, individual classification was clustered according to their population of origin only for the individuals of Tantauco, but not for the individuals from Huilo Huilo and Queulat; however, 93.37% of the individuals were correctly classified to the individual they belonged to (Fig. 4B).

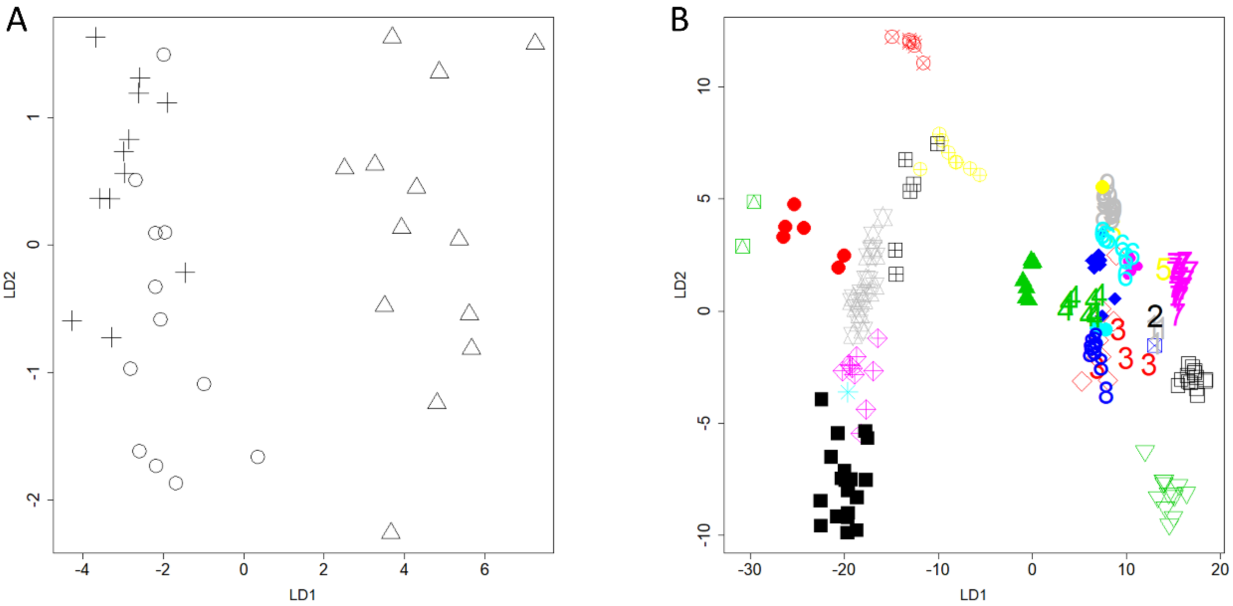

Figure 4. Linear discrimination functions representing (A) inter-population and (B) individual distinctiveness of advertisement calls of *Rhinoderma darwinii*. In (A) symbols identify the three populations (+: Huilo Huilo, Δ: Tantauco, ○: Queulat). In (B) each symbol type represents a call of different individuals from Huilo Huilo (filled symbols), Tantauco (numbers) and Queulat (empty symbols).

5. Discussion

The analyses of advertisement call variation among individuals and within and between geographically distant populations of *R. darwinii* show that call duration, dominant frequency and chaos components vary significantly at the individual, intra- and inter-population levels, and that such variation is associated to variations in body size. An inverse relationship between body size and dominant frequency occurs typically in birds, mammals and anurans (e.g. Penna and Veloso 1990; Digby et al. 2013, García-Navas and Blumstein 2016). In *R. darwinii*, differences in the relative duration of chaos, revealed that this NLP occurs in higher proportions in smaller individuals within each population, suggesting that body size has an influence on this acoustic component at both intra- and inter-population levels. Such influence of body size on variation of acoustic NLP has not been previously demonstrated on any living organism.

The relationship between body size and NLP has been previously proposed to occur in mammals (Fitch et al. 2002; Tokuda et al. 2002; Cazau et al. 2016), but not corroborated by significant correlations. In contrast with the variation in spectral features that are known to occur among populations of anuran species (Gerhardt and Huber 2002; Velásquez 2014), to the best of our knowledge no evidence of geographic variation of NLP has been reported for any animal taxa. In *R. darwinii*, body size is influenced by the climatic conditions affecting each population (Valenzuela-Sánchez et al. 2015). Our results suggest that variation in chaos, as well as other temporal and spectral call features could be determined by individual body size differences occurring among populations.

The influence of temperature on call structure is evident for call duration, which covaries inversely with the environmental variable. This agrees with a number of studies in anurans showing similar relationships of temperature with temporal variables between (Platz and Forester 1988) and within (Gayou 1984; Márquez and Bosch 1995) species. Other relationships between ecological factors and the occurrence of NLP is an aspect that remains to be explored; for example, signals having larger energy dispersion like those containing NLP could be more appropriate for transmission in environments that have high noise levels in the range of communication signals (Tokuda et al. 2002; Suthers et al. 2006; Zhang et al. 2015). In fact, *R. darwinii* calls during the day in environments with high levels of interfering biotic noise from calling birds, especially during early morning and late evening hours. A comparative analysis of background sound levels and structure at the three localities studied could unveil potential differences in acoustic environmental characteristics related to inter-population differences in the calls of *R. darwinii*.

The analysis of CVs indicates that note duration, dominant frequency and SPL correspond to static variables while the rest correspond to dynamic variables (Table 4). The CVip/CVi ratios obtained for most of the variables, including chaos are >1, except for subharmonics. These results coincide with previous studies showing the relevance of non-linearities for individual distinctiveness in the goitered gazelle (*Gazella subgutturosa*; Volodin et al 2011), the rhesus macaque (*Macaca mulatta*; Fitch et al. 2002) and the frog *O. tormota* (Feng et al. 2009a, 2009b). Although other characteristics of the call of *R. darwinii* may have an individual signature (e.g. dominant frequency or the durations of call and note), NLP are important factors to facilitate individual recognition as they increase the attention of receivers (Blumstein and Récapet 2009) and prevent signal habituation (Karp et al., 2014).

In contrast with the results of the analysis of CVs showing a similar variation at individual and population levels, DFA showed that distinctiveness of acoustic traits among individuals is larger than among populations, as estimated errors were higher for population than for individual discrimination. The individual variability involves seven variables used for the analysis: dominant frequency, chaos, call duration, note duration, subharmonics, delta frequency and inter-note intervals. These results agree with studies showing that individual distinctiveness is not based on a unique call feature but instead on multiple variables acting concurrently (Asquith et al. 1988; Feng et al. 2009a; Pettit et al. 2013; Gambale et al. 2014). On the other hand, population distinctiveness only involved dominant frequency, in contrast with other anuran studies showing population distinctiveness based on multiple calls variables (Funk et al. 2009; Velásquez et al. 2013). Interestingly, the differences in acoustic features between populations disappeared after performing body size corrections. Since DFA were calculated with dataset independent of body size, it is apparent that population and individual distinctiveness in *R. darwinii* are mostly driven by differences in body size. Altogether, the results of the CV and distinctiveness analyses indicate that population variation depends mainly on dominant frequency, while individual distinctiveness depends on multiple acoustic components altogether within populations.

Darwin's frogs have limited ability to disperse (Uribe et al. 2017), and therefore maintain long-term stable groups in patched areas (Valenzuela-Sánchez et al. 2017), where males occur in overlapping home ranges, not showing evidence of territorial behaviour (Valenzuela-Sánchez et al. 2014). Under these circumstances, no clear role can be attributed to individual distinctiveness of the calls for this species. Furthermore, individual distinctiveness of vocalizations does not necessarily imply that signals are useful for individual recognition, as males of the strawberry dart-poison frog (*Oophaga pumilio*) produce calls with high individual distinctiveness (Pröhl 2003) but do not discriminate neighbours from strangers (Bee 2003).

Most studies describing the occurrence of NLP in mammals and birds have not evaluated the role of these acoustic variables in social interactions, and the adaptive role of NLP in calls of ectotherms remains totally unexplored. Also, many studies on the variables affecting the occurrence of NLP among vertebrates are focused on alarm calls (Tokuda et al. 2002; Blumstein and Récapet 2009; Fitch et al. 2002; Karp et al. 2014; Labra et al. 2013; Townsend and Manser 2010), while rarely considering the occurrence of non-linearities in signals of quality and social significance (Feng et al. 2009a; Digby et al., 2013). Overall our study shows unique evidence on the role of NLP on individual distinctiveness based on body size, and its possible involvement in communication on quality and social status. Furthermore, this variation encompasses the individual and geographic levels, pointing to an evolutionary potential of the acoustic dissimilarities exhibited by a species having a unique reproductive strategy.

CAPÍTULO 2. WHO IS CALLING WHEN DARWIN'S FROGS BREED?

Serrano, J.M., M. Penna, M. Méndez & C. Soto-Azat.

1. Abstract

Signal display by males promotes female mate choice in different organisms. However, when males and females signalize it is not clear how different the signals can be between the sexes. In this study, we characterized the advertisement calls of males and females of Darwin's frog (*Rhinoderma darwinii*), a species in which males carry-out parental care, brooding the larvae inside their vocal sacs. The goal of this study is to determine the sexual differences in acoustic signals of the Darwin's frog. To do this we compared the acoustic properties and occurrence of advertisement calls recorded from females and pregnant and non-pregnant males in a population located in the island of Chiloé, Chile. We found that female calls produce a similar call repertoire relative to males and differed from pregnant and non-pregnant males in having lower dominant frequency and longer note and call durations. Calls between pregnant and non-pregnant males did not differ, but the amplitude of calls of pregnant males is inversely related to the number of brooding larvae carried. Although different variables contributed significantly in the distinctiveness model of the sexual status of calls (dominant frequency, call rate, note duration and aggregated entropy), only dominant frequency contributed significantly to differentiate them. Call repeatability was higher within individuals than within sexes, which points out the relevance of individual traits of acoustic signals for the dynamics of chorusing aggregations. Overall, our results suggest that mutual selective pressures could be involved in the evolution of the advertisement calls of the Darwin's frogs since calls produced by females and males show a low sexual dimorphism.

2. Introduction

Vocalization provides a valuable communication channel through which behaviour can be coordinated and controlled by conveying and acquiring social information. Under the perspective of sexual selection theory, sexual dimorphism and large variation in communicative signals is expected to occur in most animals (Snowdon 2004; Wilkins 2013). So, variation could be related to the quality of individuals of the chosen sex (Candolin 1999; Forsman and Hagman 2006), which typically corresponds to males (Darwin 1871; Ryan and Rand 1993; Snowdon 2004; Price 2015). However, recognition of conspecifics and sexual signalling can be regarded as a unitary problem (Ryan and Rand 1993), particularly when one of the sexes is dishonest in communicating its sexual identity (Sherman et al. 1997). In addition to convey sexual information, signals also mediate individual and social recognition (Gheusi et al 1994; Keen et al. 2016), and in a context of social interaction, individually distinct signals allow identification of social status or breeding categories (e.g. individuals with and without breeding experience, Keen et al. 2016).

In social species of insects, birds and mammals, the evolutionary role of social signals produced by females has been widely discussed (West-Eberhard 1983, 2014; Wasser 1983; Tobias et al. 2012); however, since in anurans the proportion of species in which females vocalize is low (less than 1% of the known species; Emerson and Boyd 1999), these questions have been addressed to a rather limited extent. Rand (1988) for instance, considered the call of *Eleutherodactylus grandis* males in response to the high pitch call of females (described in Dixon 1957) to be a call of "subordination", even though such behaviour has not been described in anurans. Instead, calls displayed by anurans are mainly related to mate attraction and are produced mostly by males (Wells 2007; Köhler et al. 2017). However, in species in which females produce calls, three patterns have been reported: 1) female advertisement calls are sexually dimorphic with males (Schlaepfer and Figueroa-Sandi 1998; Emerson and Boyd 1999; Goyes-Vallejos et al. 2017), 2) females and males produce sexually monomorphic advertisement calls (Serrano and Penna 2018); and 3) females produce aggressive calls towards males during mating events (Cui et al. 2010; Preininger et al. 2016) or even in non-mating contexts (Capranica 1965; Stewart and Rand 1991).

The southern Darwin´s frog (*Rhinoderma darwinii*) is an anuran endemic of the temperate forest of South America (Soto-Azat et al. 2013a). This frog is worldwide known not just by its history and discovery by Charles Darwin in the nineteenth century, but also by its peculiar reproduction mode known as neomelia, namely brooding of larvae in the male’s vocal sac (Goicoechea et al. 1986). The mouth brooding ends when metamorphosis is completed, a process lasting for 4 to 6 weeks (Jorquera et al. 1972; Goicoechea et al. 1986). Vocal activity of Darwin’s frog is mainly diurnal, and its vocalization is restricted to the warmer months of the southern hemisphere, when breeding takes place: October to March (Penna and Veloso 1990; Crump 2002; Valenzuela-Sánchez et al. 2017). In the field, males usually call isolated, in pairs or in small groups on moss mounds on undergrowth in temperate forest environments (Crump 2002) and pregnant are distinguishable from non-pregnant males by their larger body size and the bulge larvae in the belly area (Crump 2002; Valenzuela-Sánchez et al. 2014). Calling behaviour has been exclusively attributed to non-pregnant males although in the field males of both conditions are commonly observed in close vicinity (Crump 2002; Valenzuela-Sánchez et al. 2014). Furthermore, Busse (2003) described exchanges of kicks, jumps and calls between males and females during mating episodes in captivity, including the emission of the typical multi-note calls by males and single-note calls by females. A first detailed description of the advertisement calls of the species focused on vocalizations produced by non-pregnant males appears in Chapter 1, however calls by pregnant males and females were not analyzed. The goal of this study is to report advertisement calls produced by non-pregnant males, pregnant males and females in a natural population of *R. darwinii*, and analyze the variation of these acoustic signals in terms of its potential for individual and sexual recognition in the context of the unusual paternal involvement in the reproduction of this species.

3. Materials and methods

3.1.General procedures

This study was done in Tantauco private park (43° 21´ S; 74° 6´ W) located on Chiloé Island, where some of the largest known populations of *R. darwinii* occur (Soto-Azat et al., 2013; Valenzuela-Sánchez et al. 2017). During monthly visits to the study site from October 2015 to February 2016, the population was monitored in an area dominated by native forest. On each month, we recorded the number of males (pregnant and non-pregnant), females, juveniles and post-metamorphs found within a ~250 m^2 area during three succesive days with a total monthly effort of 12 person-hours. Vocalization was observed from November to February and recordings of individual calls were conducted during five additional days per month. So, frog and vocalization abundance were summarized monthly. The data obtained for each individual registered were: snout-vent length (SVL) and mass as measures of body size, the number of larvae incubated (counted by gently pressing with a sterile cotton swab the ventral area of the pregnant males), and photographs of the unique ventral black and white coloration patterns to identify individuals (Soto-Azat et al. 2013). Thereafter each frog was released at the original point of capture. Calling individuals present in the area were recorded as described below. All the individuals captured were included in the monthly estimation of abundances.

3.2.Individual recordings

Vocalizations produced by 32 adult individuals belonging to the three different sexual status of *R. darwinii* were recorded. These individuals corresponded to 11 non-pregnant males, 11 pregnant males and 10 females. Some of the individuals were recorded successively on different days, and these recordings were employed to estimate individual call repeatitivity (see below). The recordings were conducted with a directional microphone Sennheiser ME-66 positioned at ~90 degrees in front of the frog's head and connected to a digital recorder Tascam DR-100 at a sampling rate of 44.1 kHz and 16-bit resolution. To measure the root mean square (RMS) sound pressure level (SPL re 20 µPa, C frequency weighting and fast time weighting) of the calls, the microphone of a sound level meter (Extech 407780) was placed adjacent to the tip of the directional microphone. The sound level meter was calibrated each day before recordings by introducing the microphone of the sound level meter inside the opening of a sound calibrator (Brüel and Kjaer 4231) which delivers a continuous 1-kHz tone at 94 dB SPL RMS.

3.3.Acoustic analysis of individuals

Calls were analyzed using the software Raven Pro 64 1.4, employing a Hamming window and 256-sample fast Fourier transform (FFT). By selecting manually notes and calls from oscillograms and spectrograms, the following measures were analyzed: note duration, call duration, dominant frequency, average entropy, aggregate entropy and call repetition rate. Note duration, dominant frequency and the two measures of entropy were measured for all the notes recorded, while call duration was obtained from calls with four notes only, to compare the most common call emitted by individuals of the three sexual status (58, 44 and 64% of calls produced by females, non-pregnant males and pregnant males corresponded to four note calls, respectively). Average and aggregate entropy were measured as a proxy of spectral complexity related to the non-linear phenomena contained in advertisement calls of this species (Chapter 1). Call repetition rate was calculated as the ratio of the number of calls recorded during the 5 min following the first call emitted.

3.4. Statistical analyses

All analyses were performed using R software v.3.5.0 (http://www.r-project.org). Simple Pearson correlations were used to explore the association between acoustic variables of the calls with temperature and morphometric (SVL and weight) variables. Additionally, Spearman correlations were used to evaluate the association between acoustic properties of the calls of pregnant males and the number of larvae incubated. Acoustic features and body size measures were compared using analysis of variance (ANOVA) to determine whether call properties varied among individuals of different sexual status (non-pregnant males, pregnant males and females). The Tukey's honest significant difference method was used to estimate *post hoc* analyses in those cases where ANOVA yielded significant results. To discard whether sexual differences in acoustic variables were supported by body size differences among populations, datasets were re-analysed correcting the data by SVL (following Platz and Forester 1988) for those variables at which significant relationships were detected (see Results) and ANOVAs were repeated afterwards. When required, Box Cox transformations were performed with the package

MASS to fulfil normality assumptions and the package car was employed to estimate ANOVA.

The chance to distinguish the three sexual status by their calls were estimated with a discriminant function analysis (DFA) performed for each of the measured acoustic variables. The data for these analyses included only four-notes calls, which were produced by 22 individuals (eight non-pregnant males, seven pregnant males and seven females). However, call rate values corresponded to the total number of all call types (from one to six notes) emitted by each individual. Average entropy was deleted from the model due to its collinearity with aggregated entropy, in order to reduce the set of variables and accomplish the assumptions of DFA method. To evaluate the contribution of the different variables to the sexual status distinctiveness model, we conducted a stepwise forward variable selection using the Wilk´s Lambda criterion. To do this, an overall model was defined first, to establish which variables contributed to discriminate the groups. Subsequently a differential resampling was applied to evaluate each independent variable putting it into the model and then taking it out, calculating a Wilk’s Lambda statistic. Wilk´s Lambda values range from 0 to 1, where 1 means no discrimination, and 0 total discrimination. The significance of the change in the lambda statistic was evaluated with a *F*-test, and if the *F*-value was greater than the critical value, the variable was kept in the model, indicating which variables support differentially the significance of lambda values. MASS and klaR packages were used for DFA and Wilk´s Lambda statistical analyses.

Total variation attributable to individuals and sexual status was quantified estimating the intra-class correlation coefficient (ICC) of acoustic features from all the notes and calls emitted by those individuals recorded on different days, and for all the subjects of each sexual status, respectively. Repeatability estimation of ICC was supported by the calculation of confidence intervals, number of measurements expected, and variance associated with these estimates following to Wolak et al. (2012). The package ICC was applied to estimate repeatability calculations.

