# Demografía de *Melanophryniscus montevidensis* en un área protegida de Uruguay, posibles amenazas y estrategias de conservación.

Tesis de Doctorado en Biología

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# Resumen general

*Melanophryniscus montevidensis* es un anfibio amenazado cuyas poblaciones se han reducido en varias localidades de Uruguay. El estudio de la demografía de esta especie permitiría una aproximación a sus amenazas locales, así como el establecimiento de medidas adecuadas para su conservación. Este trabajo tuvo como objetivo la estimación de los parámetros demográficos de esta especie en Laguna de Rocha (un área protegida de Uruguay), a efectos de entender su dinámica poblacional utilizando modelos matriciales, además de evaluar la importancia relativa de cada parámetro sobre dicha dinámica. La estimación de estos parámetros permitió: 1) una aproximación a las amenazas que podrían constreñir la dinámica poblacional, por su efecto sobre los diferentes estadios de vida, y 2) el modelaje de diferentes medidas de conservación para la misma. Con el fin de mejorar los métodos de estimación de las tasas de supervivencia de juveniles y adultos, también se profundizó en la aplicabilidad de la fotoidentificación como método de marcaje para estudios poblacionales de esta especie. En cuanto a los estadios larvarios, se utilizaron encierros *in situ* en cuerpos de agua del área protegida, logrando estimar las tasas de supervivencia desde la desova hasta la metamorfosis (1.2–4.8%) y la importancia relativa de la calidad del agua y las variables bióticas sobre dicha supervivencia y el tamaño corporal. Si bien la conductividad fue la variable abiótica con mayor efecto negativo, la densidad larvaria y el tamaño corporal tuvieron efectos negativos y positivos más importantes, respectivamente. Los metamorfos obtenidos en el campo fueron criados *ex situ* para monitorear mediante fotografías el desarrollo del patrón de coloración característico de la especie. Éste se desarrolló progresivamente y se estabilizó a una mediana de 220 días después de la metamorfosis, con una longitud máxima hocico-cloaca de 13.2 mm, tamaño considerado como el mínimo para fines de fotoidentificación. Durante tres años de muestreos de campo se aplicó un método de captura-marcaje-recaptura (CMR, utlizando la fotoidentificación como método de marcaje) para estimar las tasas de supervivencia de juveniles y adultos, tasas de madurez sexual, probabilidades de detección y abundancias. Las medianas de supervivencia fueron altas para adultos y juveniles (>80%), sin embargo, se detectaron ligeras disminuciones de otoño a invierno y disminuciones drásticas de primavera a verano (<50%). Utilizando las estimaciones de las tasas vitales (supervivencias, transición, reproducción), se construyeron modelos matriciales de Lefkovitch para determinar las tendencias poblacionales y las probabilidades de extinción en el área, y para evaluar la eficacia del manejo de hábitat y la suplementación por cría *ex situ* como estrategias de conservación. Se probó la hipótesis de que la disminución estacional en la supervivencia de las hembras adultas aumenta la probabilidad de extinción a corto plazo al reducir las tasas de crecimiento específicas durante estas estaciones. Evidencias de ello fueron: una tasa de crecimiento de la población (λ) mayormente sensible a la supervivencia de las hembras, que λ disminuyó cuando la supervivencia de las hembras disminuyó, y que los modelos con variación estacional en la supervivencia de las hembras tuvieron una probabilidad de extinción >90%. Las simulaciones de estrategias para revertir esta tendencia indicaron que el manejo de hábitat durante estaciones cálidas sería la práctica más efectiva. Finalmente, la protección de los cuerpos de agua donde esta especie habita y se reproduce es importante para su conservación, siendo recomendable el monitoreo de los parámetros de calidad de agua de estos sitios para cuidar el adecuado desarrollo larvario. No obstante, dada la sensibilidad reportada de λ hacia los estadios adultos de la población, se sugiere con mayor énfasis profundizar en el conocimiento de aquellos factores que estacionalmente disminuirían las tasas de supervivencia de los estadios terrestres, para la mitigación efectiva de estas amenazas.

# Main abstract

*Melanophryniscus montevidensis* is a threatened amphibian whose populations have been reduced in the last years at several localities of Uruguay. Studying the demography of this species would allow an approach to local threats, as well as the establishment of appropriate measures for its conservation. This work aimed to estimate demographic parameters of this species in Laguna de Rocha (a protected area of Uruguay); in order to understand the population dynamics using matrix models, assessing the relative importance of each parameter on such dynamics. Estimation of these parameters allowed: 1) approaching the threats constraining population dynamics, by their effect on different life stages; and 2) modelling different conservation strategies. To improve the methods of estimating juvenile and adult survival rates of this species, the applicability of photo-identification as a marking method was further investigated. Regarding to larval stages, *in situ* enclosures were used in water bodies of the area to estimate survival rates from spawning to metamorphosis (1.2-4.8%). Also, the relative importance of water quality and biotic variables on such survival and body size were assessed. Although conductivity was the abiotic variable with the greatest negative effect, larval density and body size had heavier negative and positive effects, respectively. Metamorphs obtained in the field were raised *ex situ* to photographically monitor the development of the characteristic color pattern of the species. The pattern developed progressively and stabilized at a median of 220 days after metamorphosis, with a maximum snout-vent length of 13.2 mm, this size was considered the minimum for photo-identification purposes. Along three years of field surveys, a capture-mark-recapture method (CMR, using photo-identification as a marking method) was applied to estimate juvenile and adult survival rates, sexual maturity (transition) rates, detection probabilities, and abundances. Survival medians were high for adults and juveniles (> 80%). However, slight decreases from fall to winter and drastic decreases from spring to summer (< 50%) were detected. Using estimations of vital rates (survival, transition, reproduction), Lefkovitch matrix models were constructed to determine population trends and probabilities of extinction in the area, and to assess the efficacy of habitat management and supplementation by *ex situ* breeding as conservation strategies. It was hypothesized that the seasonal decline in adult female survival increases the probability of extinction in the short term by reducing specific growth rates during these seasons. In agreement with this hypothesis, we found that population growth rate ($\lambda$) was mostly sensitive to female survival, $\lambda$ decreased when female survival declined, and models with seasonal variation in female survival had an extinction probability > 90%. Simulations of strategies to revert this trend indicated that habitat management during warm seasons would be the most effective practice. Finally, the protection of water bodies where this species inhabits and breeds is important for its conservation, being advisable monitoring programs of water quality parameters to ensure a proper larval development in these environments. However, due to the reported sensitivity of $\lambda$ to the adult stages of the population, the improvement of the knowledge of those factors that seasonally decrease the survival rates of terrestrial stages is suggested for effective mitigation of these threats.

# Introducción general

Desde los primeros registros de declinaciones en las poblaciones de anfibios en los años cincuenta y sesenta, los reportes han continuado hasta la actualidad a una tasa anual de alrededor del 2% (Houlahan *et al.* 2000), siendo un fenómeno global que ha colocado a los anfibios como el grupo taxonómico con mayor proporción de especies amenazadas (Stuart *et al.* 2004; IUCN 2017). Este grupo es particularmente susceptible a las alteraciones de origen antrópico debido a características propias de las especies que lo integran, tales como sus ciclos de vida complejos (con estados larvarios acuáticos y adultos terrestres, que los expone a un amplio rango de ambientes), su respiración cutánea que requiere gran vascularización y mantenimiento de la humedad de la piel (que los vuelve sensibles a la desecación, contaminantes y enfermedades cutáneas), y el hecho de ser termo conformes (que los expone a las variaciones de temperatura consecuencia del cambio climático) (Wake y Vredenburg 2008). El conocimiento de las dinámicas poblacionales, y el impacto de las actividades humanas sobre las mismas, permite diseñar estrategias para reducir este impacto y conservar las especies de anfibios. La conservación de este grupo, como parte de la biodiversidad en su conjunto, no debe ser desatendida ya que aporta bienes y servicios ecosistémicos que contribuyen a sostener la vida humana (Myers 1996).

El género *Melanophryniscus* pertenece a la familia Bufonidae y está compuesto por 29 especies de pequeña a mediana talla con una distribución que se limita al sur de Sudamérica (Frost 2017). Son conocidos por exhibir coloraciones intensas en el vientre y producir sustancias tóxicas en la piel, principalmente hidroquinona y alcaloides del grupo de las pumiliotoxinas (Mebs *et al.* 2005, 2007), algunas de las cuales poseen potencial valor medicinal (Daly *et al.* 2000; Mebs *et al.* 2005). Seis especies de este género ocurren en Uruguay (Maneyro y Carreira 2012), cuatro de ellas poseen algún grado de amenaza a escala global y/o nacional  (IUCN 2017; Carreira y Maneyro 2015), entre las que se encuentra *Melanophryniscus montevidensis*. Particularmente para esta especie se ha registrado una reducción del número de poblaciones en varias localidades de Uruguay (Carreira y Maneyro 2015), además de figurar con tendencia poblacional decreciente en la Lista Roja de Especies Amenazadas de la IUCN (2017). No obstante, no se cuenta con información demográfica de *M. montevidensis* (*i.e.*, tasas de crecimiento y tamaños poblacionales, tasas de sobrevivencia) que permitan cuantificar esta tendencia. Tampoco se dispone de información específica sobre las amenazas, si bien se postula que la urbanización, el desarrollo agrícola y la deforestación y reforestación con especies exóticas son las principales amenazas para la especie (Langone *et al.*, 2004). Estas lagunas de información dificultan el establecimiento de medidas adecuadas de conservación para *M. montevidensis*.

Los efectos de las amenazas antrópicas mencionadas para *M. montevidensis* (especialmente la urbanización y el desarrollo agrícola) suelen reflejarse sobre la calidad del agua dulce (Vörösmarty *et al.* 2000; Cazzolla Gatti 2016). Los estadios larvarios de los anfibios son especialmente susceptibles a los cambios ambientales (Halliday 2008), existiendo diversos reportes sobre la respuesta larvaria a diferentes niveles de parámetros de calidad de agua tales como: nitritos, nitratos, amoníaco (Marco *et al.* 1999; Rouse *et al.* 1999; Marco *et al.* 2001; Ortiz-Santaliestra *et al.* 2006), turbidez, conductividad y oxígeno disuelto (Schmutzer *et al.* 2008), pesticidas (Peltzer *et al.* 2008), o acidez (Freda y Dunson 1985). Los niveles alterados de calidad de agua (por fuera del rango óptimo para la vida acuática, *sensu* USEPA 1997) pueden reducir la supervivencia y reclutamiento de individuos, mediante la alteración de las tasas de depredación, la disponibilidad de alimento, la prevalencia de enfermedades y parásitos, o la incidencia de anomalías (Johnson y Chase 2004; Rohr y Crumrine 2005; Peltzer *et al.* 2008; Relyea y Hoverman 2008; Hayes *et al.* 2010; Egea-Serrano *et al.* 2012). El estudio *in situ* del efecto de diferentes parámetros de calidad de agua sobre el desarrollo larvario de *M. montevidensis,* y su importancia respecto a variables bióticas (*e.g.*: depredación, densidad larvaria) permite identificar amenazas potenciales para esta especie.

Si bien la supervivencia y el adecuado desarrollo larvario son fundamentales para el reclutamiento y mantenimiento de las poblaciones (Hayes *et al.* 2010), la supervivencia de las hembras reproductivas ha sido reiteradamente documentada como la tasa vital con mayor importancia relativa para el crecimiento poblacional de los anfibios (Cairo 2009; Homyack y Haas 2009; Unger *et al.* 2013; Kissel *et al.* 2014). La estimación de las tasas vitales (supervivencia, tasas de maduración, reproducción) de estadios larvarios, juveniles y adultos, permite establecer un vínculo cuantitativo entre las reducciones de ciertas tasas vitales y la reducción de la tasa de crecimiento total (λ) de la población, que es lo que verdaderamente conlleva a una declinación poblacional (Biek *et al.* 2002; Conroy y Brook 2003). En particular, los modelos estructurados por estados de vida, como las matrices de Lefkovich (Caswell 1989), son los más adecuados para modelar integralmente la dinámica poblacional de los anfibios, quienes poseen ciclos de vida complejos en los que cada estado tiene una ecología, fisiología y morfología diferentes (Werner 1988). Estos modelos permiten calcular λ y estimar la tasa vital de mayor importancia relativa sobre λ mediante análisis de sensibilidad, así como proyectar el futuro de las poblaciones para estimar probabilidades de extinción y simular diferentes medidas de conservación para estimar su eficiencia (Caswell 1989; Heppell *et al.* 2000; Morris y Doak 2002; Kissel *et al.* 2014). La mayor dificultad para la aplicación de los modelos matriciales radica en el requerimiento de estimaciones completas de las tasas vitales, incluyendo sus varianzas temporales (Caswell 1989). Sin embargo, formulaciones sencillas, con información mínima de la especie a modelar, o usando información demográfica disponible en la bibliografía sobre especies similares (corporal, filogenética y/o ecológicamente), permiten aproximaciones aplicables para la conservación (Heppell *et al.* 2000).

A efectos de estimar las tasas de supervivencia y tamaños poblacionales, los métodos de captura marcaje recaptura (CMR) son los más adecuados, ya que ajustan los datos de recuento sistemático a las probabilidades de detección de los animales, evitando el sesgo intrínseco de los conteos aislados (Schmidt 2003, 2004). Dependiendo del tipo de datos de recaptura obtenidos utilizando métodos de CMR, existen diferentes formulaciones matemáticas adecuadas para la estimación de las tasas vitales (White y Burnham 1999; Cooch y White 2015). El software MARK permite la construcción y evaluación de diferentes modelos candidatos (hipótesis) para la estructura temporal de los datos contemplando la biología de la especie a modelar (White y Burnham 1999). En cuanto a *M. montevidensis*, la única dinámica temporal que se conoce es el aumento de los patrones de actividad motivados por las precipitaciones locales, que están relacionados con la estrategia explosiva de reproducción de la especie (Maneyro y Carreira 2012; Pereira y Maneyro 2016a). Sin embargo, la alta probabilidad de captura durante la actividad reproductiva puede enmascarar patrones demográficos a largo plazo, especialmente en anfibios de pequeña talla (Vasconcellos y Colli 2009). Los patrones temporales de supervivencia, reclutamiento y abundancia de los anfibios pueden verse afectados por los patrones de reproducción de la especie, al igual que en otros bufónidos (Vasconcellos y Colli 2009), pero también pueden ser afectados por patrones de actividad estacional independientemente de la actividad reproductiva (Guimarães *et al.* 2011), variar anualmente o incluso no tener un patrón definido (Hiert *et al.* 2012).