4. Results

4. Abundance and calling behaviour

The number of non-pregnant males and females showed a gradual increase in their abundance from October to February but a decrease in number of pregnant males and juveniles occurred in February relative to January (Fig. 5A). A marked decrease in the number of non-pregnant males vocalizing was observed from November to February. The number of calling pregnant males increased from November to January but decreased in February, while the number of females vocalizing decreased from November to January and increased again in February (Fig. 5B).

When vocalizing, frogs produced advertisement calls spontaneously for periods of 3 to 20 minutes with irregular silent intervals between calling bouts. In general, individuals were observed vocalizing during up to three continuous days (see below). No aggressive calls or physical attacks were observed for any of the sexes during this study.

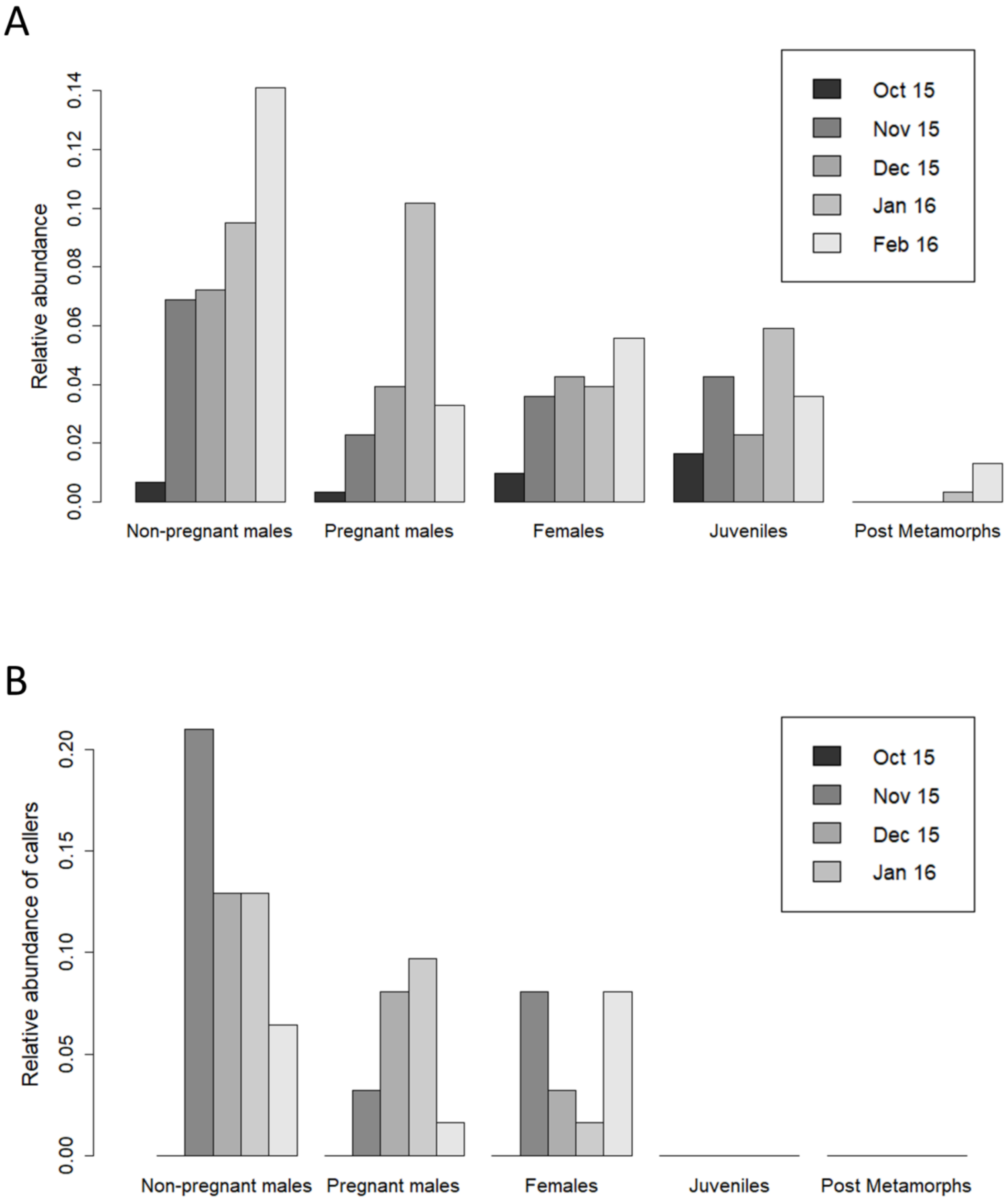

Figure 5. Relative number of individuals (A) and callers (B) for the different sexual status and age ranges of *Rhinoderma darwinii* per month along the period of study (October 2015 to February 2016) on Chiloe Island.

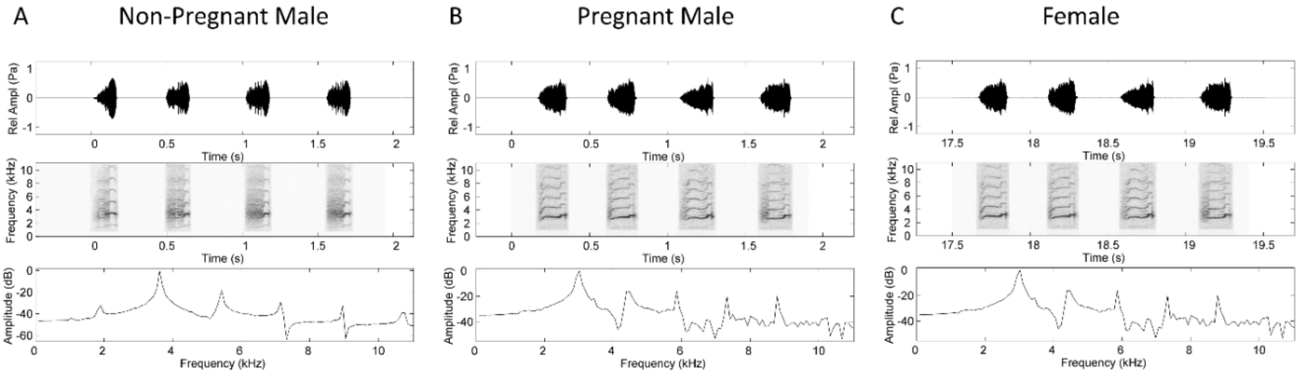

Figure 6. Oscillograms, spectograms and power spectra of typical advertisement call for a non-pregnant male (A), a pregnant male (B) and a female (C) of *Rhinoderma darwinii* recorded in Chiloé island, Chile. Sample rate was 44.1 kHz for all the recordings. Snout-vent-length of A, B and C were 21.3, 20.9 and 23.6 mm, respectively. Air temperatures during recordings were 14.6, 21.6 and 16.2 °C, respectively.

4.2.Call differences and distinctiveness

Advertisement calls characteristic of the different sexual status were not recognisable to the observer’s ear. Examples of typical advertisement calls from the three sexual status are shown in Fig. 6. Subjects from distinct sexual status differed in body size (ANOVA; F= 6.583; p< 0.01; Fig. 7A), being females larger than males. Also, individuals differed in body mass (F = 25.431; p< 0.001; Fig. 7B) among the three sexual status-. In addition, females had longer note (F= 8.122; p< 0.01; Fig. 7C) and call (F= 4.970; p< 0.05; Fig. 7D) duration than males. Furthermore, females had lower dominant frequency than non-pregnant and pregnant males (F= 3.998; p< 0.05; Fig. 7E). However, no statistical differences were found among the three sexual status in aggregated and average entropy, call rate and SPL (p> 0.05; Figs. 7F-I).

When datasets were re-analysed correcting the data by SVL, ANOVAs showed that all the acoustic variables that initially showed differences among sexes were rendered non-significant (ANOVA Tests, call duration, F= 3.226, p= 0.057; note duration, F= 2.618, p= 0.091; dominant frequency, F= 2.467, p=0.104), indicating that differences among populations in these acoustic variables were influenced by body size.

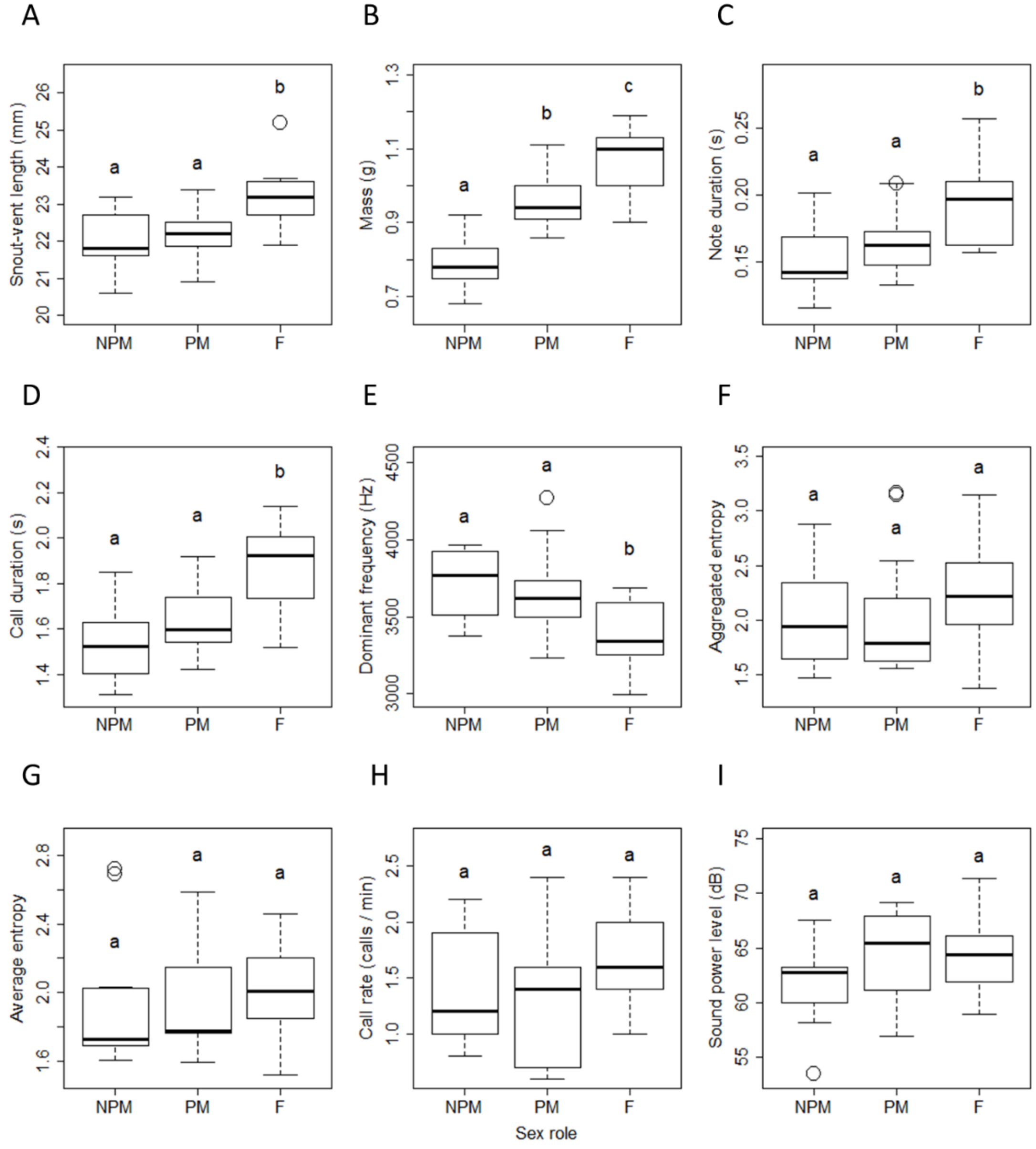

Figure 7. Body measurements (A-B) and acoustic features of the calls (C-I) in the three sexual status of *Rhinoderma darwinii*. Lowercase letters (a, b, c) indicate significant differences between sexual status in post-hoc analysis (p <0.05).

Comparisons of SPLs of the calls among the different sexual status were not affected by the distance from the focal individula at which this variable was measured, as no significant correlation between this amplitude measure and recording distance (range: 18 – 68 cm) occurred (n= 18; r= -0.13; df= 16; p= 0.612). When we compared body size among calling and silent individuals, no differences were found between pregnant males and females; however, calling non-pregnant males were larger than silent ones (Mann-Whitney Test; U= 521.5; p <0.001). The number of larvae observed was between two and nine (n= 58, mean= 3.4 ± SD= 1.7). However, the number of larvae observed in calling pregnant males (n= 11) was between two and five, being four larvae the most frequent count. The number of larvae in calling pregnant-males was significantly related to SPL only, as males brooding lower numbers of larvae called louder (r=-0.79; df=6; p=0.019; Fig. 8.).

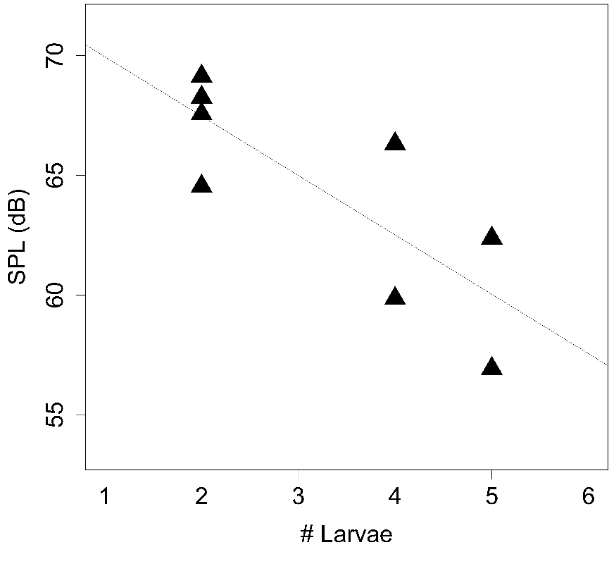

Figure 8. Relationship between the number of larvae and sound pressure level (SPL) in calls of pregnant males of *Rhinoderma darwinii*.

Collinearity from the seven acoustic variables studied was visually explored to assess model assumptions and incorporate pertinent variables to DFA analysis. Call duration and average entropy were omitted from the DFA given their collinearity with note duration and aggregated entropy, respectively. All the five acoustic variables incorporated in DFA were chosen due to their contribution to the whole model; however, only dominant frequency was significant in the differential model (differential F), which means that only this variable was relevant after re-sampling procedures of Wilk's Lambda criterion and therefore the distinctive model was formed by this variable only (Table 6). Two linear discriminant vectors were projected explaining 96% (LD1) and 4% (LD2) of the data (Fig. 9). The graphic distinctiveness between males and females was more noticeable than between the two male status, as females were separated from both types of males by LD1 and LD2. A priori probabilities of discrimination were 35, 35 and 30% for non-pregnant males, pregnant males and females, respectively. With these probabilities, 90.9% of the data were correctly assigned to the respective sexual status, as only the mean values of calls from one female and one non-pregnant male were assigned erroneously to pregnant males.

Table 6. Relative importance of acoustic variables for sexual status distinctiveness in *Rhinoderma darwinii*. Linear discriminant coefficients (LD), eigenvalues, and explained variance percentages of discriminant function analysis. Overall F indicates the contribution of the acoustic variable for the overall model. Differential F indicates the contrast comparing the model including and excluding the corresponding acoustic variable Significance levels for F values= *: $p < 0.05$, **: $p < 0.01$.

	LD1	LD2	Wilk's lambda	Overall F	Differential F
Dominant Frequency	0.952	-0.060	0.625	5.690*	5.690*
Call Rate	-0.513	-0.393	0.474	4.076**	2.882
Note Duration	-0.565	-0.095	0.420	3.080*	1.093
Aggregated Entropy	-0.451	-0.130	0.388	2.419*	0.646
Sound Pressure Level	-0.385	0.737	0.349	2.080	0.852
Eigenvalues	4.010	0.786			
Explained %	96.3%	3.7%			

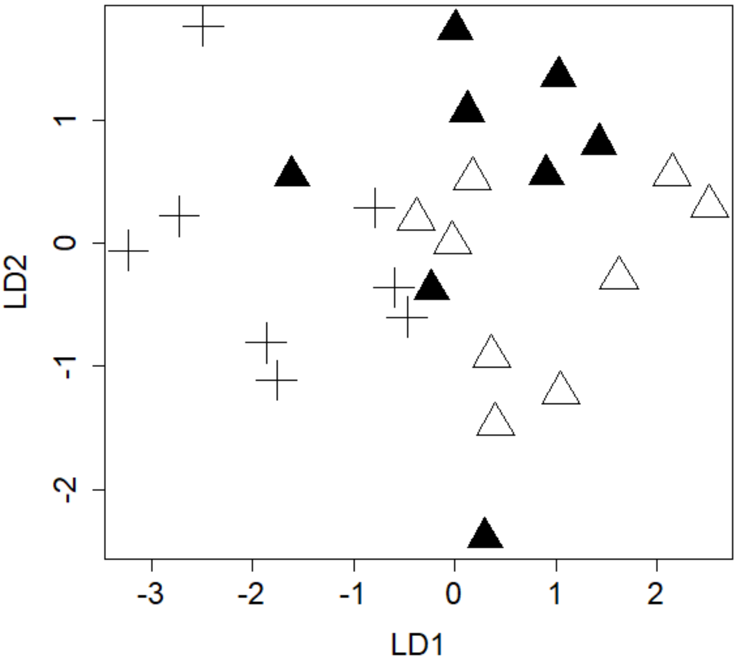

Figure 9. Linear discrimination functions representing sexual status distinctiveness in *Rhinoderma darwinii* advertisement calls. Symbols identify non-pregnant males (Δ), pregnant males (▲) and females (+).

4.3.Sexual status and individual repeatability

Thirteen individuals (10 non-pregnant males, two pregnant males and one female) were recorded repeatedly two to four times with intervals between recordings of 1 to 62 days. Most of these repeated individual recordings were obtained during sessions spaced one to three days apart. Among these individuals, only one male was recorded while having different pregnancy status (Fig. 10).

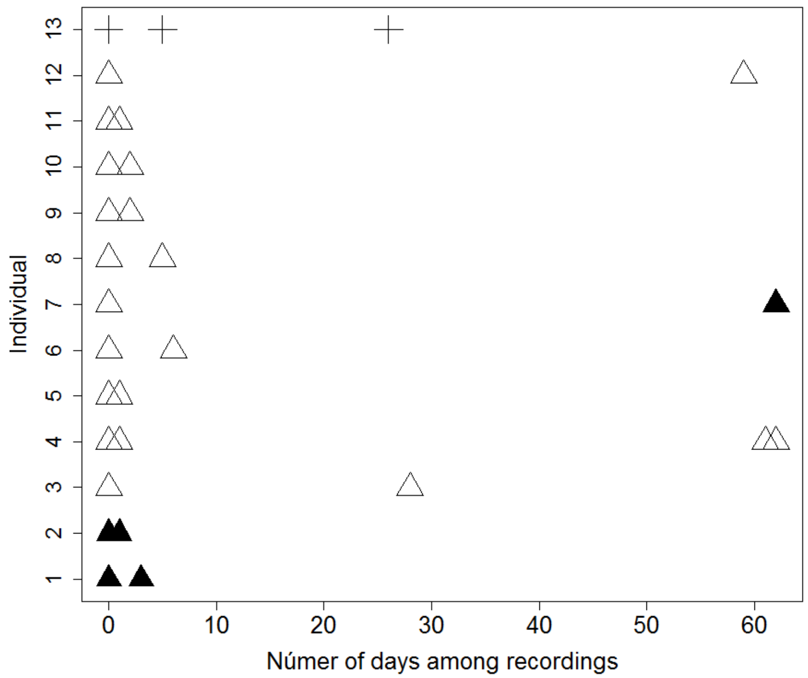

Figure 10. Intervals between days in which calls of 13 different individuals of *Rhinoderma darwinii* (10 non-pregnant males, 2 pregnant males, 1 female) were recorded on two to four opportunities. Symbols identify individuals of the three sexual status: Δ: non-pregnant males; ▲: pregnant males; +: females. Only one non-pregnant male was recorded on a second opportunity while he was pregnant.

Repetitiveness of acoustic features was higher among individuals than among sexual status groups. Note and call duration had values of mean repetitiveness among sexual status of 0.45 and 0.40 ICC, respectively, while the rest of the acoustic features had low values (<0.25). Among individuals, note duration, call duration, aggregated entropy, average entropy and SPL had high repetitiveness with values above 0.61, while dominant frequency repetitiveness had a medium value of 0.50 and only call rate had a low value (0.19). The confidence intervals of these estimates were higher for individuals than for sexual status groups. Also, the estimated number of measurements (k) for reach repeatability was not reached for sexual status, but close to reach for individuals registered (Table 7), since the average number of call measurements was 82 (range: 17 – 338) for individuals and 824 (range: 525 – 1268) for sexual status. In agreement with these results, the variance within and between the ICC values was higher for sexual status than for individuals.

Table 7. Intra-class correlation (ICC) values for acoustic features of sexual status and individuals. Estimations of confidence intervals (CI), number of expected measurements (k), variance within (VarW) and among (VarA) classes for the corresponding number of classes (n) are shown.

Class	Acoustic feature	n	ICC	CI	k	VarW	VarA
Sexual status	Note Duration	3	0.45	0.18 – 0.97	763.70	0.0010	0.0008
	Call Duration	3	0.40	0.15 – 0.96	109.99	0.041	0.027
	Dominant Frequency	3	0.23	0.07 – 0.92	108836.2	70167.4	32171.8
	Aggregated Entropy	3	0.01	0.01 – 0.34	723.70	0.493	0.006
	Average Entropy	3	0.01	-0.01 – 0.13	763.70	0.221	0.0006
	Call Rate	3	0.16	-0.01 – 0.91	14.89	0.324	0.0604
	Sound Pressure Level	3	0.14	0.03 – 0.86	113.90	23.50	3.7146
Individual	Note Duration	30	0.62	0.51 – 0.75	80.29	0.0006	0.0009
	Call Duration	28	0.66	0.54 – 0.79	11.71	0.021	0.040
	Dominant Frequency	30	0.50	0.39 – 0.65	80.29	66060.1	66590.3
	Aggregated Entropy	30	0.63	0.52 – 0.76	80.29	0.190	0.326
	Average Entropy	30	0.61	0.50 – 0.74	80.29	0.089	0.140
	Call Rate	30	0.19	-0.42 – 0.57	1.55	0.295	0.069
	Sound Pressure Level	24	0.67	0.54 – 0.80	14.59	8.927	17.940

5. Discussion

In this study we report sexual dimorphism in spectral and temporal characteristics of Darwin’s frog’s advertisement calls. We also found that males keep calling after they have ingested a clutch to brood. In the population studied, relative abundance and relative vocal activity of individuals from different sexual status varied along the breeding season following dissimilar patterns. For non-pregnant males, the proportion of individuals increased but the proportion of callers diminished along the reproductive season, indicating that males keep concurring to the breeding areas throughout the breeding season, but their vocal activity tends to cease as the season progresses. For pregnant males, the proportions of number and of calling individuals increased from the beginning of the season, reaching a peak in January and decreased in February, indicating that mating rates of the population are maintained for about four months and decrease in the fifth month of the breeding season. For females, the proportion of individuals increased from the beginning to the end of the season, but their vocal activity peaked in November and February, revealing two maxima in their engagement in vocal activity. For this population of Darwin's frog, the different patterns of abundance and calling displays between males and females suggest that each sexual status performs characteristic temporal reproductive behaviors as the breeding season progresses. Furthermore, the number of males is not limited at any time, as the total abundance and number of calling males approximately doubles that of females throughout the breeding season.

Male and female calls of *R. darwinii* differ in note and call duration and dominant frequency. Although discriminant model was constructed including dominant frequency, note duration, call rate, average entropy and sound pressure level, the only acoustic feature that contributed to differentiate advertisement calls of the sexual status in *R. darwinii* was dominant frequency.

Overall, sexual dimorphism in advertisement calls of *R. darwinii* is apparently influenced by body size. In size dimorphic species, vocalizations may differ because of the relationship between dominant frequency and body size, but not due to selective pressures acting on the signal itself (Snowdon 2004). Additionally, biases in female size are to be taken with caution as they may not result from male choice (e.g. Woolbright 1983). Female breeders are the larger individuals in *R. darwinii* (e.g. Crump 2002), a relationship that has also been reported in anurans and other taxa (Fairbairn et al. 2007). Alternatively to a context of sexual selection, female signaling could be related to social selection (West-Eberhard 1983, 2014; Tobias et al. 2012) as female body size sets dominance relationships within social age-structured groups in different taxa (Wasser 1983; Buston and Wong 2014). In anurans, females are usually larger because they are older (Monnet and Cherry 2002) or grow faster than males (Kupfer 2007). As such, our study suggests that acoustic social displays of Darwin’s frogs, involving females and two sexual statuses of males, could be indicating their age or experience to the each other sex.

The number of larvae brooded affects the amplitude of pregnant male’s calls. The relationship between number of brooding larvae and call amplitude may indicate that brooding interferes with the efficiency of call emission and suggests that receivers could use this acoustic feature to evaluate the condition of pregnant males. The lack of clear differences between calls of males of different sexual status in *R. darwini* is in concordance with studies on midwife toads, *Alytes cisternasii* and *Alytes obstetricans* showing no differences between the calls of males carrying and not carrying eggs (Márquez 1990). In *A. obstetricans* a relationship between body size and the number of eggs carried occurs in one population (Raxworthy 1990) but neither significant relationships of this kind nor differences between males that carry and do not carry eggs, have been reported in subsequent studies in midwife toads (Márquez 1993; Bush 1996; Lea et al. 2003). Males of Darwin's frog do not stop calling after they have obtained their first clutch and similarly to midwife toad males, are probably available to obtain multiple matings (Bürguer 1905). Also, since call properties do not differ among pregnant and non-pregnant males of Darwin’s frogs but their call variation are influenced by size (Chapters 1 and 2), it is possible that size may be a relevant cue for female choice, which would contrasts with midwife toads where female preferences are not dependent on males' size (Dyson et al. 1998; Bosch 2001; Lea et al. 2003).

Additional knowledge on sexual maturity and spermatogenesis in this species is needed to understand why pregnant male Darwin's frogs call after mating. Vocalization of sexually immature males has been detected in the South American hylid frogs *Pseudis minuta* that undergo early spermatogenesis during their second to third years after metamorphosis, as juveniles emit calls with lower number of pulses and higher frequencies calls than larger adults (Goldberg et al. 2016). In a previous study, we found that within non-pregnant males, smaller individuals have calls with higher dominant frequencies and larger proportion of non-linear phenomena (Chapter 1), however in the present study two entropy indices measuring dispersion of the spectral energy of acoustic signals did not show differences between sexes and male status. Additionally, calling activity of males differed among non-pregnant males of Darwin's frog depending on size, as larger individuals call more actively than smaller ones. This difference could be related to the maturity status of non-pregnant males (Crump 2002). It is therefore possible that calling non-pregnant males of *R. darwinii* correspond to immature individuals with experience limitations for larvae breeding.

Our results showed higher repeatability for the call of *R. darwinii* among individuals than among sexes. Darwin's frog is a strongly philopatric species that maintains a restricted home range throughout the reproductive season (Valenzuela-Sánchez et al. 2014) and for many years. This individual call stability suggests that peculiar features of calls are relevant to sustain prolonged social interactions among permanent neighbors as reported in other sound-communicating species (Sheehan and Bergman 2016). According to our daily monitoring of frog calling activity, individuals of both sexes in *R. darwinii* typically call for up to three continuous days and can resume calling after three to sixty-two days. However, females are the sex that calls less often, which may indicate that their vocal behavior is restricted to times of readiness for mating and egg laying, as observed in other anuran species (Emerson and Boyd 1999).

In species in which both sexes display courtship signals, the sex that displays intra-sexual interactions is generally the one toward which sex ratio is biased (Clutton-Brock 2007). According to our data, in *R. darwinii* although the sex ratio is biased towards males, these subjects are not observed to engage in overt competitive behavior. Vocal activity of males is constrained to one to three continuous days, after which certain days pass in silence., and apparently, the readiness to reproduce may vary broadly along the reproductive season for males as much as for females. Alternatively to intra-sexual competition, the signals may have social effects, facilitating coordination and synchronization of breeding activities among sexes (Tobias et al. 2012; West-Eberhard 2014). Furthermore, signalizing individuality of *R.darwinii* may not relate to compete for mates but for multi-purpose areas like sheltering, feeding and reproducing as it has been proposed for other taxa (West-Eberhard 1983; 2014; Tobias et al., 2012).

In these instances, calling activity can be effective in modulating the reproductive statuses of male and female anurans (Burmeister and Wilczynski 2000; Lea et al. 2001; Lynch and Wilczynski 2006). Recent studies of experimental advertisement call broadcasts in the wild have demonstrated that these signals are relevant to promote and maintain gregarious reproduction in many anuran species (Buxton et al. 2015; 2018; James et al. 2015). As such, vocalization by both sexes could concur to maintain breeding activity of natural populations. Overall, the gregarious condition of Darwin’s frog, the persistent vocal exchanges between male and female callers, the sexual dimorphism of the advertisement calls related to inter-sexual body size differences and the absence of overt contests between individuals and sexual status, suggest that the calling behaviour of males and females could act mostly in indicating their reproductive status further than sexual selection processes in this enigmatic species.

CAPÍTULO 3. SEXUAL RECOGNITION BETWEEN FEMALES AND MALES THAT CAN CALL BEING PREGNANT: WHO AND HOW DO DARWIN'S FROG INTERACT VOCALLY?

Serrano, J.M., N. Guzmán-Collao, M. Penna, M. Méndez & C. Soto-Azat.

1. Abstract

Calling behaviour of anurans in reproductive contexts is mostly performed by males, but in species in which females call it is not known how sex recognition occurs, particularly when sexual dimorphism in signals is low, as in cases in which call repertoire is identical but acoustic properties differ. To explore the mechanisms behind vocal recognition among the different sexual status of the Darwin's frog (*Rhinoderma darwinii*), a species in which males perform brood larvae inside their vocal sacs and females have higher dominant frequency and shorter calls and notes than males, we recorded natural duets between non-pregnant males, pregnant males and females and evaluated their evoked vocal response to natural calls of each sexual status from November 2015 to February 2016 in the island of Chiloé, Chile. Call rate, phase angles, sound pressure level (SPL), number of overlapped calls and delay of overlapped calls were measured to determine differential responses between natural duets and in response to stimuli consisting of natural calls of individuals with different sexual status and synthetic calls representing individuals of different body size. Spontaneous interactions observed among duets occurred mainly between males and particularly between non-pregnant males. Call ratios in response toward calls of different sexual status were similar. Females decreased their SPL in response to female calls, while females and pregnant males decreased their call overlapping and increased their call delay to each other, respectively. In contrast, non-pregnant males responded preferentially to

small and medium body size stimuli and responded with lower phase angles to medium body size stimuli. Our results suggest that that pregnant males and females of *R. darwinii* could signal sexual motivation by increasing their call alternation to the other sex and that call overlap is a key factor probably regulating interactions between males.

2. Introduction

All forms of selection driven by social interactions, including sexual selection depend on signals indicating the quality or identity of emitters (Darwin 1871; Ryan and Rand 1993; Lyon and Montgomerie 2012; Keen et al. 2016). The display of sexual signals has been mostly considered an exclusive feature of males (Price 2015; Köhler et al. 2017; Clutton-Brock et al. 2009), however, growing evidence has shown that females can display sexual signals in various taxa (insects: Bailey 2003; mammals: Wasser 1983; birds: Odom et al 2014; and anurans: Serrano and Penna 2018), questioning their role as traits that only mediate choice by females and competition between males (West-Eberhard 1983; Lyon and Montgomerie 2012; Tobias et al., 2012). Furthermore, the duets and choruses formed by males and females may contribute new explanations about the role of signal exchanges in social processes, facilitating cooperation, group cohesion and mediating conflicts (Bailey 2003, Tobias et al 2012, Price 2015, Fishbein 2018).

In complex social environments, different timing interaction patterns occur among conspecifics, ranging from strict synchrony to strict alternation (Grafe 2005). In addition to call timing patterns, other features relevant to quantify vocal interactions include leadership patterns among interacting callers (e.g. Naguib et al. 1997), switching between call types (e.g. Given 1987) and variation in signal amplitude (e.g. Stewart and Bishop 1994). The relevance of these additional features depends on the temporal patterns of signal exchanges, the social environment in which they occur, and the sensory capabilities of the subjects involved (McGregor 1992).

A largely unexplored issue in animal communication is how the timing of acoustic signals involved in recognition of conspecifics contribute to group cohesion (Moore et al. 2016; Sheehan and Bergman 2016). In birds, song overlap has been mostly considered to operate in agonistic contexts, although alternative instances such as eavesdropping and song learning are related to this interaction pattern, depending of the taxa involved (Peake 2005; Helfer and Osiejuk 2015). Cooperative and competitive hypotheses have been put forward to explain synchrony and alternation of calls in anurans, insects and birds, although the support for cooperative interpretations is relatively limited (Greenfield 1994; Bosch 2001; Grafe 2005). In the context of intersexual singing duets, some bird species have shown to emit sex-specific responses for territory defence and mate guarding purposes like the suboscine chestnut-backed antbird (*Myrmeciza exsul*) (Fishbein et al. 2018) and intrasexual female vocal exchanges mediate competitive interactions in the sexual role reversed African black coucal (*Centropus grillii*) (Geberzahn et al. 2009).

In anurans, intrasexual competition occurs widely among males but no examples of vocal competition among females have been identified (e.g. Cui et al. 2010), although females may exhibit aggressive behaviour toward males (Preininger et al. 2016) or toward nest intruders (Steward and Rand 1991). Even in species of the genus *Alytes* in which parental care is exclusively conducted by males and female vocalization has been extensively studied (Verrell and Brown 1993; Bosch and Márquez 1996; Bush and Bell 1997; Márquez and Bosch 2001) females do not show clear intrasexual vocal competition

(Bosch 2001; 2002). However, females have been observed to perform physical aggression in captivity (Bush and Bell 1997).

Darwin's male frogs brood in their vocal sac larvae collected from eggs laid by females and fertilized externally by males (Bürger 1905). In captivity and in field conditions males have been observed to get pregnant of larvae of different stages of development (Jimenez de la Espada 1872). However, the occurrence of sexual and social interactions within and between sexes in Darwin’s frogs have not yet been determined. Recently, the variation and occurrence of non-linear phenomena in advertisement calls of non-pregnant males of this frog under natural conditions have been studied (Serrano et al. in review). Here an association between non-linear phenomena and body size have been described, as well as a high potential for individual distinction of non-linear variables of male advertisement calls (Chapter 1). Additionally, in a previous study it has been found that advertisement call shows a sexual dimorphism that varies according to body size differences between males and females but lacks clear differentiation between males with different pregnancy status (Chapter 2). The aim of the current study is to understand the role of vocal signalling in a social environment conformed by individuals of distinct sexual status. In this study, we evaluate the hypothesis that calls provide sexual status identity, recording natural vocal interactions among individuals of different sexual status and performing evoked vocal response experiments to stimuli representing the diverse sexual status. Additionally, synthetic stimuli were generated to evaluate the alternative hypothesis that calls from males and females signalize information about their sexual status and body size.

3. Materials and methods

Observations and experiments reported in this study were conducted in Parque Tantauco, a private reserve located on the island of Chiloé, Chile (43° 21´ S; 74° 6´ W), during 2016 and 2017. Frogs were manipulated to obtain individual photographs and morphometric measurements of body length and body mass after recording their calls. Strict biosecurity standards were followed during the manipulation of individuals and are detailed in Chapter 1.

3.1.Duets' recordings

Natural duets were recorded with a digital recorder Tascam DR-100 at a sampling rate of 44.1 kHz and 16-bit resolution and two Sennheiser ME-66 microphone plugged to each recording channel. The distance separating the two subjects intervening in the duet was measured. Additionally, sound pressure level (SPL, re 20 µPa, C frequency weighting and fast time weighting) of calls of the individuals conforming the duet was measured with the microphone of a sound level meter (Extech 407780) placed adjacent to the tip of the directional microphone at 90 degrees relative to the frog's head. This variation in distance depended on the topography of the sites on the vicinity of the calling posts of the different subjects recorded. Air temperature was recorded after each acoustic recording by placing a thermohygrometer (Sper Scientific 850070) 5 cm above the position of each of the subjects.

3.2.Acoustic analysis of duets

Interactions between duetting individuals were evaluated by measuring the latency as the delay between the onsets of calls of one individual relative to calls of the other subject. Subsequently, interval delays were transformed to phase angles, following Klump and Gerhardt (1992). The inter-call period between successive calls of individual A was considered to correspond to a 360° reference angle, and the lag time in which individual B follows A during the inter-call interval A – A (Figure 11). This was used to calculate a lagging angle of call interaction by dividing the angle of reference 360°. So, for example, if call of B is emitted just in the middle of inter-call interval of A, it would correspond a phase angle of 180°. As individuals A and B were named randomly, the same procedure was employed to calculate the lagging angle for individual A relative to B (Klump and Gerhardt 1992; Bosch and Márquez 2001). The differences in phase angles between individuals of each pair were computed to identify the leaders and followers in duets, as leaders and followers yielded values between 180 – 360°and 0 – 180°, respectively. Additionally, correlations between the intervals between the onsets of successive calls of an individual and the interval between the preceding call of its duetting counterpart in vocal interacting pairs (A – A, A – B, B – B and B – A) were obtained to determine if the timing of the calls of the duetting pairs experienced adjustments on the course of interactions (Bosch and Márquez 2001). Furthermore, an additional way to measure interaction among duetting pairs was by counting the number of calls overlapping among pairs within each recording.