La precisión de las estimaciones de supervivencia y tamaño poblacional por CMR depende de la aplicación de formulaciones adecuadas para el tipo de datos de recaptura y del tamaño muestral (Cooch y White 2015), pero también del método de marcaje a aplicar. Un método de marcaje ideal no afecta la salud, supervivencia o comportamiento del animal, permite la individualización, perdura a lo largo de los muestreos y tiene un costo mínimo (Ferner 2007). Las coloraciones características de los estadios terrestres de los *Melanophryniscus* permiten la aplicación de técnicas de identificación a partir del patrón de coloración natural, como la fotoidentificación (Abadie 2012; Caorsi *et al.* 2012, 2014; Elgue *et al.* 2014). Estos métodos de marcaje no son invasivos (a diferencia de métodos clásicos como el “Toe clipping”) y ya han sido aplicados exitosamente en *M. montevidensis* (Elgue *et al.* 2014; Pereira y Maneyro 2016b). No obstante, al igual que en otras especies del género, los individuos recién metamorfoseados de esta especie pueden carecer de esta coloración característica, presentando coloraciones crípticas (Langone *et al.* 2008; Caorsi *et al.* 2014). Estos juveniles tempranos son difíciles de encontrar en el campo, y no se han registrado recapturas de los mismos usando fotoidentificación (Pereira y Maneyro 2016b). Por consiguiente, es posible que el uso de la fotoidentificación como método de marcaje en estudios poblacionales no sea adecuado para todos los estadios de vida de esta especie, ya que podría violar el supuesto de perdurabilidad de la marca si se aplica en estadios previos a la aparición definitiva del patrón de coloración.

Este estudio tuvo como ejes principales la estimación de los parámetros demográficos de *M. montevidensis* en Laguna de Rocha (un área protegida de Uruguay), a efectos de entender la dinámica poblacional de esta especie utilizando modelos matriciales, así como evaluar la importancia relativa de cada parámetro demográfico sobre dicha dinámica. Colateralmente, la estimación de los parámetros demográficos permitió 1) una aproximación a las amenazas que podrían afectar la dinámica poblacional, por su efecto sobre los diferentes estadios de vida de esta especie, y 2) el modelaje de diferentes medidas de conservación para la especie. Paralelamente, con el fin de mejorar específicamente los métodos de estimación de las tasas de supervivencia de juveniles y adultos, se abordó la problemática de la aplicabilidad de la fotoidentificación como método de marcaje para estudios poblacionales de esta especie.

Los capítulos de este estudio (disponibles en idioma Inglés) abordaron las problemáticas planteadas de la siguiente manera:

### Capítulo I: *In situ* larval development of the threatened South American toad *Melanophryniscus montevidensis* (Anura, Bufonidae)

Utilizando encierros *in situ* en cuerpos de agua de un área protegida (Laguna de Rocha, Rocha, Uruguay) se estimó la tasa de supervivencia y duración del desarrollo larvario, y se midió el tamaño corporal, desde la desova hasta el final de la metamorfosis. Además, utilizando modelos lineales generalizados, se evaluó qué factores de la dinámica larval (densidad, tamaño corporal, depredación, sitio, profundidad) explicaron mejor la supervivencia global de las larvas. Este capítulo fue enviado para su publicación en la revista *South American Journal of Herpetology* en febrero de 2017.

### Capítulo II: Role of water quality and biotic predictors on body size and larval survivorship of the endangered red bellied toad *Melanophryniscus montevidensis*

En base a los resultados obtenidos en el capítulo anterior, se planteó la hipótesis de que la baja calidad del agua reduce el éxito de la metamorfosis al reducir el crecimiento y la supervivencia de las larvas de *M. montevidensis*. Para poner a prueba esta hipótesis, se evaluó el efecto relativo de los parámetros fisicoquímicos de calidad de agua (pH, conductividad, turbidez, amoníaco, oxígeno disuelto, yodo, nitrato, nitrito y fosfato) y variables bióticas (densidad, tamaño corporal, depredación) sobre los datos obtenidos *in situ* de supervivencia y tamaño corporal en diferentes estadios larvarios (embriones, larvas, metamorfos) como variables de respuesta de modelos lineales generalizados. También se caracterizó a los cuerpos de agua por sus variables fisicoquímicas, determinando si los mismos poseían valores aceptables para la vida acuática o reflejaban desequilibrios típicos de eutrofización u acidificación.

### Capítulo III: Quantitative determination of the minimum body size for photo-identification of *Melanophryniscus montevidensis* (Bufonidae)

Mediante la cría *ex situ* de metamorfos obtenidos en los capítulos anteriores, se monitoreó el desarrollo de la coloración y crecimiento durante el primer año de vida de estos juveniles, hasta la estabilización del patrón ventral de coloración. Esto permitió estimar el tamaño mínimo que debe tener un juvenil de esta especie para que las técnicas de fotoidentificación provean resultados confiables cuando son utilizadas en estudios poblacionales. Este capítulo fue publicado en la revista *Herpetological Conservation & Biology* en mayo de 2017 (Apéndice 1).

### Capítulo IV: Seasonal estimations of survival rates and abundances of the threatened bufonid *Melanophryniscus montevidensis* in a protected area of Uruguay

Utilizando la fotoidentificación como método de marcaje, y un diseño robusto de captura-marca-recaptura (CMR), se realizaron muestreos estacionalmente desde 2013 a 2015 una población de *M. montevidensis* localizada en Laguna de Rocha (Uruguay). De esta manera se estimaron las tasas de supervivencia estacionales de juveniles y adultos, las tasas de madurez sexual, las probabilidades de detección y abundancias de esta población. También se analizaron los patrones de actividad (reproductiva y no reproductiva) para probar diferentes hipótesis sobre los parámetros demográficos de la especie.

### Capítulo V: Demographic projections and conservation strategies for the threatened bufonid *Melanophryniscus montevidensis*

Las estimaciones de tasas vitales (supervivencia, maduración, reproducción) de los diferentes estadios (larvas, juveniles, adultos) de la especie obtenidas en los capítulos anteriores, permitieron la construcción de modelos matriciales de Lefkovitch para determinar la tendencia poblacional (creciente o decreciente), la tasa vital con mayor peso sobre esta tendencia y las probabilidades de extinción en un área protegida (Laguna de Rocha, Uruguay). Específicamente, utilizando modelos determinísticos y estocásticos, se puso a prueba la hipótesis de que la disminución de la supervivencia de las hembras adultas durante las estaciones cálidas (detectada en el capítulo anterior), aumenta la probabilidad de extinción de la población a corto plazo al reducir las tasas de crecimiento poblacionales durante tales estaciones. Además, se realizaron simulaciones de diferentes estrategias de conservación (manejo de hábitat y la suplementación por cría *ex situ*) para evaluar la eficacia de las mismas en disminuir las probabilidades de extinción de la población estudiada.

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# Capítulo I

## *In situ* Larval Development of the Threatened South American Toad *Melanophryniscus montevidensis* (Anura, Bufonidae)

# ABSTRACT-RESUMEN

**Abstract**. Population declines are noticeable when adults disappear locally, or massive die-offs are reported. However, populations can slowly decline if recruitment is unsuccessful. We aimed to determine the recruitment rates and the biotic factors involved in the metamorphosing success of a threatened species, *Melanophryniscus montevidensis*. Using *in situ* enclosures in ponds of a protected area (Laguna de Rocha, Rocha, Uruguay) we assessed larval survivorship, body size, and developmental duration, from eggs to the end of metamorphosis. Also, we evaluated which factors of the larval dynamics (density, body size, predation, location, depth) better explained the overall survivorship using GLMs. From 25 spawns from three ponds, we reported metamorphs in only eight spawns from two ponds, accounting for a median success per spawn of 1.2%. Median development time until Gosner stage 42 was 27 days, with a median body length of 5.7 mm. The best simplified GLM for survival success included number of larvae (recorded ten days before sampling) and depth as significant negative predictors and body length as a positive predictor. Depth and body length had the greatest effect. However, larval density was important either directly on the metamorphosing success or indirectly on the ability to respond to pond desiccation. In spite of the potential plasticity of the species to respond to pond desiccation, we predicted that ponds that dry in less than 22 days might not yield any offspring. The *in situ* data provided by this study may help understanding the processes behind the decline of *Melanophryniscus montevidensis*, thus helping to establish proper conservation actions for the populations and the ponds where they breed.

**Resumen**. Las declinaciones poblacionales son evidentes cuando los adultos desaparecen localmente, o son reportadas muertes masivas. Sin embargo, las poblaciones pueden disminuir lentamente si el reclutamiento es bajo. Nuestro objetivo fue determinar las tasas de reclutamiento y los factores bióticos involucrados en el éxito de la metamorfosis de una especie amenazada: *Melanophryniscus montevidensis*. Para ello medimos la supervivencia larvaria, el tamaño corporal y la duración del desarrollo, desde la desova hasta el final de la metamorfosis, utilizando encierros *in situ* en charcas de un área protegida (Laguna de Rocha, Rocha, Uruguay). Además, se evaluó cuáles factores de la dinámica de las larvas (densidad, tamaño corporal, depredación, sitio, profundidad) explicaron mejor la supervivencia global usando GLMs. Registramos 25 desovas en tres charcas, de ellas, procedentes de dos charcas, registramos metamorfos en solo ocho desovas, lo que representó un éxito por desova del 1.2%. La mediana del tiempo de desarrollo hasta la etapa 42 de Gosner fue de 27 días, con una mediana del tamaño corporal de 5.7 mm. El mejor GLM simplificado para el éxito de supervivencia incluyó el número de larvas (registrado diez días antes) y la profundidad como predictores negativos significativos, y la longitud del cuerpo como predictor positivo. La profundidad y el tamaño corporal fueron los factores de mayor peso. No obstante, la densidad larvaria fue importante, ya sea directamente en el éxito de la metamorfosis o indirectamente sobre la capacidad de responder a la desecación de la charca. A pesar de la posible plasticidad de la especie para responder a la desecación de las charcas, aquellas con una duración menor a 22 días no permitirían el reclutamiento en estas poblaciones. Los datos *in situ* proporcionados por este estudio pueden ayudar a entender los procesos que están detrás de los declives de *Melanophryniscus montevidensis*, así como establecer acciones de conservación apropiadas para las poblaciones y las charcas donde se reproducen.

# INTRODUCTION

In the context of the current amphibian declines, death and decreased recruitment are the immediate results of ultimate declining causes, such as atmospheric change, environmental pollutants, habitat loss, invasive species and pathogens (Stuart *et al.* 2004; Hayes *et al.* 2010; Egea-Serrano *et al.* 2012; Carvalho *et al.* 2017). Although population declines are more noticeable when adults disappear or massive die-offs are reported, populations can decline slowly if healthy adults are not breeding or if offspring do not develop properly (Hayes *et al.* 2010). As most anurans have complex life cycles, to assess if non-conspicuous decline of a given population is occurring (*e.g.*, by using stage structured models of the population dynamics), vital rates of both larval and adult stages in nature should be assessed first (*e.g.*, Biek *et al.* 2002; McCaffery and Maxell 2010).

Regarding larval stages, survivorship is the most direct measure of recruitment (Cecil and Just 1979; Berven 1990). However, body size at the end of metamorphosis plays an important role in the post-metamorphic fitness of many anuran species, being larger sizes at the end of metamorphosis advantageous for later terrestrial stages (Werner 1986; Berven 1990; Altwegg and Reyer 2003; Cabrera-Guzmán *et al.* 2013). Both survivorship and growth of the larvae are usually density-dependent, with competition for food being one of the most relevant factors leading to the decrease of both parameters at high densities (Wilbur and Collins 1973; Semlitsch and Caldwell 1982). On the other hand, density can have positive effects on survivorship by reducing predation risk in aggregations (DeVito 2003). Also, in order to avoid predation anuran larvae are selected to grow fast (Werner, 1986 and references therein), with a minimum threshold body size to be reached for successful metamorphosis (Wilbur and Collins 1973; Werner 1986). Few studies have addressed the relative importance of these biotic factors on the metamorphosing success that leads to recruitment in natural populations of amphibians (*e.g.*, Rowe and Beebee 2003; Touchon *et al.* 2013). Moreover, most of the studies focusing on the effects of biotic parameters on larval survivorship were carried out in laboratorial conditions, where survival rates may be biased (Melvin and Houlahan 2012, 2014).

*Melanophryniscus montevidensis* (Philippi, 1902) is a diurnal explosive breeder (*sensu* Wells 2007) that lays eggs in ephemeral ponds after heavy rains, and has a fast larval development. It takes about 30 days from hatching to complete metamorphosis (Garrido-Yrigaray 1989; Prigioni and Garrido 1989). These features make it an excellent species for *in situ* monitoring of the complete larval development. Some information about the development of *M. montevidensis* in captive conditions is available (Garrido-Yrigaray 1989; Alonzo *et al.* 2002; Maneyro and Achaval 2004), as well as the development time in semi-natural conditions (Bardier *et al.* 2017). However, there are no detailed data about the development or the biotic conditions that may affect survival success in nature. Also, as it is a threatened species (*Vulnerable* in IUCN 2017; *Critically Endangered* in the Uruguayan red list, Carreira and Maneyro 2015; *Near Threatened* and *Endangered* in Brazilian national and state-level lists, respectively, Fundação Zoobotânica 2014; ICMBio 2014), metamorphosing success (recruitment) in non-altered habitats is an important vital rate that may help understanding population declines, either by comparing with future reports or by including them in stage-structured models.