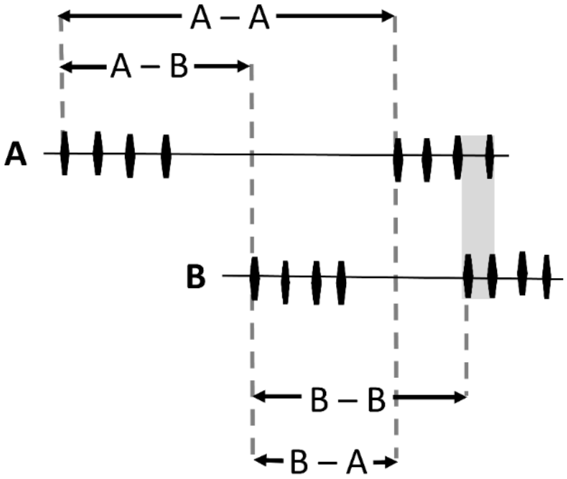

Figure 11. Schematic diagram of vocal interactions measurements for natural duets and playback experiments between leading (A) and lagging (B) individuals or between stimulus (A) and responding experimental subject (B), respectively. An overlap between duetting individuals or between stimulus and call response is illustrated in grey shadow and it corresponds to the time interval between the offset of the second call or stimulus in A and the onset of the call in B. This interval in natural duets corresponded to the overlap delay (A-B) for individual B relative to A, and in playback experiments to the overlap delay of the calls of experimental subjects relative to stimulus. Modified from Klump and Gerhardt 1992.

Finally, when overlap of calls between the duetting individuals occurred, the latency was quantified and referred to as overlap delay from the onset of individual A to the onset of individual B. As such, overlap delay corresponded to a measure of time between pair A-B when call of B began before the call A ended (Fig. 11). By measuring overlap delay we registered the readiness of callers to respond to predecessor calls and to stimuli, while by measuring phase angles we registered the rhythm of call alternation between two callers and from callers to the bouts of stimuli. Therefore, call overlapping depends on the readiness to response of a focal subject, while phase angles show the interaction of focal subjects with natural and playback inter-call intervals.

3.3.Playback stimuli and experimental design

Natural stimuli consisting of vocal sequences of individuals of the different sexual status produced within social aggregations were constructed. Isolated four-note calls recorded from different individuals were edited excluding transient environmental noises present in the recordings. Examples of natural and calls used to build bouts of stimuli are shown in Fig. 6.

Call bouts were composed of 10 natural calls from individuals of the same sexual status having a high signal to noise ratio. The time intervals between successive calls within a call bout were generated with random intervals of silence lasting 5 – 60 s, which correspond to the inter-call intervals that occur naturally in call sequences of individuals of this species (Chapter 2). Using this procedure, bouts of calls having different call rates and lasting 138 – 399 s were obtained. This randomization in call timing allowed to evaluate the temporal relationship of the evoked vocal response to the stimuli, discarding that responses correspond to a rythmic calling behaviour based on an internal oscillator (Zelick and Narins 1985; Klump and Gerhardt 1992). Three-minute silent intervals were included between call bouts of the different sexual status (Fig. 12). The order of presentation of call bouts of each sexual status was randomized.

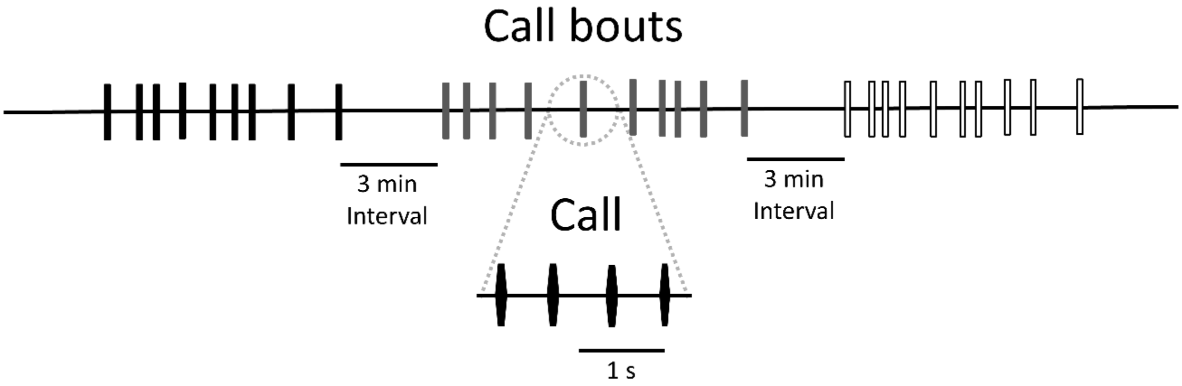

Figure 12. Diagram representing the composition of a sequence of call bouts presented in evoked vocal response experiments. Call bouts containing stimuli of different characteristics (sexual status or size category) are indicated by different gray shades.

As calls of individuals from different sexual status differed in some acoustic variables related to body size (see below), two experiments were carried out: one with natural stimuli to evaluate the evoked responses of individuals from each sexual status to calls from their own and the other sexual status, and a second experiment using synthetic stimuli to evaluate the dependence of evoked vocal response from the ranges of body size approximating those of each sexual status. As such, synthetic calls differing in their dominant frequency, note and call duration, representing small, medium and large subjects of the three sexual status indistinctly, were built using the information (reported in Results) on relationship among body size and acoustic features within each sex (Table S1). Procedures for randomizing call timing as those employed to build natural stimuli playbacks and stimuli presentation order were used to build bouts of synthetic calls.

In total, 44 call bouts were built, 32 corresponding to natural calls of non-pregnant males (N=14), pregnant males (N=12) and females (N=6), and twelve synthetic bouts of calls corresponding to small (N=4), medium (N=4) and large (N=4) individuals of the three sexual status. Call bouts were randomly combined in 21 trials, corresponding to 14 trials of natural stimuli and seven trials of synthetic stimuli (Fig. 13; Table S2). Each trial was presented only once to different individuals. The total stimulation time for each individual was 26 to 32 minutes. All the natural and synthetic stimuli were managed and synthetized using Adobe Audition 3.0.

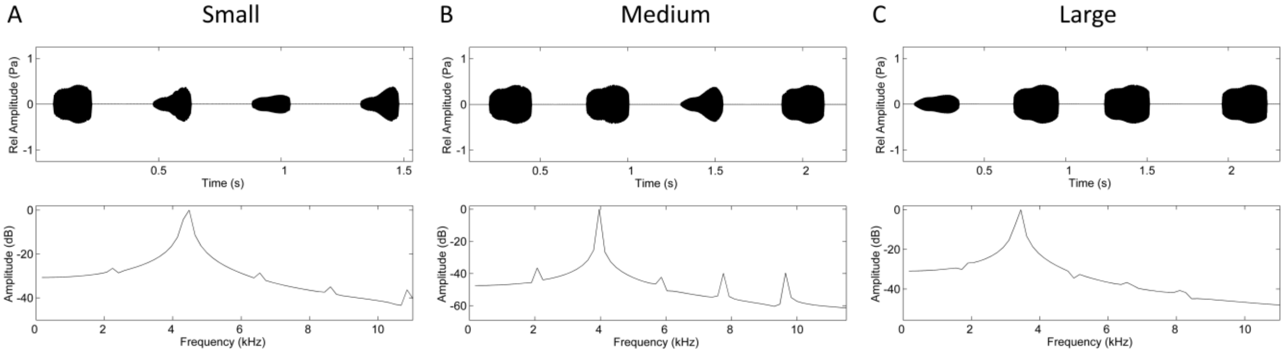

Figure 13. Oscillograms and power spectra of synthetic stimuli for individuals of small (A), medium (B) and large (C) body size. Sample rate was 44.1 kHz for all the recordings.

Amplitude of stimuli (maximum dB SPL RMS) was checked for each sequence at the study site on a silent time interval. Maximum SPLs of playbacks ranged originally between 61.0 – 71.9 and were adjusted to a SPL as constant as possible, by modifying the gain settings of the loudspeaker for each playback, resulting in values of 61 – 64 dB SPL. These values approximate the average of 63 dB SPL measured at 18 – 68 cm from calling individuals of this population (Chapter 1; see Results). For these measurements, stimuli were delivered through a portable loudspeaker (i.Sound 5464) and the microphone of the sound level meter (Extech 407780) was placed one meter away from the loudspeaker in an area with no interfering vegetation.

3.4.Playback protocol

Playback experiments were carried out during January and February 2016 and February and March 2017. Twenty-one relatively isolated and vocally active subjects were exposed to acoustic playbacks. Stimuli were presented with a Samsung J1 WAV player connected via Bluetooth to a portable loudspeaker (i.Sound 5464). The loudspeaker was placed on moss vegetation at 1 m from the subject and at an angle of about 90 degrees relative to the frog's head. The loudspeaker was placed avoiding interfering vegetation or trunks between this device and the subject. Evoked vocal responses were recorded as described above. Upon completion of each playback experiment, body size and sex of individuals were registered.

3.5.Acoustic analysis of evoked vocal responses

Responses to natural and synthetic stimuli were analyzed measuring call rate, latency (measured as phase angle relative to the stimulus as done for the analysis of timing between duetting individuals) and SPL. Call rate and SPL were computed for periods of silence and stimulus presentation, while latency was only measured for stimuli presentations. Additionally, as it was done for duet recordings, phase angle, number of overlaps and overlap delay between evoked calls and stimuli calls were measured (Fig. 12). Due to the small number of overlaps and the low number of repetitions of experiments with synthetic stimuli, overlap delay was quantified for natural stimuli only.

3.6.Statistical analysis

To evaluate whether individuals forming presumed duets adjusted the timing of their calls, Pearson correlations between the inter call periods of one individual and latencies from call onset of the counterpart individual to call onset of the focal individual were calculated to determine whether synchrony adjustments occurred during vocal interactions (Bosch and Márquez 2001). In addition, a Wilcoxon-test was employed to evaluate whether phase angle differences were constant among duetting individuals. Finally, general linear models (GLMs) with binomial distribution were used to evaluate the occurrence of overlapped versus non-overlapped calls between and within pairs of natural duets and from focal subjects responding to playback stimuli.

The effects of acoustic stimulations on evoked vocal responses were evaluated using GLMs with Gamma distribution tests to assess the variation resulting from testing multiple stimuli between the different sexual status of *R. darwinii*. This evaluation in search of differences among vocal responses was performed by means of two comparisons in one of which the inter-bout periods of silence between stimuli were considered and another in which they were excluded. As phase angle and number of overlaps were measured only along bouts of stimuli, these variables were analyzed considering the response to non-pregnant males as a reference to compare the responses to calls of the other sexual status. However, as call rate and SPL were measured both during bouts of stimuli and inter-bout periods of silence, these variables were compared with the pre-stimulation period of silence (i.e. S1), as it corresponds to the basal vocal activity of the experimental session. In addition, to compare the responsiveness to each stimulus, the number of call overlaps was analyzed for each sex using GLMs with Poisson distribution test. Finally, ANOVA and post-hoc tests were used to compare overlap delays to calls of the three sexual status. All these statistical analyses were performed for responses of all the sexual status to natural stimuli and for responses of non-pregnant males to synthetic stimuli only, due to the low number of pregnant males and females tested with synthetic stimuli.

4. Results

4.1.Duetting interactions

Thirteen interactions were recorded between individuals belonging to the three sexual status. Duetting individuals were positioned at distances ranging from 0.17 to 19.5 m. Duetting interactions were assumed to occur when no other callers were heard by the researchers in addition to the focal individuals. Duetting interactions were recorded between non-pregnant males (N= 5), non-pregnant males and females (N= 4), pregnant males (N= 3), and a non-pregnant male and a pregnant male (N= 1). No interactions were observed among females and among pregnant males and females (Table 8). Differences in call delays (phase angles) between duetting individuals were observed in three pairs only: two duets of non-pregnant males, and one formed by a non-pregnant male and female. In the three interactions the smaller individuals showed significant shorter response phase angles, indicating their follower status relative to the leader callers of larger size.

The correlation between the onsets of successive calls of an individual and the interval between the preceding call of its duetting counterpart in vocal interacting pairs was significant in one of 13 pairs analysed only (Table 8), indicating that most duet pairs did not adjust their calls to the timing of each other signals as the interaction progressed. In natural duets, overlapping on average was larger for non-pregnant males (13 %), followed by females (11%) and pregnant males (7%). However, overlapping was more dissimilar in females, as they overlapped with 22% of the calls of non-pregnant males but did not overlap with calls of pregnant males (Table 9).

Table 8. Relationship of call alternations among duetting individuals of *Rhinoderma darwinii*. Sexual status, body size, number of calls (n), phase angle, sound pressure level (SPL) and correlation coefficient (r) among latencies between calls of duetting individuals are shown. Significant differences ($p<0.05$) in phase angles of calls between duetting individuals and r values are highlighted in black. Abbreviations: NPM= non-pregnant males; PM= pregnant males; F= females.

Sexual status of duet	Month	Duet ID	Body size (mm)	n	Distance (cm)	Phase angle (°)	SPL (dB)	r
NPM– NPM	Nov	1 (male A)	22.7	15	193	**112.64**	61.86	0.14
		1 (male B)	21.3	26		**50.72**	–	0.03
	Nov	2 (male A)	21.8	15	143	180.51	53.45	0.08
		2 (male B)	23.2	28		100.92	–	-0.12
	Feb	3 (male A)	21.6	24	402	**41.25**	65.66	0.08
		3 (male B)	22.7	29		**213.25**	–	-0.20
	Feb	4 (male A)	21.4	40	981	64.51	–	-0.42
		4 (male B)	22.5	9		61.99	–	0.50
	Feb	5 (male A)	20.6	22	1947	295.51	58.82	-0.15
		5 (male B)	23.0	18		88.26	–	-0.24
NPM – F	Nov	1 (male A)	22.7	18	44	**36.68**	61.24	0.31
		1 (female B)	23.7	13		**124.07**	–	-0.15
	Dec	2 (male A)	21.2	14	180	83.31	65.52	0.43
		2 (female B)	22.2	9		109.80	–	**0.98**
	Feb	3 (female A)	22.7	21	790	38.18	65.58	-0.15
		3 (male B)	19.4	12		191.54	–	0.17
	Feb	4 (female A)	22.1	30	77	51.52	67.18	-0.06
		4 (male B)	23.2	20		90.29	–	0.06
PM – PM	Jan	1 (male A)	22.6	32	26	79.27	65.51	-0.37
		1 (male B)	20.9	19		121.17	–	-0.30
	Jan	2 (male A)	22.4	20	948	109.10	70.21	-0.20
		2 (male B)	21.8	14		113.65	–	-0.37
	Feb	3 (male A)	22.3	13	56	18.67	64.54	-0.54
		3 (male B)	21.7	19		77.85	–	0.42
NPM – PM	Dec	1 (male A)	23.0	27	17	35.25	60.02	-0.40
		1 (male B)	22.6	10		187.83	–	0.33

The analysis of overlap delays allowed to estimate the responsiveness to ongoing calls of other individuals. Because of the limited samples, the analysis for pregnant males and females was done considering all duetting counterparts pooled together and only for non-pregnant males the analysis was done separately for the duetting counterparts of the three sexual status. For duets in which call overlaps occurred, the overlap delays for the two types of males had a median of about 0.5s and for females it was about 1.0 s (Fig. 14A; ANOVA test, Chi^{2}= 6.134; p <0.05). Overlap delay of non-pregnant males was significantly different (ANOVA test, Chi^{2}= 6.972; p <0.05), and post hoc testing revealed that overlap delay to females were shorter than to both types of males (Fig 14B).

Table 9. Overlapping and non-overlapping calls in vocal interactions between individuals of the three sexual status in natural duets and playback experiments. Abbreviations: non-pregnant males (NPM), pregnant males (PM) and females (F). Significant differences (*: p<0.05; **: p<0.01; ns: non-significant; nc: non-calculated).

	Caller		Total number of calls			
Interaction	Focal individual	Preceding individual	Overlapping	Non-overlapping	% of Overlapping calls	Binomial test
Natural duets	NPM	NPM	22	171	11.4	**
		PM	7	36	16.3	ns
		F	25	178	12.3	**
	PM	NPM	1	26	3.7	*
		PM	24	203	10.6	**
	F	NPM	11	39	22.0	ns
		PM	0	12	0	nc
	Focal individual	Natural stimuli	Overlapping	Non-overlapping	% of Overlapping calls	Binomial test
Playback experiments	NPM	NPM	14	33	29.8	ns
		PM	20	21	48.8	ns
		F	16	22	42.1	ns
	PM	NPM	8	11	42.1	ns
		PM	8	14	36.4	nc
		F	1	19	5.0	*
	F	NPM	24	21	53.3	ns
		PM	15	23	39.5	ns
		F	19	37	33.9	ns

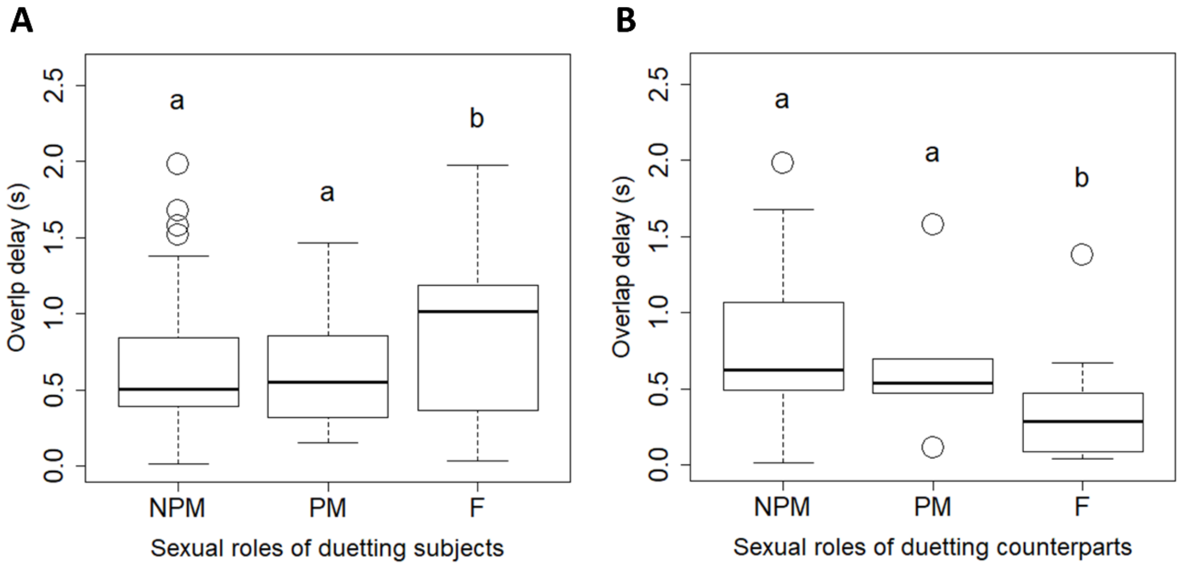

Figure 14. Overlap delays observed for duets between individuals of each sexual status and individuals of the three sexual status combined (A) and duets between non-pregnant males and individuals of the three sexual status (B). Abbreviations identify the three sexual status: non-pregnant males (NPM), pregnant males (PM) and females (F). Different low-case letters (a, b) indicate significant differences in post-hoc analyses ($p < 0.05$).