The aim of this study is twofold. First, using *in situ* enclosures we assessed *Melanophryniscus montevidensis*'s larval survivorship, body size, and developmental duration (from egg to the end of metamorphosis), in natural conditions. Second, using *in situ* metamorphosing success as a response variable, we evaluated which biotic factors of the larval dynamics (density, body size and predation) influenced more on the overall survivorship in natural ponds.

## METHODOLOGY

### General background on the species

*Melanophryniscus montevidensis* is an explosive breeder that exploits shallow ponds (less than 30 cm deep) formed over psammophilic vegetation after heavy rains, possibly all year round (Alonzo *et al.* 2002; Pereira and Maneyro 2016; Bardier *et al.* 2017). Breeding activity is mainly diurnal; males floating on the water surface or holding on to the aquatic vegetation start calling immediately after rains. The amplectant pairs lay eggs in several small masses attached to the vegetation of the pond (Alonzo *et al.* 2002). No gregarious behaviour has been described for the larvae. Although the larval diet has not yet been described, they seem to have benthic habits

(Garrido-Yrigaray 1989; Prigioni and Garrido 1989; Alonzo *et al.* 2002; Baldo *et al.* 2014; Pereira and Maneyro 2016).

### Sampling design

We studied the larval development of *M. montevidensis* at three ephemeral ponds of Laguna de Rocha (34°37'S, 54°17'W, 3–10 m a.s.l., Figure 1, description in Apéndice 2), a protected area of the Uruguayan National System of Protected Areas (SNAP). We observed three breeding events in the ephemeral ponds after heavy rains in February 2014. We accessed climate data before and during sampling days from http://www.weatherlink.com/user/lagunaderocha (Apéndice 3). Each breeding event lasted two or three days. During those days we searched for, collected, and stored amplectant pairs individually in plastic cages of about one liter, with water and vegetation from the pond), a procedure previously applied to obtain egg masses from this species (Alonzo *et al.* 2002; Pereira 2014).

We collected a total of 29 amplectant pairs, 28 were female-male and one was a male-male pair. From the 28 female-male pairs, 25 spawned inside the cages within 24 h after capture: six from pond 1, 10 from pond 2, and nine from pond 3. After that, we released the adults in the same places where they were originally captured. We recorded the number of eggs *per* spawning, and then we placed each spawning (from each amplectant pair) in an *in situ* semi-permeable enclosure at the exact position where the amplectant pair was first observed (Figure 2). Enclosures were 32-liter cages of 43.0 cm (length) x 27.4 cm (width) x 27.1 cm (height), made of polypropylene, a safe, relatively inert polymer that does not contain Bisphenol-A, and that is used as food and drinking water containers (Maier and Calafut 1998). We made perforations to allow water flow in two sides of the cages, and covered the sides with a thin plastic mesh (1 x 1 mm). We glued the mesh to the inner part of the cage using Sikaflex 1a (Sika AG, Zugerstrasse, Switzerland), which is used for drinkable water containers. To emulate natural conditions, we fixed the enclosures to the bottom of the pond and added some emergent vegetation, to provide shelter while tadpoles exploit the whole water column. As the diet of the larvae was unknown, we paid special attention to the trophic behaviour of the larvae, in order to discard carnivore or macrophage diets. None of these behaviours were observed during the study. We observed larvae feeding only on the periphyton that grew on the walls and floor of the cages or on decomposing leaves of emergent plants, and so did not add extra food. A wide plastic mesh (14 x 19 mm) covered each enclosure to avoid avian or mammalian predation but not invertebrate predators. Common invertebrate predators for amphibians such as coleopteran and dragonfly larvae were present in the three ponds inside and outside the enclosures; we did not observe ponds without predators in the area during the sampling period. Although these larvae could not pass the thin mesh, eggs smaller than 1 mm$^2$ could have entered the cages (*e.g.*, Joop *et al.* 2007), or could also have been oviposited by adults through the wide mesh cover.

We monitored the clutches once every two days, from 10:00 to 18:00 h, until the end of metamorphosis or complete desiccation of the pond (no water in the enclosures), for a maximum of 35 days. We took pictures inside the enclosures during each sampling day in order to record the number of larvae and estimate their body, using a Coolpix L810 camera (16 MP; Nikon Corporation, Tokyo, Japan). We included a 1.0 mm ruler as a scale in the picture frames, and the distance and lighting remained roughly constant to standardize images and facilitate digital measurements. Metamorphs were photographed using the same criteria. Each time we took pictures (between 10:00 and 18:00 h) we measured water temperature and level inside the enclosures, using a digital thermometer (Oakton Instruments, Vernon Hills, Illinois, USA) and a wooden yardstick. To reduce depth variation inside the enclosures, each time depth decreased below the level of the previous sampling day we moved the enclosures into the closest deeper zone of the pond if such existed (see example plotted for three of the six enclosures from pond 1 in Apéndice 4). Due to the shallow initial water level of the ponds, water level of the enclosures was usually below 13 cm (half of the cages' height; Apéndices 2 and 4).

We measured and counted larvae and eggs using the Micrometrics SE Premium (ACCU-SCOPE, Commack, New York, USA) and ImageJ 1.48v (National Institutes of Health, Bethesda, Maryland, USA) software, respectively. We considered body length (BL) from the tip of the snout to the body terminus, excluding the tail (*sensu* McDiarmid and Altig 1999). On the seventh day after oviposition we observed that survivors reached larval stages (Gosner stages 26–30), so we considered hatching success on this day. Predators became observable from day six to seven, and were observed predating on *M. montevidensis* on the following days. On those days, we drew a random square of 12 x 12 cm on the floor of the enclosures to estimate predator density (predator/cm$^2$). For the purpose of this study we considered the success of an individual to complete metamorphosis when reaching Gosner stage 42 (Gosner 1960), because at this stage the animals avoided water by climbing the net of the cages above the water level. In captivity, using proper humidity levels, specimens of *M. montevidensis* in this stage were already terrestrial (Bardier *et al.* 2017). Some specimens reached this stage on day 20 after oviposition, so we considered this day as the start of the metamorphosing phase of the development in further analysis.

## Analysis

We generated descriptive statistics for the following variables of each spawn of each amplectant pair: total number of eggs laid, hatching success (percentage of hatched larvae that survived until day seven over total eggs laid), metamorphosing success (percentage that reached Gosner stage 42 from the total eggs laid), median time of development, and median BL of larvae and metamorphs. We searched for differences in larval performance (number of eggs laid and percentages of larvae and metamorphs) between the three ponds. Whenever comparisons were possible and the assumptions of homoscedasticity (Bartlett and Fligner-Killeen tests for homogeneity of variances) were met, we used ANOVA tests. For paired comparisons between ponds, when the normality assumption of data was met (tested through Shapiro-Wilk tests), we performed a Student's *t*-test; otherwise we performed Wilcoxon rank sum test. We plotted survivorship curves for each enclosure in each pond; the death of more than 50 larvae (representing nearly 20% of the initial spawn size) between two sampling days was considered a mass mortality event. As we detected predators inside the enclosures of ponds 1 and 3, we performed a Student's *t*-test to determine if there were differences in the predator charge.

In order to determine the relative importance of biotic variables over metamorphosing success, we fitted logit quasi-binomial General Linear Models (GLMs) to the data. The daily proportion of metamorphs from the number of eggs per clutch (enclosure) was the response variable; only considering data since the beginning of the metamorphosing phase (days 20 to 34 of the development, $n = 61$). The biotic predictors chosen were the daily measurements *per* enclosure of predator density (predator/cm$^2$) on day seven, number of larvae since day 20 and ten days before that measuerement (to measure the importance of larval density in previous stages), and body length since day

20 (using the median BL of up to 15 larvae *per* enclosure). To consider inter-pond differences in metamorphosing success and water depth variation, we also included site (pond), and pond depth (measured inside each enclosure before relocation each day) as predictors in the models. As all larvae died before day 20 in three enclosures of pond 1, and as all egg masses died before day five in pond 2, data from those three enclosures of pond 1 and all data from pond 2 were excluded from the data set for this analysis.

To avoid multicollinearity between variables, we screened for highly correlated predictors based on pairwise Spearman's rank correlations matrix with $|r| > 0.7$. For each correlated pair, we retained the variable with the higher explanatory power on the survival and growth of larvae, according to a univariate generalized linear model within the "select07" procedure in R (R Foundation for Statistical Computing, Vienna, Austria; "select07" method in Dormann *et al.* 2013). As site is a factor with two levels (pond 1 and 3) it was not included in the "select07" procedure. The saturated model included site and each uncorrelated continuous predictor fitted as a covariate of site (Crawley 2007). A backward stepwise procedure was applied to the saturated model to obtain a simplification in which predictors still contributed significantly to predict the probability of success in the survival and metamorphosis of the larvae (Hosmer *et al.* 2013). Because of the overdispersion of errors in the fit, a quasi-binomial distribution of errors was assumed in the survival GLMs; there is no automatic simplification routine for this adjustment (no AIC is available), so we used ANOVA to compare the residual deviance between saturated and simplified models (Crawley 2007). To assess the relative importance of predictors within the final model, we used the student $t$ statistic: the higher the $t$ for each predictor, the higher the explanatory power of the predictor over the response variable. We considered an alpha of 0.05 in all analyses, using the statistical software R (*op. cit.*).

## RESULTS

We found no differences in spawning size between the three ponds considering the results of the ANOVA (Bartlett's K-squared = 5.2, df = 2, $P = 0.1$; Fligner-Killeen's $\chi^2 = 2.7$, df = 2, $P = 0.3$; $F = 0.0$, df = 2, $P = 1.0$), the average spawning size per amplectant pair was 134.8 ± (SD) 73.3 eggs. However, we found significant differences in hatching success per spawn between ponds (Shapiro-Wilk normality test of % of surviving hatchlings: W = 0.9, $P = 0.2$ pond 1; W = 0.9, $P = 0.2$ pond 3; $t = -5.7$, df = 12.1, $P < 0.001$) and metamorphosing success per spawn between ponds (Shapiro-Wilk normality test of % that reached Gosner stage 42: W = 0.5, $P < 0.001$ pond 1; W = 0.8, $P < 0.05$ pond 3; Wilcoxon W = 8.0, $P < 0.05$). In pond 2, all egg masses died; no egg hatched along the first five days after spawned and showed signs of decomposition by the third day. Only tadpoles from seven spawns in pond 3 and from one spawn in pond 1 completed the metamorphosis (Table 1; Apéndice 2). Considering only these ponds (with eight successful enclosures out of 15) the median percentage of eggs that reached metamorphosis per spawn in this study was 1.2%.

All enclosures suffered mass mortalities during hatching stages in pond 1 (Figure 3); the survivors of this pond completed the metamorphosis before the pond dried. In pond 3, although metamorphosing success was higher than in pond 1, mass mortalities occurred in three enclosures because the pond dried before larva completed the metamorphosis (Figure 3). Median development time was 27 days (Table 1). On the fifth day larvae reached Gosner stage 25; stages 26–30 were reached on the seventh day. Stages 31–39 were observed on days 11–12 of the development, median BL at these later stages was 4.3–4.5 mm (Table 1, Figure 4). Newly metamorphosed individuals showed a grayish coloration, lacking the brilliant coloration patterns of adults, median BL of these metamorphs was 5.7 mm (pond 3; Table 1, Figure 4).

We observed invertebrate predators (Odonata and dytiscid Coleoptera) feeding on *M. montevidensis* larvae inside the cages from ponds 1 and 3. We found significant differences in the number of predators between ponds 1 and 3 (Shapiro-Wilk normality test of number of predators: W = 0.9, $P = 0.2$ pond 1; W = 0.9, $P = 0.7$ pond 3; Student's $t = 3.4$, $P < 0.005$). In pond 2, measurements ended on the fifth day and we did not see invertebrates inside the enclosures.

Regarding to GLMs, we found a correlation between number of larvae on sampling day and number of larvae ten days before ($|r| > 0.7$, $P < 0.05$) having the latter a higher explanatory power over the response variable (metamorphosing success). Thus, only the number of larvae ten days before the sampling day was included in the saturated model (Table 2). Predator densities were dismissed during the simplification procedure, as were interactions between site and other predictors, and site as a predictor itself (Table 2). The simplified model included number of larvae ten days before and depth as significant negative predictors, and body length as a positive predictor of metamorphosing success, having depth and body length the greatest effects (Table 3).

## DISCUSSION

Information on complete larval development of amphibians in natural conditions is generally scarce (Melvin and Houlahan 2012), and has not been previously recorded for any *Melanophryniscus* species. This study provides new data of survival rates, body sizes and development times, as much as other descriptive information, in semi-natural conditions.

The range of eggs laid *per* couple did not differ between ponds, and was within the numbers reported in previous studies of *M. montevidensis* (Garrido-Yrigaray 1989; Alonzo *et al.* 2002; Pereira 2014). Later during development, hatching and metamorphosing success differed locally (*i.e.*, between ponds). Considering only ponds with metamorphs, we found local differences in predator densities, with lower percentages of metamorphs in ponds presenting higher predator densities. The presence of predators would reduce larval survivorship in *Melanophryniscus* species, as some reports indicate that the ephemeral ponds where these species breed usually do not host predators and larvae do not exhibit predator avoidance behavior (Pereyra *et al.* 2011; Laufer *et al.* 2015). Nevertheless, predator density was dismissed as a predictor of the metamorphosing success during the simplification process of the saturated model, being larval density on previous days, body length, and water depth the main explanatory variables. Larval density at intermediate stages (mainly between days 10 and 20) had a more important (and detrimental) effect on metamorphosing success than larval density by the end of metamorphosis. Densities during larval stages were higher than during the metamorphosing phase (after day 20), so intraspecific competition or disease spread were possibly stronger during larval stages, reducing larval survivorship, which lead to reduced metamorphosing success (*e.g.*, Tejedo and Reques 1994).