4.2.Evoked vocal response experiments

Thirty-two individuals were stimulated with natural stimuli, 14 of which were non-pregnant males, 12 pregnant males and six females. For all the acoustic variables analyzed, large individual variability occurred and in some cases significant differences in the responses of the three sexual status analyzed separately occurred. In terms of responsiveness, the three sexual status did not show call ratio changes in response to stimuli relative to the inter bout silent periods. The only significant differences in call rate occurred among silent periods, as pregnant males and females increased (GLM test, $t=2.022$, $p=0.0509$; $t=3.461$, $p<0.01$, respectively) and non-pregnant males decreased their call rates to inter bout periods of silence (GLM test, $t=3.461$; $p<0.01$) relative to their initial (S1) call repetition rate. In terms of SPL, the only differences observed corresponded to females for which a significant decrease in SPL (Chi^2= 17.437; p< 0.01; Fig 15A) was observed in response to female calls (GLM test, t=3.136; p<0.01) and during two periods of silence (GLM test, t=2.542, p<0.05, t=-2.387, p<0.05) relative to the initial silent period (S1). In terms of phase angle, females responded with larger phase angles to the calls of pregnant males relative to the other two sexual status (GLM test, t= -2.573; p< 0.05; Fig 15B). In terms of call overlaps, pregnant males responded with lower number of overlaps to the calls of females (GLM test, z= -1.691; p<0.05; Fig 15C) relative to the other two sexual status.

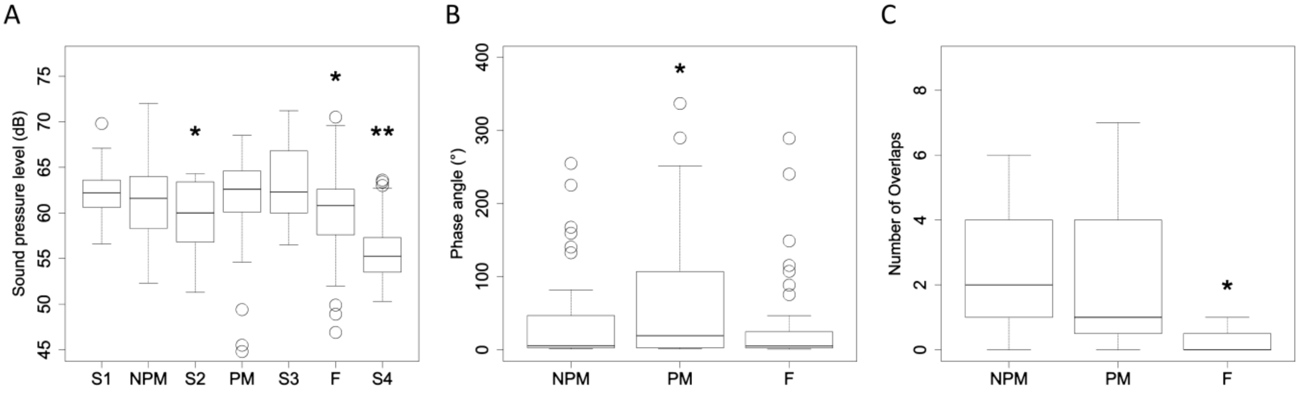

Figure 15. Sound pressure level (A) and phase angle (B) in evoked calls of females, and number of overlaps in evoked calls of pregnant males (C) in response to natural stimuli of the three sexual status. Stimuli abbreviations: NPM: non-pregnant males, PM: pregnant males, F: females. S1, S2, S3 and S4: silent intervals between stimuli presentations. Asterisks indicate significant differences in post-hoc analyses relative to S1 in A, and between stimuli in B and C (Tukey tests, *: p<0.05, **: P<0.01).

In playback experiments, overlapping produced by pregnant males was the most dissimilar, as they overlapped in a 42% with calls of non-pregnant males but only 5% with female calls, however the number of overlapped and non-overlapped calls produced by non-pregnant males and females were similar (Table 9). The occurrence of call overlaps was larger in playback experiments relative to duet interactions (z= 8.11; p< 0.001). In addition, the number of overlapped versus non-overlapped calls was significantly lower between duet interactions in contrast to playback experiments (Table 9). Finaly, overlap delay differed among sexual status ($Chi^2 = 7.107$; $p < 0.05$), and post hoc tests revealed that females responded with a shorter overlap delay to all the stimuli combined relative to both types of males (Fig. 16).

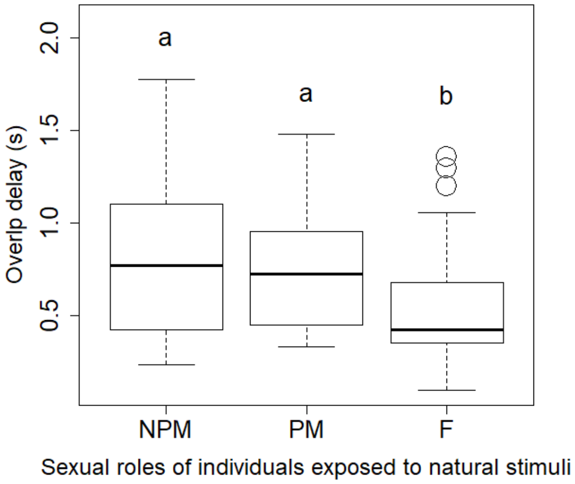

Figure 16. Overlap delays observed for duets between individuals of each sexual status and all natural stimuli combined. Abbreviations identify the three sexual status: non-pregnant males (NPM), pregnant males (PM) and females (F). Different low-case letters (a, b) indicate significant differences in post-hoc analyses ($p < 0.05$).

Synthetic stimuli simulating calls produced by individuals of small, medium and large body size were tested in a small number of experimental subjects: five non-pregnant males and two pregnant males. Statistical analyses were not applied to responses of pregnant males to synthetic stimuli due to the low number of subjects but the corresponding graphs are shown in order to provide a visual assesment of their responses relative to non-pregnant males. In terms of responsiveness, non-pregant males showed higher call rates to the stimuli of small and medium size individuals relative to the initial silent period (S1)

(GLM test, t=2.492, p< 0.05; t= 2.107, p= 0.0536; Fig. 17A) and, in addition, non-pregnant males responded with lower phase angles to stimuli representing medium body size frogs relative to the other two stimuli (GLM test, t= -2.133; p <0.05; Fig. 17B). No differences occurred in overlap of responses with stimuli.

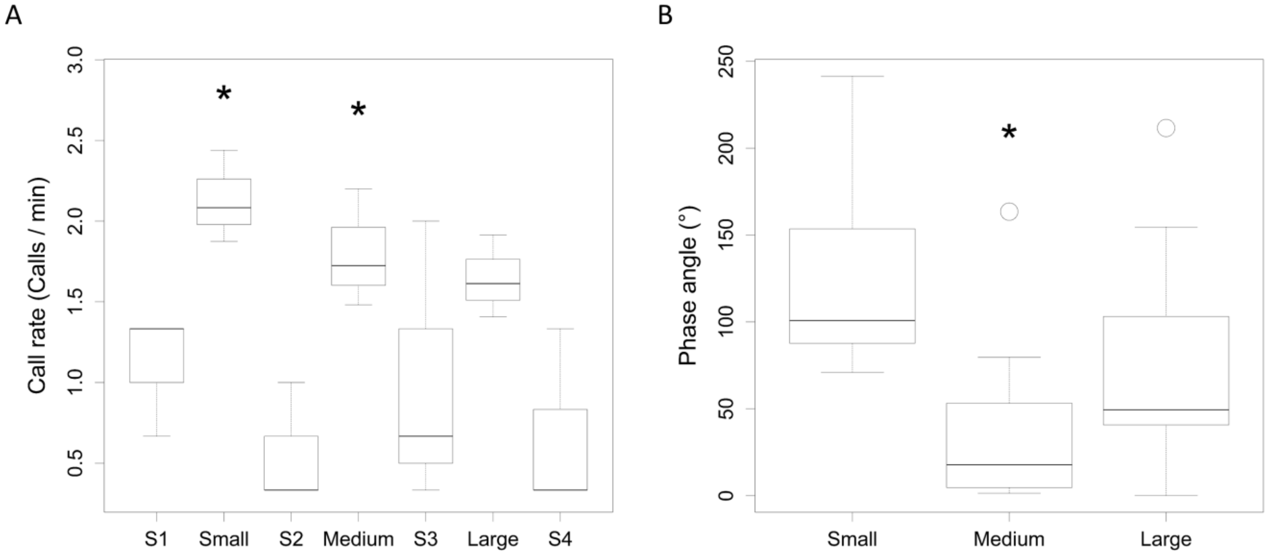

Figure 17. Call rate (A) and phase angle (B) in evoked vocal responses from non-pregnant males to synthetic stimuli. Asterisks indicate significant differences in post-hoc analyses relative to S1 in A, and between stimuli in B (Tukey tests, *: p<0.05, **: P<0.01)

5. Discussion

Darwin's frog exhibits non-stereotyped responsiveness patterns in the temporal domain in natural vocal interactions between individuals of the same or different sexual status and in response to acoustic playback experiments. Males overlap their counterparts during intrasexual vocal interactions, and predominantly alternate with a longer delay with individuals of the opposite sex. We propose that this could be an acoustic mechanism to stabilize vocal interactions, as males and females do not engage in vocal aggressive behaviours as occurs in other anurans that communicate vocally. Moreover, in contrast to the expected behaviour of intense female competition when males perform parental care (Lyon and Montgomerie 2012; Tobias et al., 2012; Stockley and Campbell 2013), vocal behaviour between females of *R. darwinii* lacks aggressions such as in males.

Duets between males are more frequently observed than between males and females, as female vocalization in *R. darwinii* occurs less frequently relative to males in the population studied (Chapter 2). In only 3 out of 13 natural duets individuals having a leader and follower status were identified based on differences in their phase angles. The leader of the duet was the largest individual in all the cases and occurred between two pairs of non-pregnant males and between a duet of non-pregnant male and female. In the rest of the natural interactions the phase angles of duetting males were similar. Furthermore, in only one of the duets recorded a caller adjusted the timing of its emissions to the timing of the calls of its partner, as shown by a significant correlation between the timing of its calls relative to the call period of its counterpart.

Individuals of the three sexual status overlapped their calls with those of their duetting partners, however significant differences occurred in the number of overlaps between non-pregnant males were higher than in duets between pregnant males and between non-pregnant males and females. In addition, the overlap delays of non-pregnant males were shorter for female counterparts than to males of both categories. This indicates that, although non-pregnant males tend to overlap to each other they also show a higher readiness to respond to females. These differences in selective call overlap between duetting pairs arise as a novel mechanism of sexual recognition instead of call responsiveness.

In birds, it has been suggested that song overlap can be perceived as an aggressive signal (Naguib and Mennill 2010; Helfer and Osiejuk 2015); however, in anurans signal overlap has been related to predator avoidance (Tuttle and Ryan 1992) and as a means to increase the distance over which calls are detected (Lörcher 1969; Wells 1977). In species in which only males vocalize or even in those where males and females are notoriously sexually dimorphic, call timing between males produce a different influence on female attraction among anurans. For example, females of the running frog *Kassina fusca* can shift their preferences between the leader and follower call depending on whether call overlap is high (75 and 90%) or low (10 and 25%), while females do not exhibit preferences when call overlap is intermediate (50%) and complete (100%) (Grafe 1999). In the case of the midwife toad *Alytes obstetricans*, precise call alternation (with phase angles lower than 180°) between males can increase female attraction (Bosch and Márquez 2001). However, call overlap does not increase attraction of females of the glassfrog *Cochranella granulosa* (Ibañez 1993). In *R. darwinii*, call overlapping could regulate preferentially intra- and intersexual vocal interactions, as each sex stablish vocal duets with different call timing with each other.

Like *R. darwinii* was not responding selectively to the stimuli in terms of call responsiveness (call rate), different species have shown similar responses in terms of call intensity and call rate to signals of different types produced by heterospecific (Eckenweber and Knörnschild 2016) and conspecific competitors (Tárano 2002) or independent of the incoming direction (Penna and Quispe 2007). Vocal response of females of *R. darwinii* to other female calls decreased in terms of SPL but females also respond more readily in terms of phase angle to calls of other females and non-pregnant males than to pregnant males, which could indicate that female can shift their SPL and promptness to respond in contexts of intra- and intersexual interactions. Because in playback experiments, pregnant males and females respectively decreased and increased their call overlapping to females only, it is likely to affirm that by means of call overlap, Darwin's frog performs a form of intrasexual vocal competence, while call alternation could be a way to engage in intersexual interactions. In addition, among all responses to natural stimuli, females responded with shorter overlap delays to other callers independent of their sexual status. This faster readiness of females to respond during playback experiments (Fig. 16) contrasts with the delayed response of females observed in natural duets (Fig. 14A) and suggests that females have higher readiness to respond to novel than to habitual neighbors.

Unfortunately, due to the low number of experiments with synthetic stimuli, our results for these playbacks are not conclusive; however, it was evident that non-pregnant males responded to medium size stimuli with higher call rate than to the initial silent period and with significant shorter phase angles to stimuli of medium size individuals relative to small and large individuals. Vocal responses of males depend on call features of the opponent have been reported in different anurans (Bosch and Márquez 1996; Penna and Meier 2011; Penna and Toloza 2015). Evoked vocal responses dependent on dominant frequency have been reported in anurans like the carpenter frog, *Lithobates virgatipes* (Given 1987) and the green frog, *Rana calamitans* (Bee et al. 2000). Since spectral properties and call duration of the Darwin’s frogs’ are strongly influenced by size (Chapters 1 and 2) and considering that in natural duets the smaller males tend to follow the leadership of the duet, it is likely that body size can be signalled by calls in natural interactions.

Overall, playback experiments with natural and synthetic stimuli showed that Darwin's frog can recognize individuals of the different sexual status through their calls and that cues leading this recognition based in the advertisement calls of each sexual status is rather unclear. Vocal responses of Darwin’s frogs in experimental and natural interactions show competitive vocal behavior by means of call delay and call overlap particularly among same-sex interactions. In species with sex role reversal such competitive intrasexual behavior is expected to occur among females only (Vincent et al. 1992; Clutton-Brock 2007). However, males and females of *R. darwinii* showed similar readiness to compete vocally with their own sex, particularly by increasing their call overlapping. However, such intrasexual competitive vocal behaviour is not evident in Darwin’s frogs in terms of call rate or SPL responsiveness. Such lack of specificity in response to male and female stimuli has been observed in *L. virgatipes*, a frog in which females call similarly to the males (Given 1993). A similar responsiveness to intensity and call-rate properties occurs in females of *Alytes cisternasii*, a species having sexually dimorphic calls (Bosch 2002). With the exception of vocal responses of non-pregnant males to synthetic stimuli of medium versus small and large size, females and pregnant males *R. darwinii* do not produce different responses to acoustic stimuli of their own sex or reproductive status, which indicates that each sex reproductive assume different vocal risks based on the own reproductive status and that of the contestant.

Aggressive vocal behavior is often performed in anurans by producing distinctive aggressive calls or manifested in graded changes of call intensity or call rate (Wells 2007). Such aggressive behavior is often performed by the sex assuming the higher reproductive costs, which is allegedly carried by males in species exhibiting sex-role reversal (Vincent et al. 1992). However, it has never been observed aggressive vocal behavior performed by males and females in Darwin's frogs (Penna and Veloso 1990; Chapters 1 and 2, This Study). The lack of both a clearly call responsiveness and an aggressive vocal behaviour in *R darwinii* suggests that instead of increasing call intensity or call rate to sustain intrasexual vocal interactions, Darwin's frog engages in intra- or inter-sexual vocal interactions regulated by call overlap adjustments. Thus, pregnant males and females of *R. darwinii* are relatively selective in their modes of synchronization with calls of different sexual status and non-pregnant males interact similarly with all the sexual status, a strategy likely to favour spatial tolerance of potential breeding partners.

6. Supplementary Information

Table S1 Mean acoustic features of natural and synthetic bout calls representing advertisement calls of *Rhinoderma darwinii* related to different sexual status and body size categories.

Stimuli category	ID	Dominant frequency (Hz)	Note duration (ms)	Call duration (s)
Non-pregnant males	1	3923	131	1.4
	2	3402	156	1.6
	3	3488	182	1.7
	4	3962	140	1.4
	5	3487	181	1.8
	6	3930	141	1.5
	7	3769	115	1.0
	8	3650	158	1.5
	9	3538	179	1.9
	10	3840	142	1.3
	11	3952	201	1.6
	12	3841	144	1.3
	13	3365	133	1.6
	14	3377	134	1.6
Pregnant males	1	3273	162	1.6
	2	3634	176	1.7
	3	4060	168	1.5
	4	3548	209	1.8
	5	3572	204	1.8
	6	3617	152	1.4
	7	3230	147	1.5
	8	4272	133	1.4
	9	3818	209	1.9
	10	3652	141	0.9
	11	3618	166	1.5
	12	3676	174	1.6
Females	1	3682	157	0.5
	2	3341	257	2.1
	3	3686	196	2.0
	4	3682	152	0.7
	5	2973	162	1.5
	6	2995	163	1.5
Small	1	4162	113	1.3
	2	4174	113	1.4
	3	4156	123	1.3

Stimuli category	ID	Dominant frequency (Hz)	Note duration (ms)	Call duration (s)
	4	4158	123	1.4
Medium	1	3596	173	1.65
	2	3590	183	1.7
	3	3592	183	1.75
	4	3576	193	1.65
Large	1	3036	253	2
	2	3050	253	2.1
	3	3087	253	2
	4	3011	233	2.1

Table S2. Sequence of call bouts for stimuli trials used in playback experiments of sexual status and body size recognition. Abbreviations: non-pregnant males (NPM), pregnant males (PM) and females (F).

		Sequence	
ID	1	2	3
A	NPM1	PM1	F1
B	NPM2	F2	PM2
C	PM3	NPM3	F3
D	NPM4	F4	PM4
E	F1	NPM5	PM5
F	PM5	F2	NPM6
G	PM6	NPM7	F3
H	NPM8	F4	PM3
I	F5	NPM9	PM7
J	PM8	F6	NPM10
K	PM9	NPM11	F3
L	NPM2	F5	PM10
M	F1	NPM13	PM11
N	PM12	F6	NPM14
O	Small1	Medium1	Large1
P	Large1	Small2	Medium2
Q	Medium3	Large 3	Small3
R	Medium4	Small4	Large4
S	Small1	Large3	Medium4
T	Large1	Medium2	Small4

CAPÍTULO 4. BENEFITS AND CONSEQUENCES IN THE SOCIAL STRUCTURE OF INTER-GROUP MOVEMENTS IN A NON-TERRITORIAL TERRESTRIAL FROG

1. Abstract

Animal aggregations can result from the distribution of resources in the environment and ecological constraints that drive individuals to group with conspecifics and carrying quantifiable costs and benefits. But the communicative processes that lead to conform those groups are generally little evident. To determine whether a population of a frog with terrestrial reproduction is socially structured, we employed social network analysis to evaluate the influence of call similarity and home range overlap in the Darwin's frog *Rhinoderma darwinii*. As males of this species brood the larvae in the vocal sacs, we also evaluated the benefits and consequences in terms of larvae produced of inter-group displacements of breeder and non-breeder adults and juveniles, and analysed whether inter-groups movements observed had consequences in the social structure of the species throughout a null model network. Our study shows that social structure of the Darwin's frog is not shaped by call similarity and home range overlap. However, reproductive benefits related with the performance of pregnant males at group and individual levels were found. We detected for the first time that a higher ratio of non-breeder and breeder males increase the reproductive output of social groups and a positive relationship of the home range area with the number of larvae brooded by males. As inter-group displacements performed mainly by larger individuals modify the social structure of the Darwin's frog, we suggest that social structure can be quite more dynamic than expected, at least for the studied population of *R. darwinii*.