Body lengths at Gosner stages 31–39 (4.3–4.5 mm) were smaller than lengths reported by Garrido-Yrigaray (1989) at stage 34 (5.6 mm). This could be due to *in situ* growth rates lower than in laboratory conditions, which is likely because of the greater food supply in laboratory compared to field conditions (Melvin and Houlahan 2014).

Median body lengths inside enclosures at the metamorphosing phase had the greatest positive weight on the metamorphosing success in our model. This positive effect has been reported in anuran and salamander species, both in laboratory and *in situ* venues (Berven 1990; Scott 1990; Morey and Reznick 2004). In these studies, body sizes also had an inverse correlation with larval densities, with higher body sizes and higher metamorphosing success reported at lower densities. Our results, however, do not support a correlation between body lengths and larval densities, as both predictors were selected for the saturated model. The relationship between these two predictors, if significant, would be indirect and more important on previous stages not included in the analysis.

Size at metamorphosis was similar to that reported for species in the *Melanophryniscus stelzneri* species group (Bustos Singer and Gutiérrez 1997; Cairo *et al.* 2008; Kurth *et al.* 2014). Such low variation in size at metamorphosis is consistent with the pattern already observed by Werner (1986) for bufonids. Small body size at metamorphosis observed within this family, regardless adult size, probably occurs due relatively low mortality of terrestrial life stages, which presents strong skin toxins protecting adults from predation (Werner, 1986). This could also be suggested for the genus *Melanophryniscus*, whose adults are known to have high concentrations of pumiliotoxin and hydroquinone in skin and internal organs (Mebs *et al.* 2005; Grant *et al.* 2012).

The experimental design was meant to describe developmental times under field conditions and we moved the enclosures in order to maximize the water availability for the larvae. However, an important negative effect of water depth inside the enclosures over metamorphosing success arose in the model. This effect can be interpreted as signal of plasticity in development time in response to pond drying. Other anuran species showed decreasing development time with higher metamorphosing rate when the water level lowered (Laurila and Kujasalo 1999; Altwegg 2002; Morey and Reznick 2004; Richter-Boix *et al.* 2006, 2011). Some studies also reported energetic costs associated to fast developments, leading to smaller body sizes at shorter development times (Altwegg 2002; Morey and Reznick 2004; Richter-Boix *et al.* 2011). Little variation in metamorph body lengths (Table 1) may indicate that those costs were not evident in our study. In spite of the variability in development time (range: 22–29 d), a median of 27 days was 10 days shorter compared with the maximum durations recorded by Garrido-Yrigaray (1989), which suggests the occurrence of a noticeable developmental plasticity in this species. Developmental plasticity has also been suggested for other *Melanophryniscus* species (Bustos Singer and Gutiérrez 1997; Cairo *et al.* 2008; Kurth *et al.* 2014). Bustos Singer and Gutiérrez (1997) reported acceleration in the development of *M. stelzneri* when the water level was artificially lowered. However, Cairo *et al.* (2008) observed mass mortality of *Melanophryniscus* sp. when ponds dried out, similarly to what we observed at some enclosures in the present study. Tejedo and Reques (1994) suggested that at low larval densities, tadpoles of *Epidalea calamita* (Laurenti, 1768) were able to grow and develop faster in short-duration ponds but slower in long-duration ponds. It was observed that, at high larval densities, competition prevented accelerated growth in response to pond desiccation, turning similar the development time for short and long-duration ponds, in some cases resulting in mass mortalities (Tejedo and Reques 1994). Similarly, the present study recorded short development time in some enclosures and mass mortalities due to pond drying in others, being the second possibly explained by a negative effect of larval density on the response to pond duration, as reported for other ephemeral pond breeders (Tejedo and Reques 1994; Newman 1998).

From this work some causal relations can be established among biotic predictors and metamorph recruitment of *M. montevidensis*. It was evident the influence of larval densities directly on the metamorphosing success or indirectly on the ability to respond to pond desiccation. Possibly, the ability to respond to pond desiccation depended on reaching a threshold of body length (Wilbur and Collins 1973), as larger median sizes inside the enclosures allowed higher metamorphosing rates. No direct effect of density over growth was observed in the present study, so either the effect is indirect or other constraints to growth at larval and metamorphosing stages may be acting, such as increased level of corticosterone secretions typical of confinement situations (Belden *et al.* 2005). Besides, some caveats in the models should be considered. In order to determine metamorphosing success, we only analyzed those enclosures with survivals further than the 20th day of the development. For this reason, the interrelation and importance of biotic predictors over survival in previous stages would not be evident. Thus, the effect of predator density over larval survivorship or larval density over growth, were not directly acting in final stages, but cannot be discarded for earlier stages. Moreover, data of unsuccessful hatching in pond 2 could not be included in models, so none of the biotic predictors could be used to explain this phenomenon.

Regarding pond 2, probably the physical characteristics of the pond, critical for embryo success (*e.g.*, dissolved oxygen or pH; see Boyer and Grue 1995), were not within the ideal range for the eggs to hatch, as no free larvae were present during sampling period. We did not observe fungal infections in the eggs, such as those caused by *Saprolegnia* spp. (presence of cotton-like masses around the eggs, following Fernández-Benéitez *et al.* 2008). Similarly, though we observed hatchlings and free larvae in pond 1, mass mortalities during hatching stages (before predators could be detected) occurred in some enclosures of this pond. Therefore, probably reduced water quality influenced the results. Further *in situ* studies would assess whether the low larval success in hatching is part of the normal variation for these populations or has anthropic causes (Boone and James 2005). These studies would also improve the estimate of an optimal survival rate for the species.

In spite of the potential response of the species to pond desiccation, ponds that dry in less than 22 days might not yield any offspring. Pond drying might intensify under global warming scenarios that would make more difficult for ponds to retain water for long periods of time, this was already suggested as an explanation of projected lossess in distribution areas for several threatened *Melanophryniscus* species, especially *M. montevidensis* (Toranza and Maneyro 2013; Zank *et al.* 2014). The *in situ* data provided by this study, combined with demographic data of the terrestrial stages, can be applied to population stage-structured models (*e.g.*, Biek *et al.* 2002). These models can be constructed not only for *M. montevidensis*, but for ecologically similar congenerics, helping to understand the processes behind their declines, as much as to establish proper conservation actions for the populations and the ponds where they breed (Conroy and Brook 2003).

# TABLES I

**Table 1**. Descriptive statistics of larval performance per spawn. "*n*" is the sample size (number of enclosures per pond) for each variable. "N eggs" is the number of eggs laid per spawn. "% hatched" and "% metamorphosed" are the proportion of survival from eggs until first larval stages (% of survivors in Gosner's stages 26-30, counted on day seven) and until Gosner's stage 42, respectively. "Days" are statistics for the median time taken to reach Gosner's stage 42 within each enclosure. "BL larvae" and "BL metamorphs" are the statistics for the median body lengths obtained in each enclosure at Gosner's stage 31 to 39 (days 11-12) and 42, respectively (based on the calculation of the median size of a sample of up to 15 tadpoles within each cage).

| | Pond 1 | | | Pond 2 | | | Pond 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | *n* | Median | min. – max. | *n* | Median | min. – max. | *n* | Median | min. – max. |
| N eggs | 6 | 124 | 79 – 221 | 10 | 124 | 51 – 216 | 9 | 95 | 26 – 317 |
| % hatched | 6 | 14.3 | 0 – 27.6 | 10 | 0 | - | 9 | 67.4 | 24.9 – 87.1 |
| % metamorphosed | 6 | 0.0 | 0 – 4.9 | 10 | 0 | - | 9 | 7.4 | 0 – 51.1 |
| Days | 1 | 25 | - | 0 | - | - | 7 | 27 | 22 – 29 |
| BL larvae (mm) | 3 | 4.3 | 4.1 – 4.6 | 0 | - | - | 9 | 4.5 | 3.5 – 5.2 |
| BL metamorphs (mm) | 1 | 6.0 | - | 0 | - | - | 7 | 5.7 | 5.3 – 6.4 |

## TABLES I

**Table 2**. Saturated and best simplified GLMs (logit) fitted to the metamorphosing success, and their contrast using ANOVA. “Site” is a nominal variable (levels: pond 1 and 3); “N(larvae-10)” is the number of larvae counted ten days before sampling day; “Dens(pred)” is predator density inside the enclosures (calculated on days six-seven); “BL” is the body length of larvae (using the median BL of up to 15 larvae per enclosure); “Depth” is water depth inside each enclosure each day.

| | | Residual Deviance | df | *P* value |
|---|---|---|---|---|
| Saturated | Site + N(larvae-10)*Site + Dens(pred)*Site + BL*Site + Depth*Site | 68.2 | 51 | |
| Simplified | N(larvae-10) + BL + Depth | 87.5 | 57 | |
| ANOVA | F = 2 | 7.6 | 5 | 0.1 |

**Table 3**. Coefficient estimation of the best simplified GLM. For a general model $y = a + b_1x_1 + .... + b_nx_n$; “*b*” are the estimates of the coefficients of each variable *x*; “SE” are the standard errors of the estimates *b*; “*t*” value are the t-statistic values of each coefficient *b*; “*P* value” are the significance values of the t-test of each coefficient *b*. “(intercept)” is the coefficient a of the model of the model (intercept of the model with the y axis); “N(larvae-10)” is the number of larvae counted ten days before sampling day; “BL” is body length of the larvae (using the median BL of up to 15 larvae per enclosure); “Depth” is water depth inside each enclosure each day.

| | *b* | SE | *t* value | *P* value |
|---|---|---|---|---|
| (intercept) | -12.4 | 3.4 | -3.6 | <0.001 |
| N(larvae-10) | -0.0 | 0.0 | -2.5 | <0.05 |
| BL | -1.6 | 0.5 | 3.1 | <0.05 |
| Depth | -0.4 | 0.1 | -3.5 | <0.001 |

## FIGURES I

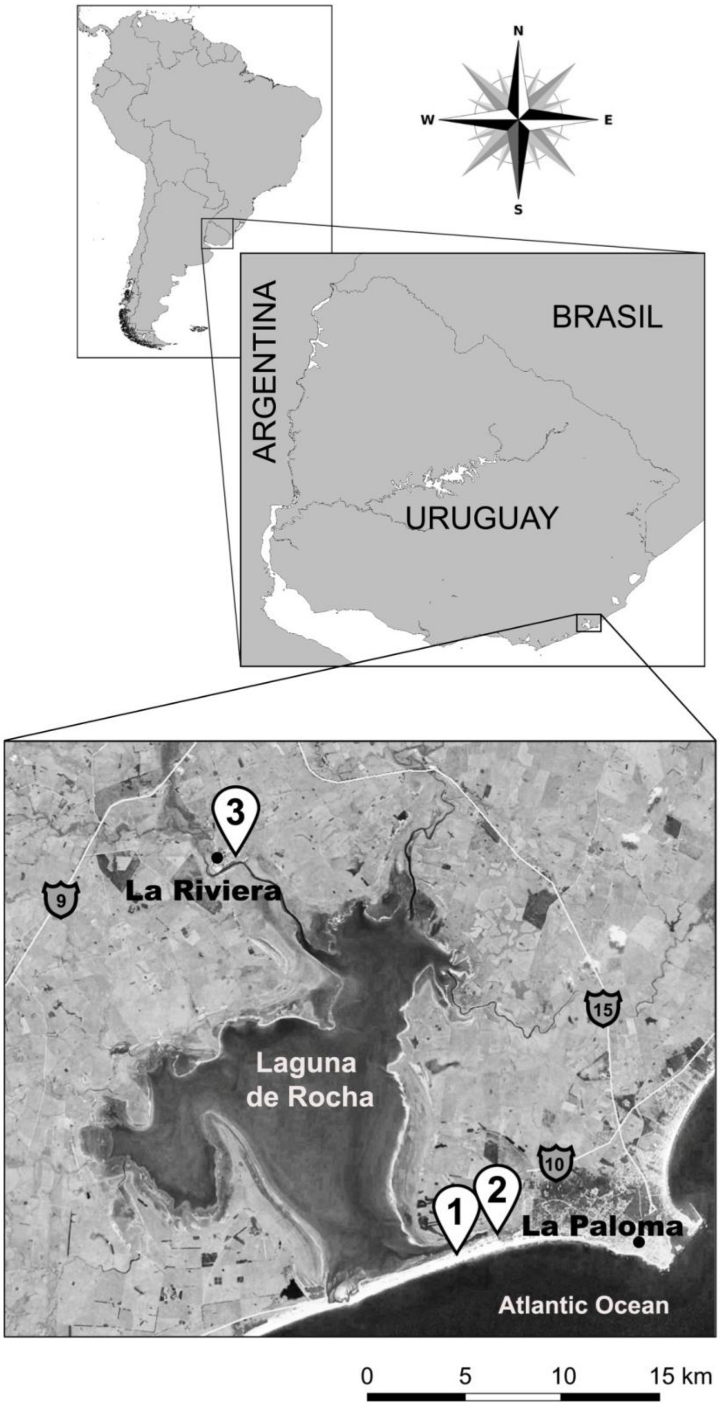

**Figure 1**. Location of sampled ponds. Numbered inverted drops represent the ponds, cities and surrounding national highways and the lagoon are also displayed. Aerial photo obtained from Google Earth (34°36'38" S; 54°17'49" W) on 18 Aug 2015.