2. Introduction

Living in groups is fundamental for the life histories of many animals. Benefits of social living comprise reduced predation risk and increased probability of finding resources as food, mate, refuge and nesting place (Tinbergen 1964; Krause and Buxton 2002; Danchin et al. 2008). However, social living could also result from ecological constraints for dispersal and acquisition of territories or refuges (Brown 1989; Danchin and Wagner 1997; Wong 2010). Until the 1970s, social structure was believed to be relevant for the biology of anthropoid species mainly (e.g. Wilson 1975). However, currently the predominantly notion is that social structure affects the evolution of sexual dimorphism (Lindenforts et al. 2002), signalling systems (Bradbury and Veherencamp 2011) and cognition processes (Byrne and Whiten 1988). As such, the study of patterns of interactions among individuals is considered relevant to explain the nature of social relationships independent of the taxa involved (Whitehead 2008; Krause et al. 2015).

Space use and ranging patterns of individuals have commonly been employed to investigate social structure (e.g. Madison 1980). This is because the amount of spatial overlap between individuals with different phenotypes provides indirect information about the probability of social interactions (Clutton-Brock 1989; Brown 1989; Godfrey et al. 2014). At the individual level, the ability to form and maintain social groups requires abilities to perform cognitive processes (Bode et al. 2015); however, knowledge about the mechanisms of information exchange that regulate and maintain social groups is still relatively little understood among different taxa, for instance: birds (Burst and Vehrencamp 2005), invertebrates (Greenfield 2005), anurans (Greenfield 2005; Jones et al. 2014) and dolphins (King and Janik 2015).

In animal groups, reproductive dominance hierarchies can influence the dispersion and migration of individuals of different sex and age, modifying the dynamic of social structure (Gauthreaux 1978). In species regulating their social relations by means of acoustic signals, dominance interactions are regulated by the characteristics of signal displays, such as repertoire size (Illes 2015), song type variability (Botero et al. 2009) or level of vocal activity (Greig et al. 2013). In sexual interactions, frogs may advertise their age by means of acoustic signals and receivers may discriminate between conspecific counterparts based on signal features (Lykens and Forester 1987; Smith 1987; Halliday and Verrell 1988; Friedl and Klump 2005; Leary et al. 2005). However, body size is a trait that is more clearly informed in signal characteristics that affect female choices (Ryan 1980; Márquez et al. 1990).

In anurans with male parental care, body size can be a good predictor of the reproductive success of males measured as number of carried offspring (Raxworthy 1990). In preliminary studies of the advertisement calls of the Darwin's frog (*Rhinoderma darwinii*) we have found that body size is relevant for geographic differentiation of calls (Chapter 1). Also, males and females present dynamic abundances and vocal patterns during the breeding season (Chaper 2) and the different sexual status potentially recognize their conspecifics by means of their calls (Chapter 3). Such signalling differentiation is likely relevant for the formation of stable social groups observed in Darwin's frogs (Valenzuela-Sánchez et al. 2014; 2019a) since females produce advertisement calls similarly to those of males and since males with different reproductive status produce indistinguishable calls (Chapter 2). The presence of diverse sexual status occupying dense home ranges increase the likely to form complex social structures based on egalitarian social interactions (Freeberg et al. 2012).

Social network analysis is a tool employed for understanding behavioural processes and their consequences at the social level in an increasing number of species (Krause et al. 2015). However, how communication signals can reflect and complement social network structures of populations is still an unexplored issue (Snijders and Naguib 2017). As behavioural traits can reveal social connections and mediate individual and social behaviour in groups formed by multiple emitters and receivers (McGregor and Horn 2015), social networks provide an excellent framework for quantifying associations among dyads of individuals conforming groups by representing a population as a series of nodes (individuals) connected by links (relationships or interactions) revealing the social structure (repeated relationships) supporting group life (Whitehead 2008; Farine and Whitehead 2015). Therefore, network analysis allows to describe ecological and evolutionary processes (e.g. Cantor and Whitehead 2013) and to test hypotheses derived from information partitions generated by complex data matrices comparing different degrees of connectivity within and between different groups or populations (Farine and Whitehead 2015).

In this study we evaluated the extent to which advertisement calls features and spatial proximity affect social structure in aggregations of the Darwin’s frog. For this, we determined spatial interactions among individuals that overlap their home ranges in chorusing aggregations formed by three different sexual status. We also explored the relationships of the reproductive outputs of frog aggregations with their sexual status ratios and the areas that they occupy. Subsequently, we combined call similarity indexes of spectral and temporal components of vocalizations to evaluate their relationships with home range overlaps. Finally, we constructed a null network model to evaluate the consequences of movements between groups of individuals with a specific body size for the social structure. By means of these analyses we evaluated the hypotheses that individual movements among groups contributes to modify the social structure of *R. darwinii*.

3. Materials and Methods

3.1.General procedures

We studied a population of Darwin's frog in the private reserve Parque Tantauco, Chiloé (43° 21´ S; 74° 6´ W), in areas of temperate forest. Monthly observations of *R. darwinii* in an area of approximately 255 m^2 were conducted based on the assumption for collecting networks data of gambit of the group, which implies that each animal in a group area is associating with every other individual in that group (Franks et al. 2010). To assess whether this assumption was useful for *R. darwinii*, we incorporated three additional criteria from the natural history known for the species (Crump 2002; Valenzuela-Sánchez et al. 2014) that would have to be accomplish each presumed group to show if they were effectively social aggregations: the number of associated individuals is higher than two, the occurrence of reproductive males and a high proportion of individuals remaining within the group area along the period of the study. So, the study area was subdivided in 10 presumed groups delimited by continuous microhabitat (e.g., adjacent patches of mossy substrate surrounded by muddy soil or pastures and herbs substrate scattered on naked soil). This approach to observe the population also facilitated us the relocation of individuals of *R. darwinii* after each sampling event

To identify individuals, we caught frogs during three continuous days using manual captures with a standardized effort of four person-hour (e.g. two hours with two people sampling). We photographed the ventral pattern of each frog, as they have unique individual designs (Soto-Azat et al. 2013), except for post-metamorphs which do not have an evident visual pattern on the ventral side, all the individuals were identified accurately every time. Sex was determined based on mass and body size measures (Chapter 2) as well as by the presence of orifices of the vocal sac underneath the tongue and pregnancy status of males (Howes 1888). The location of each capture and re-capture were marked on the ground with a small plastic flag, so that at the end of the study we knew the utilization distribution of each frog captured over five months within the area of study.

3.2.Home range estimations

Utilization distribution (UD) was calculated using the minimum convex polygon (MCP; thereafter referred also as home range) for individuals captured (Fieberg and Kochanny 2005). Due to low recapture for all sexual status (Fig. S2) and because MCP joining calculations require at least five points to run, one and two "dummy" points were added for those individuals captured four and three times, respectively. “Dummy” points were constructed adding ± 1 pixel (one pixel equals 1 cm in our MCP estimates and other spatial estimations) randomly from the original position x or y registered for one capture. Overlap indices employ the x and y axes to represent the spatial domain utilized by the animal. Some overlap indices generate a z-axis to represent the relative proportion of time spent in each spatial location (i.e., z= UD[x,y]), and therefore UD is no-uniform if there are activity centers or bias within the space used by two animals overlapping (Fieberg and Kochanny 2005). Home range area was calculated for two sets of data: first, individuals for which acoustic recordings were conducted, and second, all the individuals that were captured at least three times.

Using the home range calculations the utilization distribution overlap index (UDOI) was calculated for each dyad of captured frogs as the product of the two UDs, UD_i (x,y) x UD_j (x,y), which corresponds to the joint distribution of the dyad's UDs under the assumption that they use space independently of one another, and is calculated as follows:

$$\mathrm{UDOI} = \mathrm{A}_{1,2} \iint_{-\infty}^{\infty} \widehat{\mathrm{UD}}_1\,(x,y) \times \widehat{\mathrm{UD}}_2(x,y)\;dxdy$$

Where $A_{1,2}$ is the area of dyad's home range calculated from x and y coordinates. The UDOI equals zero for 2 UDs that do not overlap and equals to 1 if both UDs are uniformly distributed and have 100% of overlap. However, UDOI can be >1 if the two UDs are nonuniformly distributed and have a high degree of overlap (Fieberg and Kochanny 2005). Calculations of MCP and UDOI index were performed with the packages adehabitatHR and sp in R (R Core Team 2018).

3.3.Call similarities

Advertisement calls of males and females were recorded as reported in detail in Chapter 2. Because four-note calls are the vocalizations most commonly issued by Darwin's frogs (Chapters 1 and 2), a single four-note call for each frog was randomly chosen and edited in separate files using Adobe Audition 3.0. To characterize the general properties of vocalizations, such as the distribution of the frequency spectrum and envelope duration of calls of two individuals, we obtained two measures of call similarity as frequency and amplitude modulation cross-correlation indexes for each dyad using Sound Ruler 0.9.6.0 (Gridi-Papp 2007). Cross-correlation scores had values from 0 to 1 indicating the degree of similarity in frequency and amplitude modulation patterns between two calls, where values of 1 means a complete similitude of each pixel between two spectrograms or oscillograms. Preliminary mantel tests were carried out to calculate the relationships among spectral and amplitude call similarity measures and UDOI values. Mantel test analyses were carried out using the package vegan in R (R Core Team 2018).

3.4.Social network analysis

To construct social networks, we employed the call similarities and home range overlap matrixes as links connecting frogs. These measures do not represent either direct vocal or spatial interactions but are used as proxies of relationships affecting the patterning relative to each other animal's behaviour (Cramer 2013; Fieberg and Kochanny 2005). We calculated mean call similarity (adding the two matrixes of cross-correlation scores) and UDOI values separately for all possible male-male, female-female and male-female combinations in the population. Additionally, we calculated the dependence of the mean cross-correlation index of advertisement calls on UDOI to determine whether there was an effect of distance on call similarity among frogs. We then structured our analyses into three components: frequency, amplitude modulation and spatial relationships, using the multiple regression quadratic assignment procedure (MRQAP) (Dekker et al. 2007). Additionally, we determined the factors having a higher influence on social structure among frogs connected in the social networks. All the network analyses were performed using the package asnipe in R (R Core Team 2018).

3.5.Inter-group displacement

To evaluate whether the observed displacement of frogs among group areas (see Fig. 18) resulted in an increase in size of the displaced individuals relative to the original and final groups where they were captured, the mean relative body size of individuals that moved among groups were compared by paired t-tests. Finally, we determined whether the difference in relative body size of displaced individuals differs among sexes.

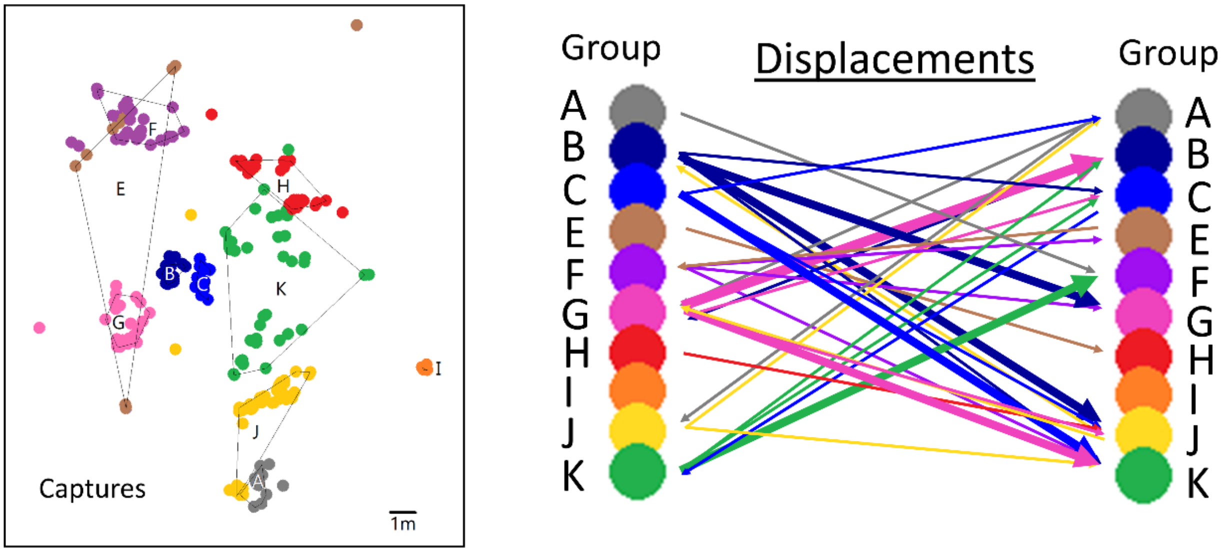

Figure 18. Spatial representation of captures of 143 individuals of *Rhinoderma darwinii* and presumed groups in the area of study is indicated on the left. Each point represents an individual capture. Colour of points indicates the group (A – K) where individuals were captured first. On the right, 36 displacements of individuals observed among groups are represented by arrows and lines. Thickness of arrows and lines are proportional to the number of displacements of one or more individuals between groups.

Focal individual observations (i.e. tracking displacements and interactions of individuals) were permuted following a randomization procedure of null hypothesis network construction (following Farine 2017) based on random associations from the original data set of individuals captured in the population. We derived a randomization to swap those individuals that moved at least once to other groups from their original focal observation. A higher probability of inter-group displacement of a weighted degree (strength) of 1.0 was assigned to pregnant males and a weighted degree of 0.5 was assigned to the rest of sexual status (females, non-pregnant males and juveniles). Such weighted degrees were assigned due to the higher number of pregnant males displacing among groups (Fig. S3; see also Valenzuela-Sánchez et al. 2014). Node permutations of focal individuals were repeated 1 to 1000 times, and the *P* values were calculated by comparing the observed slope coefficient of a mixed effects model on the randomized degree from the observed network calculated for each permutation.

4. Results

In total, 316 captures involving 143 individuals of *R. darwinii* were obtained during this study. Fifty-nine of the individuals were captured just once, and only one individual was captured nine times. The median number of captures was two. The sexual status with higher number of captures corresponded to non-pregnant males with 124 captures, followed by 62 pregnant-males, 56 females, 54 juveniles and five post-metamorphs (Fig. S2).

Not all presumed groups included pregnant males and therefore the number of brooded larvae ranged broadly from 0 to 29 larvae per group (Table 10). The presumed groups varied broadly in their area size (range: 0.02 – 62.54 m^2), and frog composition, i.e., in their total number of individuals (range: 2 – 30 individuals) and their sexual status, as well as the number of individuals moving in and out (range: 0 – 7 movements). Three of the ten presumed groups (E, I and K in Table 10) did not meet one of the three pre-established criteria to be considered as true groups (the number of associated individuals higher than two, the occurrence of reproductive males and a high proportion of individuals remaining within the group area).

Table 10. Summary of the total numbers of individuals by sexual status, the number of individuals moving in and out of presumed groups and the total number of larvae brooded by males of the 10 groups monitored. Sexual status of *Rhinoderma darwinii* are indicated as follows: F: females, PM: pregnant males, NPM: non-pregnant males, J: juveniles, P: post-metamorphs. Asterisks indicated the presumed groups accomplishing the natural history criteria to be considered as true groups.

Group	Area (m^2)	F	PM	NPM	J	P	Total Individuals	Total Larvae (range)	Individuals Moving In/Out
A*	2.38	2	2	2	3	0	9	8 (4)	2/3
B*	1.24	4	4	7	2	0	17	13 (2-4)	5/7
C*	1.60	3	3	3	2	1	12	11 (2-5)	3/5
E	62.01	5	5	5	0	0	15	28 (4-8)	1/2
F*	12.64	6	7	10	2	0	25	29 (2-7)	4/3
G*	6.73	2	7	6	5	1	21	18 (1-4)	6/7
H*	9.60	2	3	8	3	0	16	11 (2-5)	1/1
I	0.02	1	0	1	0	0	2	0	0/0
J*	14.88	3	9	10	9	3	30	29 (1-9)	6/4
K	62.54	9	0	9	6	0	24	0	8/4

No significant relationship ($p > 0.05$) was found between the composition of individuals, the number of larvae, movements and the area of the groups. But the ratio of movements in/out was negatively correlated with the proportion of pregnant males/non-pregnant males ($r = -0.67$, $p < 0.05$), and the number of larvae was positively related to the proportion of pregnant and non-pregnant males ($r = 0.64$, $p < 0.05$; Fig. S4).

4.1.Home range overlap

A spatial distribution of the home ranges estimated for two sets of individuals (recorded callers and individuals recaptured at least three times) are shown in Fig. 19 to illustrate the overlap between individuals in the population studied. Home ranges estimated for 42 individuals varied between 0.0026 and 48.17 m^2. Although home ranges of males (0.003 – 48.179 m^2) and females (0.032 – 45.177 m^2) were larger than those of juveniles

(3.452 – 4.763 m^2), the size of home range did not differ among sexes (KW Chi^2= 1.653, df= 2, p> 0.05). The positive relationship between the number of larvae and the home range area of pregnant males showed a marginal significance (r= 0.48; df= 15; p= 0.053). Furthermore, UDOI was very similar between males and females (Table 11).

4.2.Call similarities

Frequency and amplitude modulation similarity indexes are reported in Table 11 for the 20 individuals recorded (17 males and 3 females). Both cross-correlation measures were highly related to each other (r= 0.931; p= 0.001). However, frequency similarity and amplitude modulation similarity were unrelated to home range overlap as tested with simple (r= -0.076; p> 0.05; r= -0.021; p> 0.05; respectively) and partial Mantel tests (r= -0.153; p> 0.05).

Table 11. Mean ± standard deviation and (range) values of network edge weight measured as utilization distribution overlap index (UDOI), frequency and amplitude modulation similarities among dyads of frogs connected in the social network. N indicates numbers of dyads of males and females captured and recorded acoustically. Abbreviations: NPM= non-pregnant male; PM= pregnant male; F= female.