# FIGURES I

**Figure 2**. Enclosures in pond 1 at Laguna de Rocha, Rocha Department, Uruguay (Photographed by Cecilia Bardier).

# FIGURES I

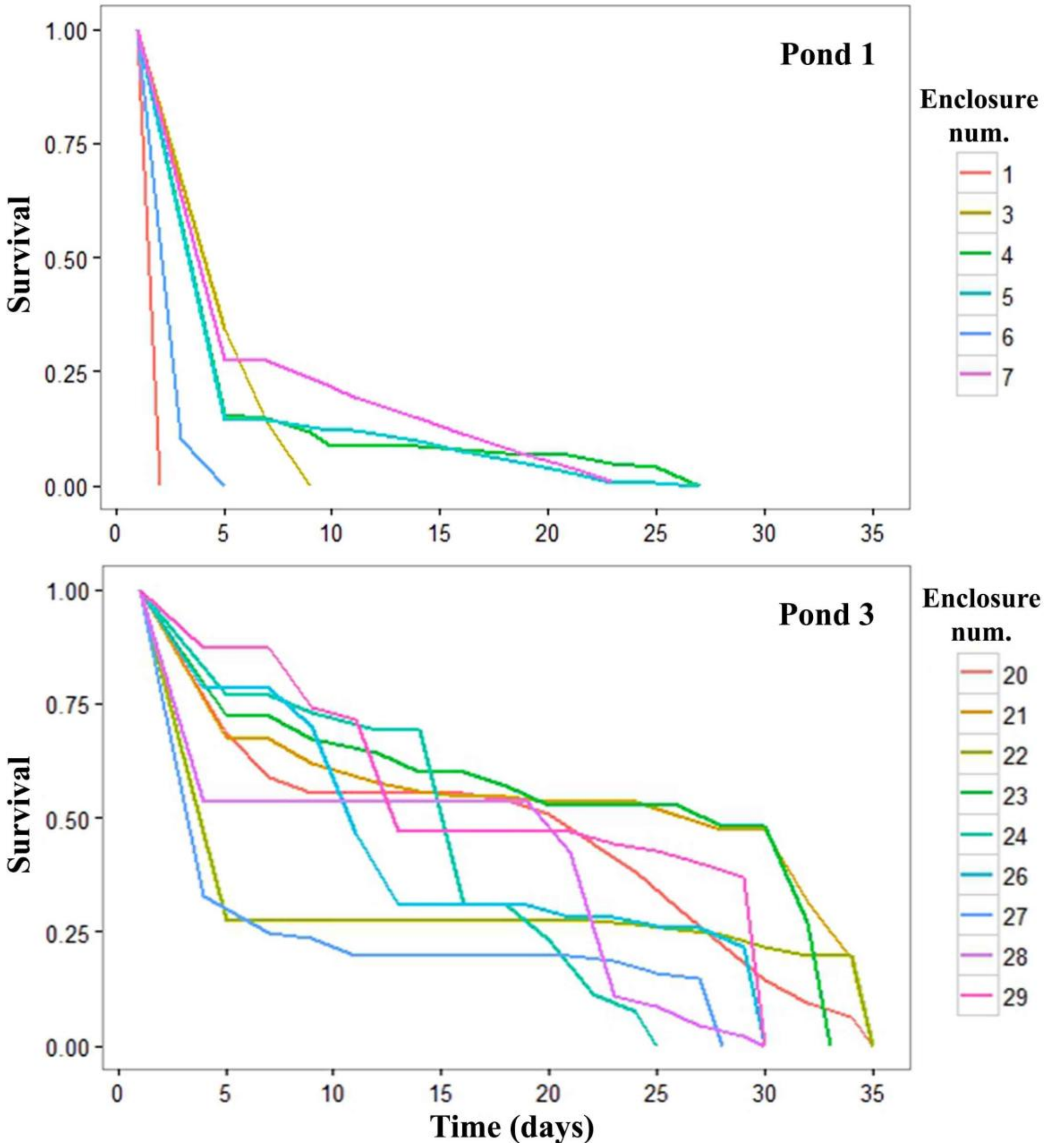

**Figure 3.** Survivorship curves in enclosures from ponds 1 and 3. Survival corresponds to the number of larvae divided by the number of eggs within each enclosure.

# FIGURES I

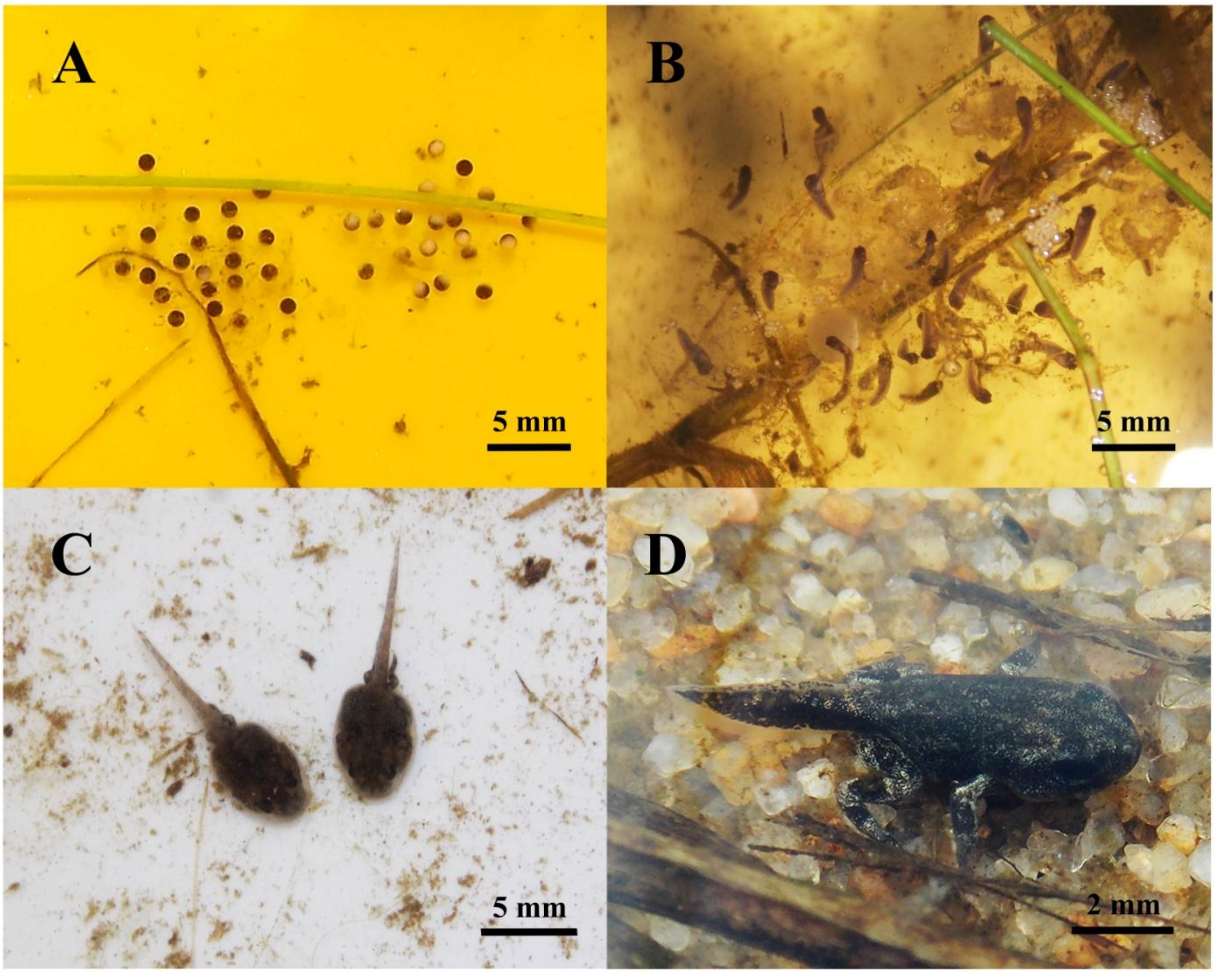

**Figure 4**. Developmental stages of *Melanophryniscus montevidensis*. A) Two egg masses five h after spawned (not a complete spawn) from pond 3; B) embryos and hatchlings two days after spawning in Gosner stages 19–21 from pond 3; C) larvae 11 days after spawning in Gosner stages 36–39 from pond 3; D) metamorph 23 days after spawning at Gosner stage 42 from pond 1 (Photographed by Cecilia Bardier).

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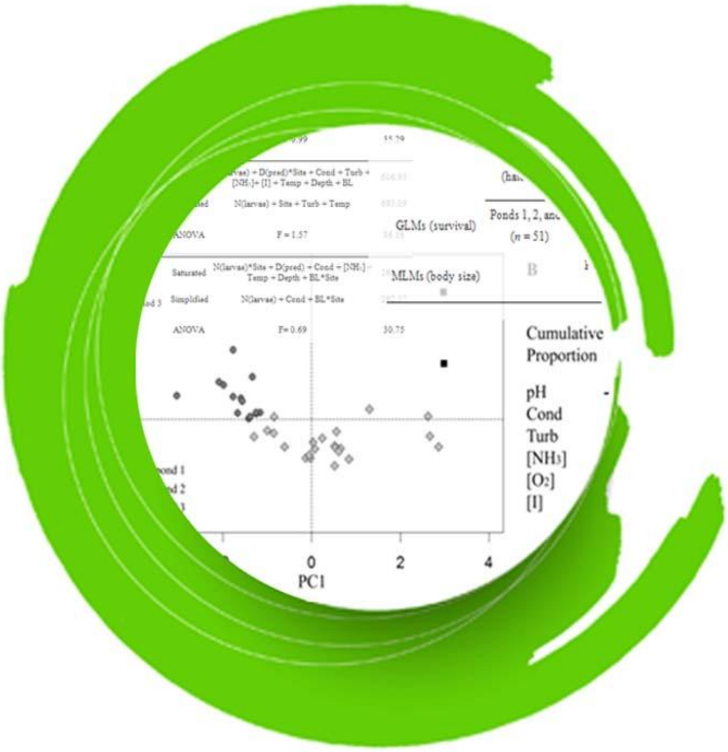
# Capítulo II

## Role of water quality and biotic predictors on body size and larval survivorship of the endangered red bellied toad *Melanophryniscus montevidensis* (Anura, Bufonidae)



# ABSTRACT-RESUMEN

**Abstract**. *In situ* studies of larval development and water quality have yielded to major understanding of the effects of pollutants on amphibians. However, they are not usually addressed to determine environmental constrains to the larval development of threatened species. We hypothesize that low water quality reduces metamorphosing success by reducing larval growth and survivorship of the threatened species *Melanophryniscus montevidensis*. Using *in situ* enclosures in ponds of a protected area we monitored the larval development, to determine the levels of water quality parameters in which the development occurs. We also tested the relative effect of physicochemical parameters (pH, conductivity, turbidity, ammonia, dissolved oxygen, iodine, nitrate, nitrite and phosphate) over the survivorship and body size in such conditions, considering their interrelations and their relation with other biotic variables (predation and larval density). No eutrophication processes were detected, though levels of conductivity, turbidity, ammonia and dissolved oxygen were out of the acceptable for aquatic life (fishes and amphibians), possibly due to decomposition processes. GLMs performed for embryonic, larval and metamorph survival as response variables, indicated that conductivity was the most important physicochemical variable during embryonic stages, while larval density and body size had heavier negative and positive effects, respectively, in later stages. MLMs performed for body size of larvae and metamorphs as response variables also supported the negative effect of larval density. Conductivity was highly correlated with ammonia, possibly masking its effect in all cases. Although the poor water quality levels of the ponds partially reduced larval body size and survival, our results did not support the initial hypothesis. Nevertheless, the levels reported for some water quality indicators can also be reached by anthropic causes, allowing some inferences of how polluted conditions would affect the larval development of this threatened species.

**Resumen**. Los estudios *in situ* sobre el desarrollo larvario y la calidad del agua han permitido comprender mejor los efectos de los contaminantes en los anfibios. Sin embargo, usualmente no se enfocan en la determinación de las restricciones ambientales para el desarrollo larvario de especies amenazadas. Hipotetizamos que la baja calidad del agua reduce el éxito de la metamorfosis al reducir el crecimiento y la supervivencia larvarias de la especie amenazada *Melanophryniscus montevidensis*. Usando encierros *in situ* en charcas de un área protegida, se monitoreó el desarrollo larvario la especie para conocer los niveles de los parámetros de calidad del agua en los que se produce el desarrollo. También se evaluó el efecto relativo de los parámetros fisicoquímicos (pH, conductividad, turbidez, amoníaco, oxígeno disuelto, yodo, nitrato, nitrito y fosfato) sobre la supervivencia y tamaño corporal en estas condiciones, considerando sus interrelaciones y su relación con otras variables bióticas (depredación y densidad larvaria). No se detectaron procesos de eutrofización, aunque los niveles de conductividad, turbidez, amoníaco y oxígeno disuelto estuvieron fuera de los aceptables para la vida acuática (peces y anfibios), posiblemente debido a procesos de descomposición. Los GLMs ajustados para la supervivencia embrionaria, larvaria y de los metamorfos como variables de respuesta, indicaron que la conductividad fue la variable fisicoquímica más importante durante las etapas embrionarias, mientras la densidad larvaria y el tamaño corporal tuvieron efectos negativos y positivos más pesados, respectivamente, durante etapas posteriores del desarrollo. Los MLMs ajustados para el tamaño corporal de larvas y metamorfos como variables de respuesta también apoyaron el efecto negativo de la densidad larvaria. Aunque los niveles empobrecidos de calidad del agua de las charcas redujeron parcialmente el tamaño de las larvas y la supervivencia, nuestros resultados no apoyaron la hipótesis inicial. Sin embargo, los niveles reportados para algunos indicadores de calidad de agua también pueden ser alcanzados por causas antrópicas, permitiendo realizar inferencias sobre cómo las condiciones de contaminación afectarían el desarrollo larvario de esta especie amenazada.