Dyads	N	UDOI	N	Frequency similarity	Amplitude similarity
NPM – NPM	14	0.46 ± 0.95 (4.6^{-5} – 1.74)	9	0.16 ± 0.12 (0.009 – 0.433)	0.07 ± 0.06 (0.003 – 0.230)
PM – PM	17	0.42 ± 0.53 (2.8^{-5} – 1.89)	5	0.15 ± 0.13 (0.031 – 0.483)	0.06 ± 0.05 (0.017 – 0.162)
F – F	9	0.51 ± 0.60 (3.1^{-4} – 1.73)	3	0.13 ± 0.06 (0.075 – 0.199)	0.04 ± 0.02 (0.025 – 0.071)
NPM – PM	14	0.14 ± 0.19 (9.4^{-6} – 0.79)	9	0.16 ± 0.12 (0.022 – 0.517)	0.08 ± 0.06 (0.009 – 0.201)
NPM – F	14	0.06 + 0.12 (8.7^{-5} – 0.41)	9	0.09 ± 0.05 (0.025 – 0.209)	0.04 ± 0.03 (0.010 – 0.127)
PM – F	17	0.16 ± 0.24 (2.2^{-4} – 0.93)	5	0.12 ± 0.04 (0.028 – 0.169)	0.05 ± 0.02 (0.015 – 0.087)

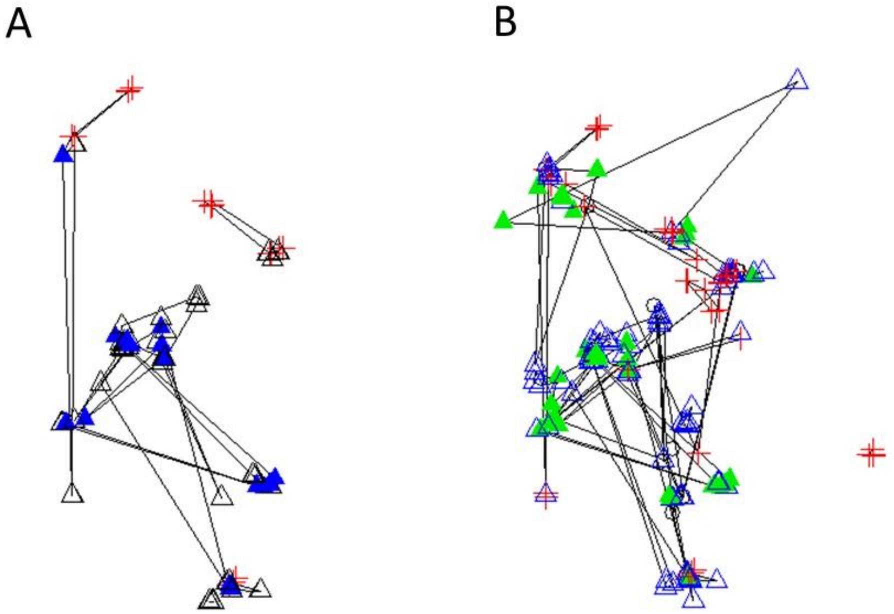

Figure 19. Home-range areas estimated for A) 20 frogs for which acoustic recordings were conducted B) 42 frogs that were captured three or four times. Coloured symbols represent position recordings and indicate the sexual status of the individuals of *Rhinoderma darwinii* (Red crosses: Females, Green triangles: Juveniles, Empty triangles: Non-pregnant males, Blue triangles: Pregnant males). Note that some polygons have empty and coloured triangles indicating transitions between different captures in the status of the same subjects (e.g. from being non-pregnant to being pregnant or from being juvenile to being non-pregnant male).

4.3.Social structure

Mean values of network links, UDOI, frequency and amplitude modulation similarities among the connected dyads of frogs recorded are shown in Table 11. The degree of networks formed by home range overlaps (UDOI) had a significant meaning (r= 70, p= 0.001) but the UDOI of dyads formed by the different sexual status did not differ (t= 1.794, df= 198; p> 0.05). Females were less represented in home range and call recordings and therefore had fewer nodes and formed fewer links in home range overlaps and call similarity networks relative to males (Table 11). The degree values did not differ among dyads of non-pregnant males – pregnant males, non-pregnant males – females and pregnant males females in both frequency ($t= 0.104$, $df=14$, $p> 0.05$; $t= 0.598$, $df=3$, $p> 0.05$; $t= 0.368$, $df=8$, $p> 0.05$, respectively) and amplitude modulation similarity indexes ($t= 0.263$, $df=17$, $p> 0.05$; $t= 1.440$, $df=5$, $p> 0.05$; $t= 0.041$, $df=8$, $p> 0.05$, respectively). The MRQAP analysis showed no significant effect ($p> 0.05$) of frequency and amplitude modulation on the UDOI of all connected dyads.

4.4. Inter-group movement

Most of the individuals captured were never observed moving away from their group (Fig. 19; Table 10). Thirty-one individuals were observed moving to another group within and between monthly samplings, and from these 90.3% (28 individuals) moved to another group only once without returning, six frogs moved two times and two frogs moved three times between groups some of the returning to their initial group and others moving to other groups. Individuals that moved were larger (mean SVL= 21.59 mm) as compared to those that did not move (mean SVL= 19.95 mm) ($t= 2.993$, $df= 60$, $p< 0.01$). However, no differences were observed between the relative body size of males and females that moved to another group ($t= 0.144$, $df= 12$, $p> 0.05$).

We found that for individuals observed moving among groups, the relative body size was larger in the final group relative to the initial group observed (Fig. 20; $t= -2.833$, $df= 29$, $p< 0.01$). The number of individuals of the different sexual status performing movements between groups were significantly lower than the individuals that did not move ($Chi^2= 9.1525$, $df= 3$, $p< 0.05$). Pregnant males were the sexual status that was recorded more times moving between groups, followed by females and non-pregnant males, and only one juvenile was recorded moving among groups (Fig. S3).

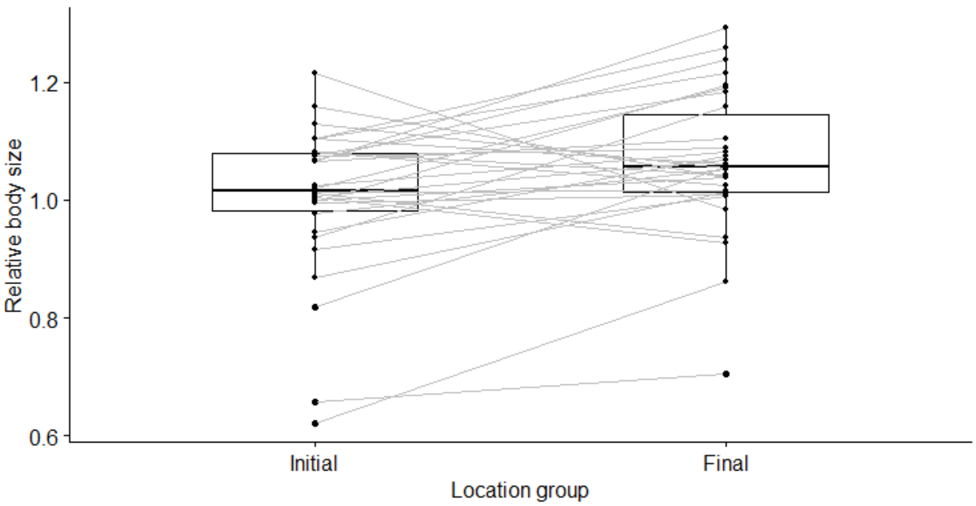

Figure 20. Relative body size in groups between which individuals of *Rhinoderma darwinii* moved. Connected dots indicate the same individual.

Observations of displacement in focal individual permutations showed that movements of individuals between groups produce significant changes in the social structure (i.e. the number of associations present) in a group of ~30 individuals. Our simulation model demonstrates that the structure of the network links is changing after each displacement event (Fig. 20). Permutation tests identified that degree distribution observed (the black histogram observed in permutations 100 and 1000 in Fig. 20) was significantly smaller (with a confidence of 95%) than that expected by chance (red vertical line in Fig. 20). After 1000 permutations, observations of two individuals swapped among sets of focal observations (~30 individuals as the largest group size listed in Table 1) results in slight changes in the edge structure in the social networks. This evaluation of hypothesis does not consider changes on the nodes (dots) but on the links, so the nodes maintain their same position but are affected by the change of links that support and modify their thickness (degree).

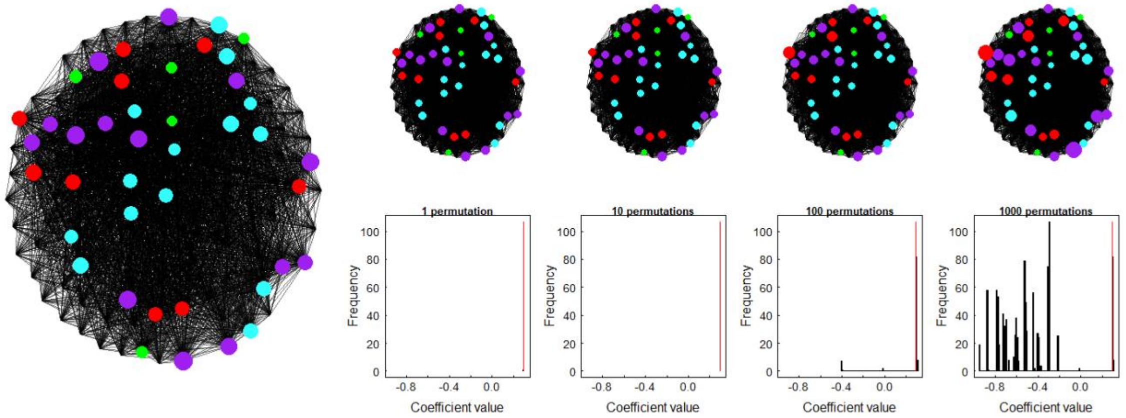

Figure 21. Network of the original dataset (left) shows a group of frogs formed (large network on the left) by juveniles (green dots), non-pregnant males (cyan dots), pregnant males (purple dots) and females (red dots). Permutation networks (above) and probabilities of network's change (below) after one of the individuals leave the group in permutation simulation. Permutations of chance in networks was adjusted by a weighted degree with the probability of moving for pregnant males was twice than that for the other sexual status (1.0 vs. 0.5). See the main text for more details.

5. Discussion

Our study shows that social structure is continually being affected by the exchange of individuals moving between groups and that social structure is maintained by such continuous inter-group movements of males and females. Larger individuals observed moving between groups increased their size relative to individuals of new groups, so the need to improve their relative size would apparently be regulating these movements. Social adjustments of this kind have been shown to occur in coral reef fishes in which sociality is driven by ecological constraints to move and improve their chances of reproducing (Colleye and Parmentier 2012). Our null hypothesis model indicates that such inter-group exchanges results from the eavesdropping on the information of body size contained in advertisement calls. The acoustic signals of Darwin's frog contain information on body size (Chapter 1 and 2) that can be relevant in duetting interactions among individuals of different sexual status and body size (Chapter 3) and inter-group movements observed during our study show how *R. darwinii* can increase its chances of reproducing by expanding its presence to groups with relatively smaller individuals.

The reproductive output of Darwin's frog was related at two levels to the ecological context of pregnant males. First, at the individual level the number of larvae in the pregnant males were directly related to the size of their home range, in concordance with the fact that pregnant males are the ones that had higher mobility between groups. Although no differences were found between home range size of pregnant and no-pregnant males or between sexes (Valenzuela-Sánchez et al., 2014; 2019a), our data suggest that mobility of pregnant males increases their chances to obtain more larvae, i.e., these males have more chances to find a fertilized egg clutch, and even more to incorporate new eggs to their vocal sac. Such behaviour could be counterintuitive about to assume the energetic costs of brooding unaware offspring, but information about paternity of *R. darwinii* is unknown, and multiple paternity is likely to occur in sexual role reversed species when external fertilization occurs (McCoy et al. 2001) as in the Darwin’s frog (Busse 2003). Alternatively, Valenzuela-Sánchez et al. (2014) suggested that brooding males need to travel more to help disperse postmetamorphs. Second, groups with a greater proportion of non-pregnant males relative to pregnant males showed a higher total number of brooded larvae, which reinforces the idea that the mating system of Darwin's frog would be based on multiple paternity. Valenzuela-Sánchez et al. (2014) detected that presence of egg nests of *R darwinii* founded in the field represented central nodes congregating pregnant and non-pregnant males. Unfortunately, nothing is known about the mating system of this speciesin wildlife. Knowledge about reproductive behaviour of R. darwinii is limited to observations in captivity (Pflaumer 1935; Busse 2003) and that in the presence of a single male and two females by terrarium the mean rate of unfertilized eggs is about 20% in captivity (O. Cabeza, Pers. Comm.). Our data suggest that reproduction of *R. darwinii* could be affected by the association between breeding and non-breeding males, e.g. by attempts to fertilize unfertilized eggs by amplectant males. The discernment of sperm competition and other aspects unknown related to the mating system should be supported by future genetic studies on this species.

The neutrality in the fitness of breeders due to the presence of non-breeders has favoured such stable association between breeders and non-breeder males in clownfish, since the non-breeder males are subordinated to the larger breeders (female and male) and help in the care of the nests(Buston 2014). In the case of *R. darwinii*, the association between pregnant and no-pregnant males occurs also in non-aggressive contests and a high overlap between males of different reproductive condition is usually observed (Valenzuela-Sánchez et al. 2014; Chapter 3; This study). The lack of aggressive behaviour could be contributing to avoid depredation risks (as in clownfishes, Buston 2014) and could act in synergy with the high mimicry of *R. darwinii* with its natural environment (Bourke et al. 2011).

Call similarity was not related to groups and to home range overlap. This indicates that in this anuran there is no “call group” or call adjustments depending on the social environment exposed as has been observed in suboscine birds (e.g. Fishbein et al. 2018). Even more, it is likely that all callers within the population studied are probably within the signal range area of propagation (Snijders and Naguib 2017) of each other (see distances among duets Table 8 and Fig. 18), i.e., any frog can hear other when calling. However, body size relationships among neighbours is apparently affecting individual decisions moving between groups since larger adults are those moving to new groups, and after moving these individuals that belong to the three sexual status are improving their body size relative to the group. Because of these movements, produce changes in the social structure constructed by call similarity parameters, they probably affect the vocal information of the relative size within groups by modifying decisions about moving or maintain their home ranges in *R. darwinii*. Such animal decisions related to sexual interactions and use of space usually occurs based on eavesdropping to signals transmitting effectively quality and position of emitters (Danchin et al. 2004; Debalsteen 2005). Since advertisement calls produced by the three sexual status contain information related to their body size and sex conditions (Chapters 1 and 2), it is likely that eavesdrop is facilitating displacement decisions of Darwin's frogs.

Although inter-group movement observed within the population was relatively low (occurs in ~20% of the individuals captured), it caused significant changes in the social structure of the groups affecting decisions about staying or leaving within groups. In aquatic frogs, the experimental alteration of group size did not affect incidence of striking by individuals or the stability of hierarchies in groups of up to 18 frogs (Boice et al. 1974). Our model suggests that the displacement between groups does not disturb the stability of *R. darwinii* groups as the exchange of sexual status and sizes of group members contributes to maintain its social structure.

From the theoretical perspective of the ideal distribution of resources (Danchin and Wagner 1997), animal aggregations are a consequence of the distribution of resources in heterogeneous environments and involve processes of habitat and partner selection, in which natural selection favours individuals transiting from an over-exploited to an under-exploited patch. The UDOI reached values >1 in all the adult sexual status, which indicates that home ranges are not uniform. This lack of uniformity in home ranges does not result from the generation of “dummy” points, as these were also generated for juveniles, but these individuals did not exceed the threshold of 1, indicating home range overlap uniformity for this group (Fieberg and Kochanny 2005). Therefore, uniformity bias of home ranges for adults of *R. darwinii* can be understood as bias of UD within groups. High mobility within their small home range areas is commonly observed among adults in different time windows (from days to interannual intervals; Valenzuela-Sánchez et al. 2019a), and agrees with the evidence showing distribution of resources in this all-use home ranges (refugee, nest, food and mates) is rather heterogeneous at least at the within population level (Valenzuela-Sánchez et al. 2019b) as occurs under ideal free distribution perspective (Danchin and Wagner 1997).

Our definition of Darwin’s frogs’ groups was related to natural history criteria to confirm that not all the presumed groups were in fact social groups. The data showed that not all groups generate reproductive output, these groups had extreme values in the proportion of individuals moving in/out (0 and 2, respectively for “groups” I and K). So those aggregations without breeders could not be considered true social groups, but ephemeral aggregations or spaces of transition between groups. Since reproduction is a benefit of animal aggregations (Danchin et al. 2008), the delimitation and definition of groups in *R. darwinii* should consider habitat continuity, occurrence of reproduction, and exchange rate of individuals.

Critically, recapture rate was low in this study possibly because the sampling effort of 12 hours person per month was insufficient to detect accurately all residents. Valenzuela-Sánchez et al. (2019a) reported better recapture levels with two additional person hours (within six months of observations distributed in three years) than in the current study. However, it is likely that the high number of unique captures detected using the capture-recapture method be related to an unusual population dynamism (formed non exclusively by residents) contrasting to other highly philopatric populations previously studied (Valenzuela-Sánchez et al. 2014; 2019a). Therefore, mobility of *R. darwinii* populations should be evaluated specifically to detect non-resident individuals in a study that includes a larger sampling area and remote displacement sensing. The traditional view of the home range is based on the notion that animals have well-defined home ranges (Fryxell et al. 2014), however, the predominance of single captures in all age ranges and sexual status that we observed in this species suggests that juveniles are not the only individuals that disperse, as has been suggested to other populations of this anuran (Valenzuela-Sánchez et al. 2019a).

Behavioural ecologists refer to the absence of aggression and competition as sociality (e.g. Brown 1987). Social behaviour of anurans mediated by vocalization has been largely studied in terms of intrasexual competition (Wells 1977; Bates et al. 2010; Bee 2016). The presumed lack of learning skills in anurans has precluded investigating social behaviour affecting social complexity and communication processes other than habituation and sensitization (Bee 2016). However, anurans constitute a group that provides vast opportunities to investigate the complexity of communication networks due to the diversity of reproductive strategies and chorusing modalities (Grafe 2005). This study shows for the first time that body size associations affects Darwin’s frogs’ spatial displacements and maintain the social stability under certain population parameters. Our results also point out how the association of breeders and non-breeders in a ratio larger than one improve the reproductive output of a frog with terrestrial reproduction.