# INTRODUCTION

Over the last decades amphibians have experienced major population declines as well as recent species extinctions (Stuart *et al.* 2004; Beebee and Griffiths 2005; Pounds *et al.* 2006). Main causes referred for these declines are habitat destruction, pollution, invasive species, diseases, climate change, and overexploitation (Blaustein *et al.* 2011). Regarding to larval stages, water pollution (or altered quality) is one of the ultimate causes of amphibian population declines, because it reduces the survival and recruitment of individuals, mostly by altering predation rates, food availability, prevalence of diseases and parasites, growth rates, or incidence of abnormalities (Johnson and Chase 2004; Rohr and Crumrine 2005; Peltzer *et al.* 2008; Relyea and Hoverman 2008; Hayes *et al.* 2010; Egea-Serrano *et al.* 2012).

Several *in situ* studies of amphibian larval communities or populations tested the effects of diverse local sources of pollution on the survival, growth and health of individuals. Some of these studies also accounted for biotic factors such as density, body size or parasitic infections (Warner *et al.* 1991; Ralph and Petras 1998; Kiesecker 2002; Peltzer *et al.* 2008; Schmutzer *et al.* 2008). These studies are usually focused on the effects of pollution over the organisms via eutrophication, acidification, and direct or indirect toxicity of compounds, which are the main threats faced by freshwater aquatic organisms (Camargo and Alonso 2006). Eutrophication is caused by elevated ammonia, nitrite, nitrate or phosphate concentrations, from anthropic sources such as animal farming, urban and agricultural runoff, industrial wastes, and sewage effluent (Maidment 1993; Camargo and Alonso 2006). These compounds enhance the proliferation and maintenance of primary producers, such as phytoplankton, benthic algae, and macrophytes (Carpenter *et al.* 1998; Camargo and Alonso 2006). The resulting unbalance has many ecological and toxicological effects, specially a drastic decrease of dissolved oxygen concentration, causing hypoxia and promoting the formation of reduced compounds, such as hydrogen sulphide, and mass occurrences of toxic algae, resulting in significant adverse effects on aquatic animals (Carpenter *et al.* 1998; Camargo and Alonso 2006). These phenomena impact anuran larvae by reducing the overall health and feeding activity, and promoting parasitic infections; which result in low survivorship and/or decreased growth and development (Johnson and Chase 2004, Peltzer *et al.* 2008; Schmutzer *et al.* 2008).

Although *in situ* monitoring of larval development and water quality variables using aquatic enclosures has yielded to major understanding of the effects of pollutants on amphibians, this approach is usually employed to answer broad ecotoxicological questions without considering the conservation status of the model species. Such experimental design is not commonly addressed to determine environmental constrains to the larval development of threatened species (reviews in Boone and James 2005; Melvin and Houlahan 2012). Indeed, this approach may be useful to unravel the ultimate causes of declines of endangered species, especially when anthropic causes, like agriculture or urbanization, are suspected to be responsible for those declines (Hayes *et al.* 2010).

*Melanophryniscus montevidensis* is a threatened species from the coastal region of Uruguay and a small portion of the southern coast of Rio Grande do Sul (Brazil) (IUCN 2017). It is an explosive breeder (*sensu* Wells 2007) that lays eggs in ephemeral ponds after heavy rains and has a fast development: about 27 days from hatchling to complete metamorphosis (Capítulo I). It is currently listed as *Vulnerable* by IUCN (2017); though regional assessments assigned higher threat levels (Fundação Zoobotânica 2014; ICMBio, 2014; Carreira and Maneyro 2015). Uruguayan assessments were based on drastic alteration in the geographic distribution of the species, many populations have disappeared and the distribution is currently highly fragmented (Langone *et al.* 2004; Núñez *et al.* 2004; Maneyro and Carreira 2012; Carreira and Maneyro 2015). Although agriculture and urban development of the Uruguayan coastal region in the past 30 years have been identified as responsible for these declines (Langone *et al.* 2004; Achaval and Olmos 2007; Maneyro and Carreira 2012), no studies tested their specific effects over the populations of *M. montevidensis*.

In a previous study we described the larval development of *M. montevidensis* in the protected area Laguna de Rocha, and analyzed the effect of different biotic factors (density, body size and predation) on the metamorphosing success in *in situ* conditions (Capítulo I). Although the importance of larval density and body length for the metamorphosing success was highlighted, some questions about the interrelations between survivorship, body length, larval density and predation in early stages of the development were raised. Moreover, we reported the death of entire egg masses before hatching, suggesting that low water quality may also be involved in the survival rates of *M. montevidensis* in a protected area (Capítulo I). Here we aimed to determine the parameters of water quality in which the development of the species occurs; and the relative effect of the physiochemical parameters over the rates of survivorship and growth in such conditions, considering their interrelation as much as their relation with biotic variables (predation and density). We hypothesize that low water quality conditions reduce metamorphosing success by reducing growth and survivorship of larvae of *M. montevidensis* in Laguna de Rocha. Predictions of this hypothesis are: 1) the water quality will be poor in ponds of Laguna de Rocha where the species breeds, and 2) larval body size and survival will be negatively correlated with poor levels of water quality.

# METHODOLOGY

## Study site

The study was achieved at three ephemeral ponds from Laguna de Rocha, a protected area of the national system of protected areas, Departamento de Rocha, Uruguay (34°37′59″ S, 054°17′40″ W; 3-10 m a.s.l., see description in Apéndice 2). This protected area is mainly composed by a microtidal coastal lagoon of 72 km$^2$, which is intermittently connected to the Atlantic Ocean, and a watershed area of 1312 km$^2$ (Conde *et al.* 1999). The climate is subtropical, the mean annual air temperature is 16.4 °C, rainfall is well distributed throughout the year with a mean of 1277 mm (data from 1980 to 2009; Fernández 2011). This area belongs to Bañados del Este Biosphere Reserve, an area that harbors a high richness of endangered species, has some populated localities and has been exploited for agricultural, commercial forestry and touristic purposes (Soutullo *et al.* 2003).

## Experimental design

We observed three breeding events that lasted two or three days at three ephemeral ponds (pond 1, 2, and 3) after heavy rains in February 2014. During those days we searched, collected, and stored amplectant pairs in plastic cages of about one liter, putting one amplectant pair per cage, with water and vegetation from the pond. We collected a total of 29 pairs, 25 of which spawned inside the cages within 24 h after capture, six from pond 1, 10 from pond 2, and nine from pond 3. After that, we released the adults in the wild. We recorded the number of eggs per spawn, and then we placed each spawn (from each amplectant pair) in an *in situ* semi-permeable enclosure in the exact point where their parents were first observed. Enclosures were 32-liter cages of 43.0 cm (length) x 27.4 cm (width) x 27.1 cm (height), made of polypropylene, a safe polymer as it is relatively inert, does not contain Biosfenol-A, and is used for food and drinkable water containers (Maier and Calafut 1998). We made perforations in two sides of the cages, allowing water passage, and covered the sides with a thin plastic mesh (1 x 1 mm). We glued the mesh to the inner part of the cage using Sikaflex 1a (Sika AG, Zugerstrasse, Switzerland), which is indicated for drinkable water containers. To emulate natural conditions, we fixed the enclosures to the bottom of the pond and added some emergent vegetation, this provided shelter while tadpoles could exploit the whole water column. As the diet of the larvae was unknown, we paid special attention to the trophic behavior of the larvae, in order to discard carnivore or macrophage diets. None of these behaviors were recorded in this study. We observed the larvae exclusively feeding on algae that grew on the walls and floor of the cages or on decomposing leaves of the plants, and so did not add extra food. A wide plastic mesh (14 x 19 mm) covered each enclosure to avoid avian or mammalian predation. Usual invertebrate predators for amphibians (coleopteran and dragonfly larvae) were present in the three ponds inside and outside the enclosures, as in the rest of the ponds in the area during the sampling period. Although predatory larvae could not go through the thin mesh, they could be present inside the cages due to the income of eggs in the case of those smaller than 1 $mm^2$ (*e.g.*, Joop *et al.* 2007); or they could also be oviposited by adults through the wide mesh.

We monitored the clutches every two or three days, from 10:00 to 18:00 h, until the end of metamorphosis or complete desiccation of the pond (no water in the enclosures). We took pictures from inside the enclosures during each sampling day to obtain body length (BL) from the tip of the snout to the body terminus excluding the tail (*sensu* McDiarmid and Altig 1999) and number of larvae, using a Nikon Coolpix L810 camera of 16.0 mp (Nikon Corporation, Tokyo, Japan) and a scale of 1.0 mm of precision. Metamorphs were photographed using the same scale. We measured water temperature (°C; Oakton Instruments, Vernon Hills, Illinois, USA) and water level inside the enclosures (using a wooden yardstick) each time we took pictures. To reduce the depth variation, each time the depth decreased below the level of the previous sampling day we moved the enclosures into the closest deeper zone of the pond (in case of existing any, see example plotted for three of the six enclosures from pond 1 in Apéndice 4). Due to the shallow initial water level of the ponds, water level of the enclosures was usually below 13 cm (half of the cages' height; Apéndices 2 and 4). Median larval density on day seven varied from 2.1 to 5.3 larvae/liter.

We measured and counted larvae and eggs using the software Micrometrics SE Premium (ACCU-SCOPE, Commack, New York, USA) and ImageJ 1.48v (National Institutes of Health, Bethesda, Maryland, USA), respectively. The end of metamorphosis was considered as the Gosner stage 42 (Gosner 1960), because at this stage the larvae started climbing the net out of the water (Capítulo I). Density of predators was measured since day six to seven of the larval development of *M. montevidensis*, when they became large enough to be digitally counted by photos. A square of 12 x 12 cm was randomly drawn on the floor of the cages and the predators were counted there using ImageJ 1.48v (*op. cit.*).

Water physicochemical variables were measured during larval sampling (10:00 – 18:00 h). Variables recorded were pH and conductivity ($\mu S\ cm^{-1}$) in the proximity of cages (Hanna Instruments, Woonsocket, Rhode Island, USA). Once per day/pond we also recorded: ammonia nitrogen concentration ([$NH_3$-N]; 0.00–4.00 mg $L^{-1}$), dissolved oxygen concentration ([$O_2$]; 0.00–10.00 mg $L^{-1}$) , iodine concentration ([I]; 0.00–14.00 mg $L^{-1}$), nitrate concentration ([$NO_3^-$]; 0.00–60.00 mg $L^{-1}$), nitrite concentration ([$NO_2^-$]; 0.00–0.80 mg $L^{-1}$), phosphate concentration ([$PO_4^{3-}$]; 0.00–70.00 mg $L^{-1}$), and turbidity (FAU). For these measurements, we used a colorimeter Smart 3 (LaMotte Company, Chestertown, Maryland, USA), which performs photoelectrical measures of the amount of colored light absorbed by a colored sample in reference to a colorless sample (blank), and uses pre-designed kits of reagents for every physicochemical test (LaMotte Company 2012). Results for ammonia nitrogen were expressed as un-ionized ammonia ($NH_3$) by multiplying ammonia nitrogen ($NH_3$–N) by a 1.2-conversion factor (LaMotte Company 2012). Median values of water physicochemical variables were compared with data available in bibliography on water quality and nutrient criteria for aquatic and amphibian life (pH, conductivity, turbidity, ammonia, dissolved oxygen, nitrites and nitrates: USEPA, 1986, 1997, 2000, 2005; turbidity: NYCRR 2017; ammonia for amphibians: Jofre and Karasov 1999; dissolved oxygen and phosphates: Allen Burton Jr. and Pitt, 2001; dissolved oxygen for amphibians: Mann and Bidwell, 2001; Apéndice 6).

## Analysis

We calculated descriptive statistics of the physicochemical variables measured, all variables with a median concentration below 0.02 mg $L^{-1}$ were considered uninportant and were not included in the following analysis. A principal components analysis (PCA) was applied to pH, conductivity, turbidity, [$NH_3$], [$O_2$] and [I], to explore the grouping of the ponds.

To determine the predictive capacity of biotic and abiotic variables on the survival probability, we fitted logit quasi-binomial Generalized Linear Models (GLMs), where the logarithmic odds of survival were the response variable. In the case of body size (BL) of the larvae, we fitted Multiple Linear Models (MLMs) to determine the predictive capacity of the aforementioned variables, as the response variable was continuous. For these models we included the variables: site (pond), predator density (predator $cm^{-1}$; measured on days 6–7), number of larvae each day, BL, depth (measured inside each enclosure before relocation each day), temperature, pH, conductivity,[$NH_3$], [$O_2$], [I] and turbidity. In the case of the MLMs the variable days of development was also included as a predictor. These analyses were achieved separately for the different stages of development. For the first five days of development it was observed that individuals were still embryos (< Gosner stage 25), so we considered these early stages as "Period 1". The following period ("Period 2": larvae) lasted from day six, when survivors reached larval stages (Gosner stages 26–30), until day 19 (Capítulo I). On day 20 we considered the beginning of the "Period 3" as metamorphs (Gosner stage 42) were first recorded (until day 34, the last sampling day). Response variables of GLMs were the log-proportion of surviving hatchlings from the number eggs (Period 1), log-proportion of surviving larvae at the end of the period from the number of hatchlings (Period 2), and log-proportion of metamorphs at the end of the period from the number of larvae at the beginning (Period 3). MLMs with BL within each period as the response variable were conducted only on periods 2 and 3, because growth was more noticeable during these stages. A scheme of how analyses were conducted on each period and associated data sets (pond) is available in Table 1.

To avoid multicollinearity between variables, we searched for highly correlated predictors based on pairwise Spearman's rank correlations matrix with $|r| > 0.7$ (Dormann *et al.* 2013). For each correlated pair, we retained the variable with the higher explanatory power on the survival and growth of larvae, according to a univariate generalized linear model within the “select07” procedure in R (R Foundation for Statistical Computing, Vienna, Austria; “select07” method in Dormann *et al.* 2013). As site is a categorical variable with three possible values: pond 1, 2 and 3; it was not included in the “select07” procedure. We searched for interactions among the three levels of this factor by fitting linear models to the response variable in each case (survival and growth) with the predictors retained by the “select07” procedure. Whenever interactions were detected, the predictor was fitted as a covariate of site in the survival GLM and growth MLM saturated models (Crawley 2007). For the uncorrelated variables obtained by the “select07” procedure, some interactions with the different levels of site were detected: in survival *vs.* predator density in Period 2; in survival *vs* number of larvae in Period 3; in survival *vs.* BL in Period 3; and in BL *vs.* conductivity, [$O_2$] and [I] in Period 2 (Apéndice 5). These predictors were included as covariates of site in the saturated GLMs and MLMs.

A backward stepwise procedure was applied to each saturated model to determine which variables contributed significantly to predict the probability of success in the survival and metamorphosis of the larvae (Hosmer *et al.* 2013). In the case of larval growth, the Akaike’s Information Criterion (AIC), through the “step” routine in R, was ran to obtain better simplifications of saturated models (Johnson and Omland 2004). Because of the overdispersion of errors in the fit, a quasi-binomial distribution of errors was assumed in the survival GLMs; there is no automatic simplification routine for this adjustment (no AIC is available), so we used ANOVA to compare the residual deviance between models (Crawley 2007). To assess the relative importance of predictors within each model (GLMs and MLMs), we used the *student* “*t*” statistic provided by the summary of each model. As it is the coefficient of each predictor divided by its standard error, it can be considered a measure of the accuracy with which the regression coefficient is measured; the higher the *t* for each predictor, the higher will be the explanatory power of the predictor over the response variable within each model.

## RESULTS

### Physicochemical parameters

We found that most of the nutrient parameters measured during this study ($[NO_2^-]$, $[NO_3^-]$ and $[PO_4^{3-}]$) were within the values of a not eutrophicated aquatic environment (according to USEPA 1986, 1997; Allen Burton Jr. and Pitt 2001, information reviewed in Apéndice 6). However, conductivity, turbidity, $[NH_3]$ and $[O_2]$ were out of the acceptable levels for vertebrate aquatic life, especially in pond 2 (Apéndice 7). We found no information about normal levels of iodine in aquatic environments.

Ponds could be grouped by their physicochemical parameters (Figure 1). The first factor of the PCA was mainly correlated with pH, turbidity, $[NH_3]$ and $[O_2]$, and the second mainly correlated with conductivity, [I] and $[NH_3]$; the cumulative proportion of variance of the first two factors combined was 0.73 (Figure 1).

## Larval survivorship and body size models

Conductivity, number of larvae in the cages and depth were the most important predictors of survival during Period 1 of development (Table 2), the number of larvae showing a positive effect whereas that of conductivity and depth were negative (Table 3). As conductivity had the highest *t* value (-6,718), it was the most important variable of the model; being number of larvae and depth only marginally significant. However, this model had the weakest effects of predictors and was the less fitted to data (residual deviance = 902.27; Table 2). Site effect, positive in pond 3 and negative in pond 1, was only noticeable on Period 2, with the highest predictive capacity (*t* values, Table 3). On this medium-fitted model (residual deviance = 719.61; Table 2), negative effects of number of larvae and turbidity, and positive of temperature also appear, being the number of larvae the second most important variable within this model. During Period 3 number of larvae and conductivity had negative effects, and BL had a greater positive effect in pond 3 than in pond 1 (Table 3). Simplified GLM of this Period 3 was the best survival model fitted (residual deviance = 292.37; Table 2). The most important predictors of survival within this model were number of larvae and conductivity levels.

Best simplified MLMs accounted for 73 and 67% of the variation in the larval BL during periods 2 and 3 respectively (Table 4). During Period 2, the days of development were the most important predictors and the only positive, while number of larvae and conductivity were less heavy negative predictors of body size (Table 5). Nevertheless, days of development did not appear as predictors during Period 3. Only number of larvae and depth seem to have strongest effects (the first with higher importance), being positive and negative respectively (Table 5).

## DISCUSSION

Our results indicate that no eutrophication processes occurred in the ponds during our study period. In the absence of human induced eutrophication or acidification, other processes may produce the observed physicochemical profile of the ponds. Natural changes occur in the physical environment due to local climatologic conditions, which can lead to sudden or gradual fluctuations of physicochemical conditions. For example, heavy rainfalls or prolonged droughts may increase siltation and decomposition processes (Chapman 1996). As the weather previous to this study was exceptionally rainy (Capítulo I, Apéndice 3), ponds were filled long before the cages were set up in the field. This hydrological phase would explain the apparently altered values of water quality observed (low levels of dissolved oxygen, high levels of ammonia and turbidity, Apéndice 6–7), which correspond to decomposition processess occurring in the ponds (Chapman 1996). Although pesticide levels were not analyzed, given the low levels of nutrients reported (except for ammonia) agricultural runoff impact would be unimportant, making unlikely the presence of significant levels of pesticides (Maidment, 1993). Though no direct records are available, studies on pesticide exposure in shorebirds from Laguna de Rocha found no evidence of the presence of organophosphorus or carbamate pesticides in the coastal part of the area, where two of the ponds are located (Strum *et al*. 2008; 2010).

Nevertheless, natural fluctuations of some water parameters can reach harmful levels for aquatic life (Chapman 1996). In the case of ammonia, high levels ($[NH_3] > 0.6$ mg $L^{-1}$), were signaled as responsible for hatching failure and deformities in amphibians (Jofre and Karasov 1999), and were detected in the three ponds. Indeed, in pond 2, where the levels were more than 10 fold the minimum acceptable, the hatching failure was total. Low dissolved oxygen levels are also lethal for most aquatic species when they fall below 2.0 mg $L^{-1}$ (Mann and Bidwell 2001); and literally zero levels can be reached in still waters when decomposition of sediment occurs (Chapman 1996). Besides that, ammonia concentration increases when pH levels surpass 7.5 and temperatures are higher than 30 °C (Chapman 1996). Also, tolerance levels of the organisms depend on the local pH and temperature, *e.g*, tolerance of salmonids increases with pH and temperature (USEPA 1999, 1986). Thus, levels of pH, conductivity, turbidity, ammonia, oxygen, and temperature are usually intercorrelated, so they cannot be considered separately (Chapman 1996).

Survival and body size models in our study suggest that mainly conductivity, followed by temperature, turbidity and depth, seem to be the most important abiotic parameters for *Melanophryniscus montevidensis*. Conductivity and turbidity, given the method of uncorrelated variables selection and the results of the PCA analysis, summarized the effect of ammonia in both cases, and pH and dissolved oxygen in the case of turbidity. Conductivity may vary with the mineral salts and the acid/base equilibrium of the solution, as much as turbidity, both depend on the amount of dissolved matter (Camargo and Alonso 2006; Line 2003; Chapman 1996). Some studies suggest that conductivity may act as an environmental stressor by rising corticosterone levels, which (among other effects) reduce larval growth rates of amphibians (Belden *et al.* 2005; Chambers 2011). However, high conductivity levels were expectable in our study area, particularly in ponds 1 and 2, because of the presence of salts in the soil given by the proximity to the Atlantic coast. Conductivity levels reported in those ponds were within the range for aquatic life (USEPA 2005), and higher levels were reported for the lagoon (Bonilla *et al.* 2005). Given the typically coastal environments exploded by the species, a relative high tolerance to conductivity would be expectable for *M. montevidensis* as well (Maneyro and Carreira 2012). Considering these facts, the correlation between conductivity and ammonia, and the levels reported for both parameters, we suggest that the negative effect of conductivity was mainly reflecting the effect of ammonia, which was out of the range tolerable for aquatic life. Similarly, in the case of turbidity levels, which were out of the acceptable range for aquatic life (USEPA 2000; NYCRR 2017), they might also be reflecting the negative effects of the correlated parameters ammonia and dissolved oxygen. Conductivity and turbidity, but also ammonia and dissolved oxygen levels, might have acted together to reduce the survival and growth of *M. montevidensis* in our study.

Regarding to the positive effect of temperature on survival during the second period, optimal levels should be needed for larval success, possibly because low temperatures may delay the metamorphosis, as in other amphibian species (Morey and Reznick 2004; Álvarez and Nicieza 2002); this would be an advantageous adaptation for a species that breeds in such ephemeral ponds, as *M. montevidensis* (Morey and Reznick 2004). The negative effect of water depth on the overall survivorship reported in Capítulo I was not so evident in this analysis, though some negative effects were observed on the hatching success. Since we applied a finer scale in the current analysis (survival rates in each developmental stage instead of rate of metamorphs from the total eggs laid), we suggest that this effect was masked by other correlated variables, and it is only observable by itself at a higher scale of analysis such as the applied in Capítulo I.

No studies on amphibian larval success tested the effect of natural iodine levels. Our interest in this parameter resided in the possibility of reporting the range in which the species develops because of the proximity of the study area to the Atlantic coast. As main pond differences were translated in terms of physicochemical differences (see PCA), we did not include site as a predictor itself (it was included whenever interactions with other predictors were detected). However, an important unidentified site effect was reported during the procedure of model simplification of the second period, which increased survival in pond 3, but reduced it in pond 1. This effect can be attributed to the effect of one or more unmeasured variables, or to different tolerances of each population to the unmeasured variable. Intrinsic pond differences would be expectable since ponds 1 and 2 were coastal and pond 3 had a more inland location. Inner and marginal vegetation composition and the dominant landscape were different among coastal and inland ponds (Apéndice 2). Coastal ponds were located in psamophilic grasslands within dunes, while pond 3 was an abandoned agricultural grassland. No evidences of pond selection by vegetal coverage for spawning behavior was reported in other *Melanophryniscus* (Goldberg *et al*. 2006); thus, how vegetation or landscape management would affect larval survivorship should be addressed in further studies.

Larval density within the cages appeared in both survival and body size models, usually to a greater extent of importance than physicochemical variables. A negative effect of larval density over survivorship of *M. montevidensis* was already documented in Capítulo I. However, the scope of analysis in that study did not allow the detection of an effect of larval density over larval and metamorphic body sizes, which was detected in the current study. These negative effects of density over both survival and body size were already reported for other amphibian species in laboratory and field conditions (Berven and Chadra 1988; Berven 1990; Scott 1990; Warner *et al.* 1991; Melvin and Houlahan 2014). We highlight the importance of analyzing separately the periods of the development, because different effects of predictors over the same response variable may arouse, being larval density a clear example of this variation. Density acted positively over survivorship during the first period of the development, possibly because of the absence of inter-individual competence, small size and low mobility of the hatchlings (Wells 2007). Therefore, the more eggs were laid, the more hatchlings became larvae. However, in the latter developmental stages, when the body sizes increased and inter-individual competence for food became stronger, the detrimental effect was accentuated. This was especially true in the last two periods of the development regarding to survival and also in body size models.

The importance of body size as a positive predictor of survivorship of recent-metamorphosed individuals, already reported in Capítulo I, was also evident in this study. Nevertheless, its effect in the current study was relativized because it was lighter than larval density or conductivity. Its importance for metamorphosing success supported the idea that a threshold body size must be reached to allow higher recruitment rates (Wilbur and Collins 1973; Capítulo I). Besides, this effect of body size was site-specific, indicating that body lengths were more important for pond 3 than for pond 1. *Sensu* Tejedo and Reques (1994), at high larval densities, larval competition would prevent proper growth from responding to pond desiccation, resulting in some cases in mass mortalities. Thus, it is possible that larval densities were involved in the site-specific effects of body sizes, as larval densities were higher in pond 3 than in pond 1 (Capítulo I), being body size more determinant for survival in pond 3 than in pond 1.

Regarding to body size models, the importance of time (days of development) as a predictor during the larval period disappeared on the metamorphosing period, having larval density (negative) and depth (positive) the heaviest effects. On one hand, it reinforces the idea of detrimental effects of high larval densities on the last stages of the development (already discussed). On the other hand, it also suggests the detrimental effects of pond desiccation over body sizes of metamorphs (*i.e.*, the lower the pond depth, the lower the body size), in spite of the low variation in metamorph body size recorded (Capítulo I). As growth and survival should not be considered separately, because of the importance of bigger body sizes on tadpole survival (Tejedo and Reques 1994; Melvin and Houlahan 2014), we are possibly observing the inner part of the nonrandom survival mechanism where high larval densities or high conductivity levels reduce growth, and, consequently small sized individuals are less prone to survive.

Predator density was dismissed as a predictor of survivorship in our models due to non-significant effects, as already documented in Capítulo I.  One possible selective pressure that forces species, such *Melanophryniscus*, to strictly develop in ephemeral water bodies is the susceptibility to predators, which would be smaller and less abundant in temporary water bodies (Wellborn *et al.* 1996). Although the presence of predators was not expected during this study, the hydrological moment of the ponds could induce an invertebrate composition that resembled more the communities of permanent ponds than the expected for temporary ponds. Selection for ponds free of predators was reported for *M. stelzneri* (Pereyra *et al.* 2011), while neither selection for ponds free of predators by amplectant pairs nor predator avoidance by larvae was reported for *M. rubriventris* (Laufer *et al.* 2015). One interpretation of these reports would be that non-avoidant spawning or larval behavior would lead to a high susceptibility for predation (Capítulo I). Although the lack of effect of predation over survivorship in our study would suggest the contrary, our results do not allow further inferences given the design of the cages, which partially avoided predation and simultaneously enhaced the catchability of the larvae for predators that could enter the cages.

Finally, given the conditions of this study, we cannot primarily support the initial hypothesis, because the second prediction was not fully confirmed. However, levels of some physicochemical water parameters partially reduced larval growth and survival. Although the levels reported are more likely the result of decomposition processes as a part of natural hydrological fluctuations of the ponds, in the context of climate change, a higher frequency of these fluctuations would represent a problem for the recruitment of *M. montevidensis*. Moreover, similar levels can be reached in eutrophicated conditions (USEPA 1986, 1997; Allen Burton Jr. and Pitt 2001; Chapman 1996). It should not be overlooked the fact that the study area has an incipient degree of urbanization, with unsewered domestic sanitation; so domestic wastewaters are also a potential source of pollution. Besides the sanitary problems given by the presence of pathogenic organisms, wastewaters are known to cause increased nitrate concentrations in the underlying groundwater, and phosphates when detergents are present (Maidment 1993; Chapman 1996). More studies in human-altered zones of the distribution of the species, considering larval density and analyzing other compounds (such as pesticides), should be conducted for this species to support our initial hypothesis.