6. Supplementary Information

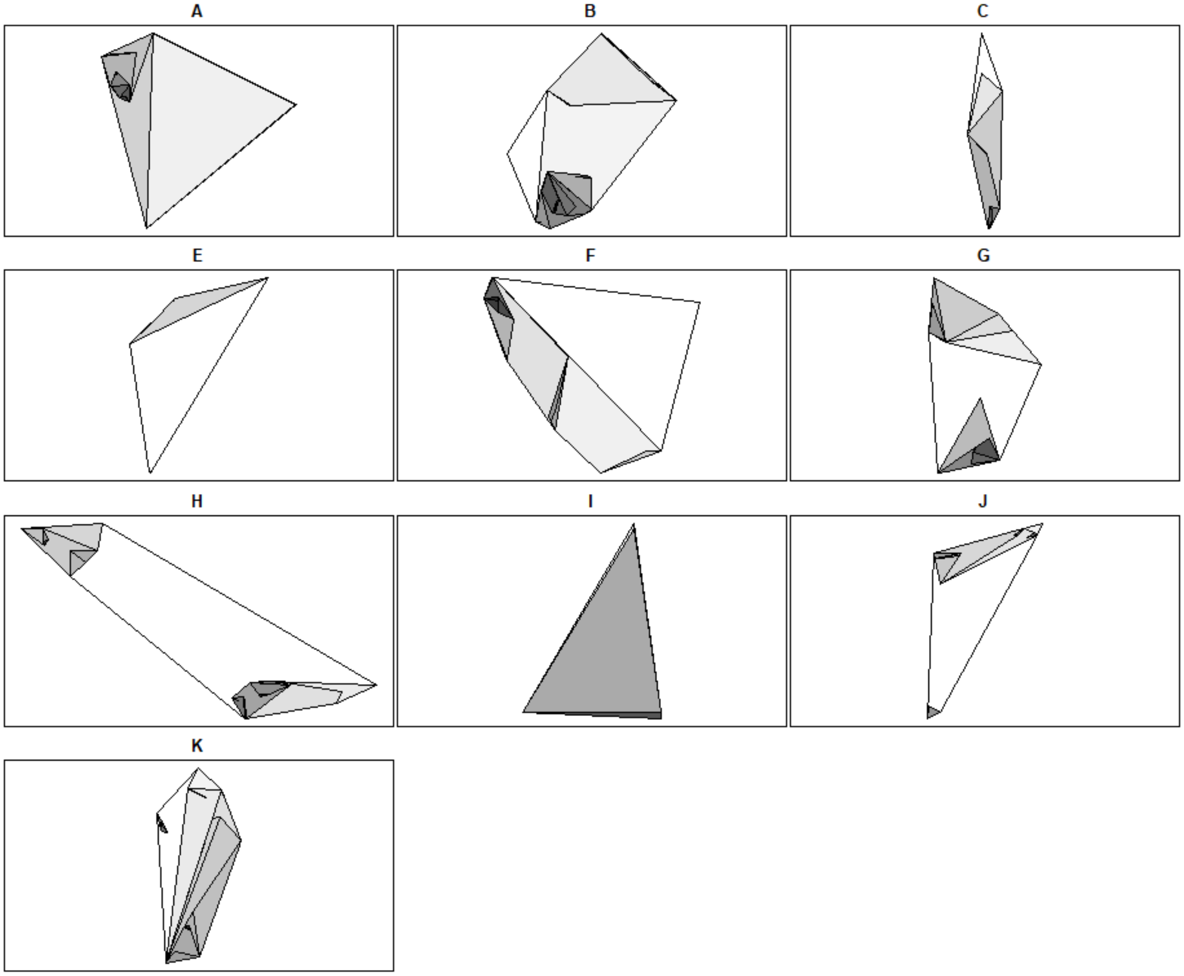

Figure S1. Clustering of individual home ranges enclose 95% of individual recaptures in the 10 groups (A - K) of *Rhinoderma darwinii* found in moss patches at the study site. Size area of each group was computed by coercing 95% of the individual captures and assigning each capture to the first group (moss patch) where each individual was captured by calculating the minimum convex polygon (MCP). MCP and spatial clusters were calculated using the package adehabitatHR in R (R Core Team 2018).

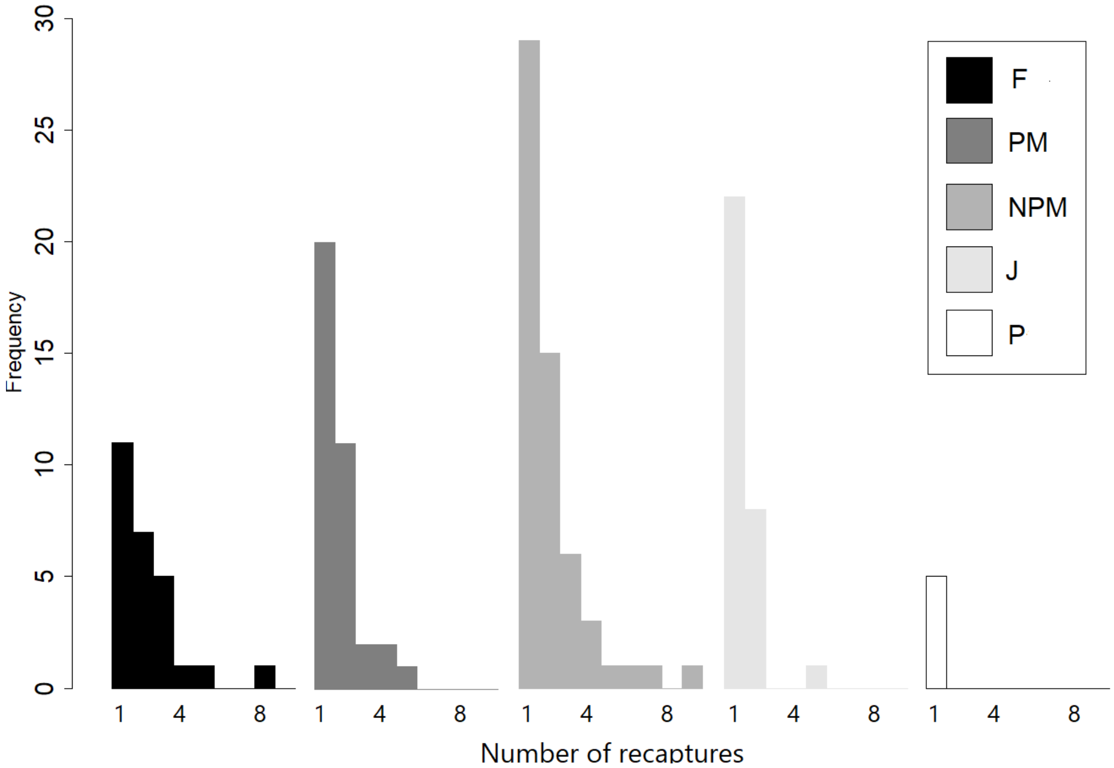

Figure S2. Histograms indicate the number of recaptures by sex and age in the population studied. Sexual status of *Rhinoderma darwinii* are indicated as follows: F: females, PM: pregnant males, NPM: non-pregnant males, J: juveniles, P: post-metamorphs.

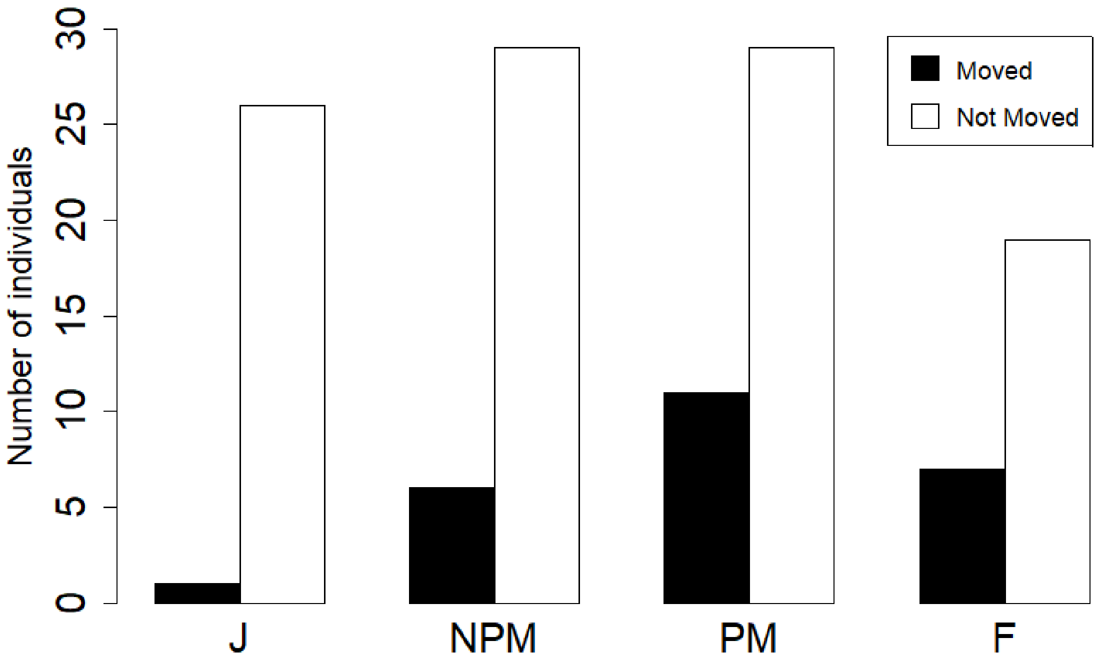

Figure S3. Number of individuals of *Rhinoderma darwinii* captured and detected performing inter-group movements and without moving. Sexual status are indicated as follows: F: females, PM= pregnant males, NPM= non-pregnant males, J= juveniles.

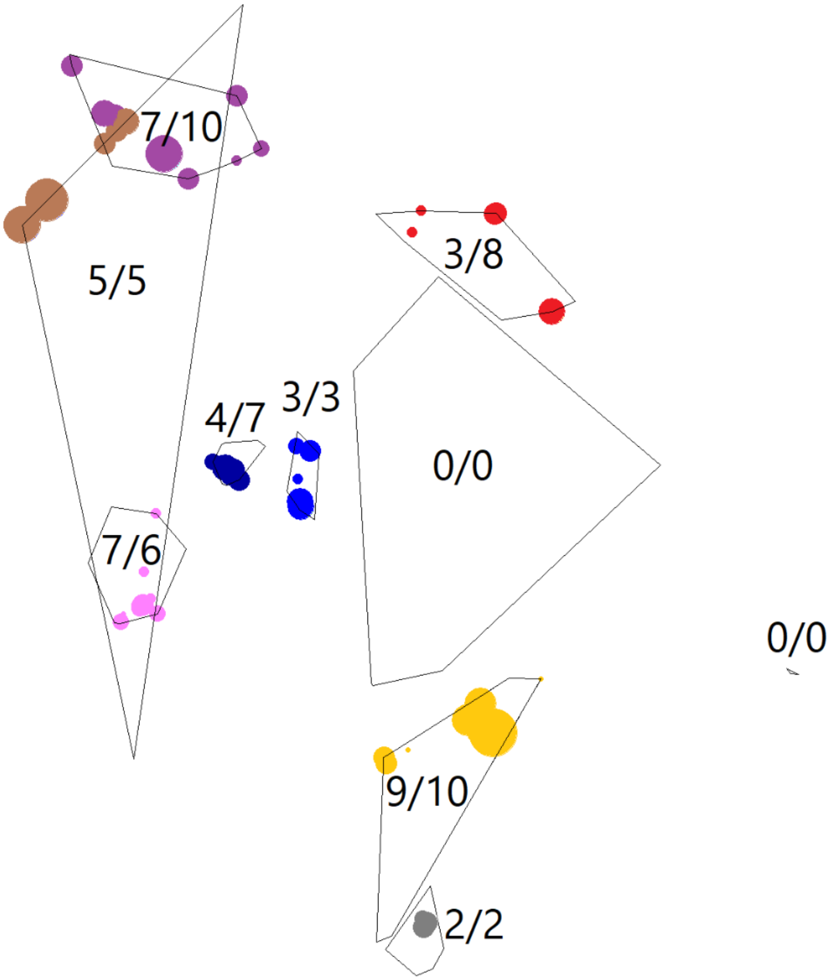

Figure S4. A spatial representation of the relationship among the number of larvae observed and the proportion of pregnant males/non-pregnant males in the groups indicated in Table 10. Colour and polygons represent pregnant males in the different groups and circle sizes indicate the number of larvae brooded by each pregnant male.

DISCUSIÓN GENERAL

Comprender los contextos en que se producen señales es relevante para identificar los procesos sociales que subyacen en la comunicación. La señalización de hembras de anuros es un fenómeno que puede o no diferenciarse de las señales masculinas dependiendo del escenario ecológico y evolutivo en que se emiten (Kokko y Johnstone 2002). Evaluar el reconocimiento de las señales es relevante para las interacciones entre sexos y permite plantear hipótesis acerca de la intensidad de la selección sexual (Ryan y Rand 1993; Johnstone 1997). Por ejemplo, en especies donde ambos sexos producen señales llamativas es posible que ambas especies elijan mutuamente al otro sexo con distintos grados de intensidad, proceso dependiente de diversos factores ecológicos (Trail 1990). El presente estudio sugiere que el repertorio y el contexto de emisión de señales acústicas de la ranita de Darwin *Rhinoderma darwinii* es más complejo de lo que se conocía previamente (Penna y Veloso 1990; Busse 2003). La descripción que realizamos del canto indica, por ejemplo, que el canto mantiene una alta variación individual y una estrecha relación con la variación del tamaño (Capítulos 1 y 2) además de que las hembras vocalizan produciendo un repertorio similar al de los machos y en particular de los machos preñados continúan vocalizando luego de haber obtenido crías (Capítulo 2), lo que implicaría costos adicionales al cuidado paternal realizado en el saco vocal (Goicoechea et al. 1986).

En la ranita de Darwin, las diferencias en las características de los cantos de machos y hembras están relacionados con las diferencias de tamaño entre los sexos. Un ejemplo similar, donde se sugiere que esta diferencia en las señales, relacionada con la diferencia en el tamaño, se ha identificado en la rana chirriadora del río Grande *Eleutherodactylus cystignathoides*, otra especie con vocalización femenina (Serrano y Penna 2018). No obstante, en aquel caso los machos no exhiben respuestas diferentes hacia los cantos de machos y hembras. En el caso de *R. darwinii*, machos preñados y hembras mostraron respuestas diferencias frente a los estímulos del otro sexo (Capítulo 3). Por esta razón, es posible que algunas características del canto que no fueron evaluadas a fondo (los fenómenos no lineales) podrían contener diferencias que cada sexo está reconociendo en los estímulos.

En general se ha sugerido que las hembras que producen señales llamativas (con múltiples notas e intensidad alta o similar a los machos) y el dimorfismo sexual con respecto a los machos, compiten por acceder a los machos cuando el cuidado parental es exclusivamente proporcionado por los machos (en aves: Goymann et al. 2004; en peces: Rosenqvist y Berglund et al. 2011; y en anuros: Goyes-Vallejos et al. 2017). En especies con rol sexual revertido, es decir que el cuidado parental es llevado a cabo principal o exclusivamente por los machos (Kokko y Johnstone 2002) se han detectado diferentes fenotipos de hembras que difieren en la competitividad de su comportamiento vocal, como ocurre en la jacana *Jacana spinosa* (Lipshutz 2017). Estas diferencias vocales y de tamaño también se han observado en *E. cystignathoides*, donde las hembras pequeñas son las que vocalizan (Serrano y Penna 2018). De acuerdo con Emerson y Boyd (1999), las hembras de anuro en general producen llamadas de advertencia cerca del momento en que están listas para desovar y aparearse. En *R. darwinii*, las interacciones entre machos son más frecuentes que con hembras y además presentan mayor interferencia dada la alta proporción de solapamiento de cantos en interacciones de dúos naturales.

En las interacciones vocales de la ranita de Darwin no se observaron comportamientos agresivos en contextos intra e intersexuales (Capítulos 2 y 3). Esta conducta tolerante es concomitante en *R. darwinii* con un camuflaje altamente mimético con su microhábitat (Bourke et al. 2011). Una condición monomórfica y coloración críptica en ambos sexos prevalece en especies de aves donde machos y hembras contribuyen al cuidado de nidos (Wallace 1889). Sin embargo, dado que el continuo de diferenciación de las señales entre monomorfismo-dimorfismo sexual está relacionado con el costo energético de cada sexo (Trail 1990; Kokko y Johnstone 2002) es posible que la mantención del dimorfismo sexual ligado al tamaño de *R. darwinii* podría ser un indicio de que ambos sexos mantienen un grado de elección sobre el otro sexo.

Los distintos experimentos de playback que realizamos sugieren que las interacciones vocales subyacen procesos de competencia intrasexual más significativos que la interacción intersexual. Otros estudios han mostrado que las señales sexuales producidas por los dos sexos pueden oscilar en función del ambiente (Prudic et al. 2011), de la evaluación de los ornamentos del sexo contrario (Berglund et al. 2005) y es en especies con cuidado paterno y señales que tienden a ser monomórficas, donde se esperaría que ocurriera elección mutua (Kokko y Johnstone 2002). Nuestros análisis de redes sugieren que la producción de señales juega un rol relevante en procesos de la organización grupal estrechamente relacionados con la variación del tamaño tanto de machos como de hembras y que estas circunstancias tienen repercusiones reproductivas a nivel individual y grupal. En el futuro, debería evaluarse si existen procesos de preferencia sexual por rasgos dentro de la variación de las señales de cada sexo (por ejemplo, frecuencia dominante, presión de sonido, número de notas producidas, etc.) por parte de ambos sexos en *R. darwinii* y si éstos varían en función del complejo ambiente social en el que cohabitan.

CONCLUSIONES

- La descripción de los cantos de advertencia de la ranita de Darwin era hasta ahora poco detallada y se había realizado en pocos individuos de una sola población. Nuestro estudio muestra que estos cantos están compuestos por señales de varias notas que pueden contener fenómenos no lineales, entre los que destaca el caos, que varía de manera amplia entre poblaciones e individuos concomitante con variación en la frecuencia dominante y la duración de los intervalos entre notas. La variación geográfica e individual de los cantos de machos no preñados está asociada a la variación del tamaño de estos anuros.
- Los cantos de machos y hembras son distintos. Las hembras tienen notas y canos de mayor duración y menor frecuencia dominante que los machos. Estas diferencias en el canto están asociadas a diferencias en el tamaño de los sexos, por lo que nuestra hipótesis acerca de que los rasgos morfológicos de los sexos se reflejan en las características del canto, fue confirmada. Los machos en estado de preñez vocalizan sin que se puedan detectar diferencias entre el canto de machos preñados y no preñados. Los cantos de las hembras son similares en su composición de notas a los de los machos y son producidos en contextos similares a los observados en otras especies (i.e. en ausencia de otros conespecíficos y en interacción con machos). Además, la distintividad y repetitividad son mayores entre individuos que entre los

sexos, por lo que es probable que en la ranita de Darwin el reconocimiento de individuos sea más efectivo que el del estatus sexual.

- No obstante, los principales emisores de cantos durante toda la estación reproductiva son los machos no preñados y las mayores interacciones espontáneas son observadas entre éstos. Las interacciones espontáneas no muestran un patrón distintivo de alternancia de los cantos entre los sexos. Sin embargo, los experimentos de playback indican que las hembras y los machos preñados se reconocen mutuamente, respondiendo con mayor ángulo de fase y menor número de cantos solapados a los cantos del otro estatus sexual respectivamente, por lo que nuestra hipótesis inicial de que machos y hembras de *R. darwinii* interactúan vocalmente de manera distinta con los cantos de cada sexo, no fue rechazada. En el mismo sentido, se demostró que ambos sexos tienden a solapar más rápidamente a los cantos de su propio sexo, lo cual podría ser un mecanismo de competencia no agresiva en la especie.
- Los análisis de redes sociales mostraron que el solapamiento de ámbitos de hogar y la similitud espectral y temporal de los cantos de *R. darwinii* no están relacionados con su estructura social, por lo cual se rechaza la hipótesis planteada. Sin embargo, evaluando la relevancia de los desplazamientos entre grupos encontramos que la proporción de machos preñados y no preñados, así como el tamaño relativo de estos individuos en las agregaciones son relevantes en la mantención de la estructura social de la especie. Son los adultos quienes tienden a desplazarse más entre grupos y son los machos preñados con mayor ámbito de hogar quienes producen mayor cantidad de larvas. En conjunto, nuestro estudio sugiere que, a diferencia de otros

anuros, las interacciones sociales de los individuos son relevantes para su adecuación en contextos no agresivos.

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