# TABLES II

**Table 1**. Data sets used in statistics (GLMs and MLMs). For the GLMs, survival represents the living hatchlings and larvae on periods 1 and 2 respectively; in Period 3 only metamorphs were considered as survivals. As eggs from pond 2 never hatched (see Capítulo I), we only fitted data from this pond in the survival GLMs of Period 1. We fitted MLMs only for the last two periods, because we considered growth as more important during these periods; body sizes considered in each case were of the living larvae in Period 2, and only of metamorphs in Period 3. Because of the few metamorphs recorded in Pond 1 (five animals), only data from Pond 3 were applied to the growth MLMs of Period 3.

| | Period 1 (hatchlings) | Period 2 (larvae) | Period 3 (metamorphs) |
|---|---|---|---|
| GLMs (survival) | Ponds 1, 2, and 3<br>(*n* = 51) | Ponds 1 and 3<br>(*n* = 80) | Ponds 1 and 3<br>(*n* = 47) |
| MLMs (body size) | | Ponds 1 and 3<br>(*n* = 80) | Pond 3<br>(*n* = 53) |

# TABLES II

**Table 2**. GLMs (logit) fitted to the survival data for the three periods of the development. N(larvae) is number of larvae inside the enclosures; Cond are the conductivity levels; [I] is the iodine concentration; Temp is water temperature inside the enclosures; Depth is water depth inside the enclosures; D(pred) is predator density inside the enclosures; Site is a nominal variable (levels: pond 1, 2 and 3); Turb are the turbidity levels; $[NH_3]$ is the ammonia concentration; BL is the larval body length.

| | | | Residual Deviance | df | *P* value |
|---|---|---|---|---|---|
| Period 1 | Saturated | N(larvae) + Cond + [I] + Temp + Depth | 867.31 | 45 | |
| | Simplified | N(larvae) + Cond + Depth | 902.60 | 47 | |
| | ANOVA | F = 0.99 | 35.29 | 2 | 0.38 |
| Period 2 | Saturated | N(larvae) + D(pred)*Site + Cond + Turb + $[NH_3]$+ [I] + Temp + Depth + BL | 606.93 | 68 | |
| | Simplified | N(larvae) + Site + Turb + Temp | 693.09 | 75 | |
| | ANOVA | F = 1.57 | 86.16 | 7 | 0.16 |
| Period 3 | Saturated | N(larvae)*Site + D(pred) + Cond + $[NH_3]$ + Temp + Depth + BL*Site | 261.62 | 42 | |
| | Simplified | N(larvae) + Cond + BL*Site | 292.37 | 36 | |
| | ANOVA | F= 0.69 | 30.75 | 6 | 0.66 |

# TABLES II

**Table 3**. Best simplified GLMs. For a general model $y = a + b_1x_1 + .... + b_nx_n$, (intercept) is the coefficient *a* of the model of the model (intercept of the model with the y axis); *b* are the estimates of the coefficients of each variable x; SE are the standard errors of the estimates b; *t* value are the *t*-statistic values of each coefficient *b*; *P* value are the significance values of the *t*-test of each coefficient *b*. N(larvae) is number of larvae inside the enclosures; Cond are the conductivity levels; Temp is water temperature inside the enclosures; Depth is water depth inside the enclosures; Site is a nominal variable (levels: Pond 1, 2 and 3); Turb are the turbidity levels; BL is the larval body length.

| | | *b* | SE | *t* value | *P* value |
|---|---|---|---|---|---|
| Period 1 | (intercept) | 1.348 | 0.516 | 2.613 | <0.05 |
| | Cond | -0.023 | 0.003 | -6.718 | <0.001 |
| | N(larvae) | 0.003 | 0.002 | 2.199 | <0.05 |
| | Depth | -0.071 | 0.034 | -2.127 | <0.05 |
| Period 2 | Pond 1 (intercept) | -3.323 | 0.640 | -5.191 | <0.001 |
| | Pond 3 | 1.937 | 0.268 | 7.242 | <0.001 |
| | N(larvae) | -0.009 | 0.002 | -6.020 | <0.001 |
| | Temp | 0.117 | 0.026 | 4.517 | <0.001 |
| | Turb | -0.001 | 0.000 | -3.146 | <0.01 |
| Period 3 | (intercept) | -7.825 | 3.371 | -2.321 | <0.01 |
| | N(larvae) | -0.063 | 0.011 | -5.725 | <0.001 |
| | Cond | -0.015 | 0.005 | -3.277 | <0.001 |
| | BL Pond 1 | 1.487 | 0.577 | 2.577 | <0.01 |
| | BL Pond 3 | 2.013 | 0.572 | 3.519 | <0.01 |

## TABLES II

**Table 4**. MLMs fitted to the body size data (body length) for the last two periods of the development. N(larvae) is number of larvae; Days are the number of days since oviposition; Cond are the conductivity levels; Site is a nominal variable (levels: pond 1, 2 and 3), Turb are the turbidity levels; $[O_2]$ is the dissolved oxygen concentration; [I] is the iodine concentration; Depth is water depth inside the enclosures; $[NH_3]$ is the ammonia concentration.

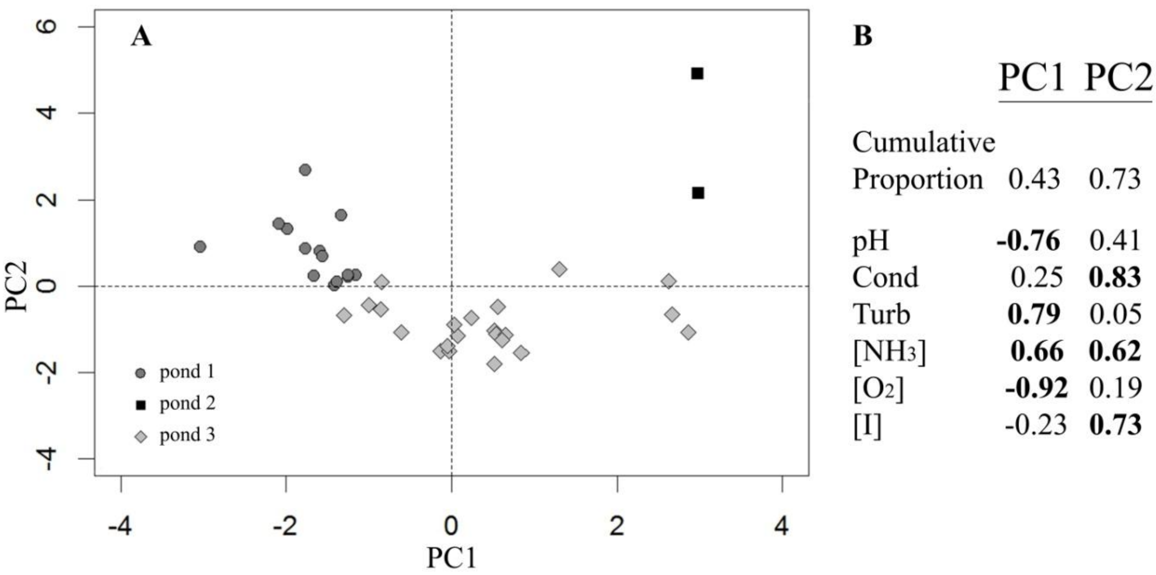
| | | | $R^2$ | AIC | df | *P* value |
|---|---|---|---|---|---|---|
| Period 2 | Saturated | N(larvae) + Days + Cond*Site + Turb + $[O_2]$*Site + [I]*Site + Depth | 0.75 | 145.19 | 68 | <0.001 |
| | Simplified | N(larvae) + Days + Cond | 0.73 | 133.57 | 76 | <0.001 |
| | ANOVA | F = 0.49 | | 11.62 | 8 | 0.87 |
| Period 3 | Saturated | N(larvae) + Days + Cond + Turb + $[NH_3]$ + $[O_2]$ + [I] + Depth | 0.74 | 50.78 | 44 | <0.001 |
| | Simplified | N(larvae) + Depth | 0.67 | 50.71 | 50 | <0.001 |
| | ANOVA | F = 1.85 | | 0.07 | 6 | 0.11 |

# TABLES II

**Table 5**. Best simplified MLMs. For a multiple model $y = a + b_1x_1 + .... + b_nx_n$, (intercept) is the coefficient *a* of the model of the model (intercept of the model with the y axis); *b* are the estimates of the coefficients of each variable x; SE are the standard errors of the estimates b; t value are the *t*-statistic values of each coefficient *b*; *P* value are the significance values of the t-test of each coefficient *b*. Days are the number of days since oviposition; N(larvae) is number of larvae; Cond are the conductivity levels; Depth is water depth inside the enclosures.

| | | *b* | SE | *t* value | *P* value |
|---|---|---|---|---|---|
| Period 2 | (intercept) | 2.737 | 0.261 | 10.461 | <0.001 |
| | Days | 0.206 | 0.016 | 13.183 | <0.001 |
| | N(larvae) | -0.006 | 0.001 | -4.026 | <0.001 |
| | Cond | -0.007 | 0.002 | -3.860 | <0.001 |
| Period 3 | (intercept) | 6.320 | 0.123 | 51.563 | <0.001 |
| | N(larvae) | -0.016 | 0.002 | -7.885 | <0.001 |
| | Depth | 0.114 | 0.027 | 4.209 | <0.001 |

# FIGURES II



| | PC1 | PC2 |
|---|---|---|
| Cumulative Proportion | 0.43 | 0.73 |
| pH | **-0.76** | 0.41 |
| Cond | 0.25 | **0.83** |
| Turb | **0.79** | 0.05 |
| [$NH_3$] | **0.66** | **0.62** |
| [$O_2$] | **-0.92** | 0.19 |
| [I] | -0.23 | **0.73** |

**Figure 1**. Physicochemical characterization of the ponds studied in the protected area Laguna de Rocha, based on the principal two components. A) Graphic representation by ponds; B) cumulative proportion of the variance explained by each principal component and loadings of each physicochemical variable. Cond and Turb are the conductivity and turbidity levels respectively; [$NH_3$], [$O_2$] and [I] are the ammonia, dissolved oxygen and iodine concentrations respectively. In bold are displayed those variables with loadings >0.5 within each principal component.

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# Capítulo III

## Quantitative determination of the minimum body size for photo-identification of *Melanophryniscus montevidensis* (Bufonidae)

# ABSTRACT-RESUMEN

**Abstract**. Toads of the genus *Melanophryniscus* possess unique color patterns on the belly, which allows for individual recognition. Photo-identification has proven to be an efficient non-invasive technique to identify individuals for this genus. However, such color patterns are absent in newly metamorphosed individuals. We studied the development of the ventral coloration pattern and evaluated its persistence in *Melanophryniscus montevidensis* to determine the minimum size and age at which use of the color pattern is a trustworthy (*i.e.*, as stable as in adults) identification method for this species. From spawns raised in the field, we obtained eight metamorphs and raised them *ex situ* to photograph their bellies. We visually analyzed the images to establish the stabilization point of the color pattern. Using the software Wild-ID, we calculated the similarity score between the images from the stabilization point with sets of images before and after stabilization. Similarity scores of adults from previous studies did not differ significantly from the scores of juveniles after the pattern stabilized, but they did differ significantly from the scores of juveniles compared to themselves at least 70 d before stabilization. The color pattern developed progressively and stabilized at a median of 220 d after metamorphosis, with a maximum snout-vent length of 13.2 mm, which we considered the minimum size for photo-identification purposes. Although we observed ontogenetic and individual variation, the pattern remained unchanged since just before the first year of age. Taking into account the threshold size we determined, photo-identification is a suitable method for ecological studies of this species.

**Resumen**. Los sapos del género *Melanophryniscus* poseen patrones de coloración únicos en el vientre que permiten el reconocimiento individual. La fotoidentificación ha demostrado ser una técnica no invasiva eficiente para identificar individuos de este género. Sin embargo, tales patrones de color están ausentes en individuos recién metamorfoseados. Se estudió el desarrollo del patrón de coloración ventral y se evaluó su persistencia en *Melanophryniscus montevidensis* para determinar el tamaño mínimo y la edad a la que el uso del patrón de coloración puede ser considerado un método de identificación confiable (es decir, estable como en adultos) para esta especie. De desovas criadas en el campo, obtuvimos ocho metamorfos que fueron mantenidos *ex situ* para fotografiar sus vientres. Estas imágenes fueron analizadas visualmente para establecer el punto de estabilización del patrón de coloración. Utilizando el software Wild-ID, se calificó la similitud entre las imágenes desde el momento de estabilización del patrón con conjuntos de imágenes antes y después de la estabilización. Las calificaciones de similitud de los adultos en estudios anteriores no difirieron significativamente de las calificaciones de los juveniles después de que el patrón se estabilizó, pero difirieron significativamente de las calificaciones de los juveniles al menos 70 días antes de la estabilización. El patrón de coloración se desarrolló progresivamente y se estabilizó a una mediana de 220 días después de la metamorfosis, con una longitud máxima hocico-cloaca de 13,2 mm, medida que consideramos el tamaño mínimo para fines de fotoidentificación. Aunque observamos una variación ontogénica e individual, el patrón se mantuvo estable desde justo antes del primer año de edad. Teniendo en cuenta el umbral de tamaño que determinamos, la fotoidentificación es un método adecuado para los estudios ecológicos de esta especie.

# INTRODUCTION

The Neotropical genus *Melanophryniscus* comprises 29 species distributed from central-southern Brazil to central-eastern Argentina, including Bolivia, Paraguay, and Uruguay (Frost 2016). The dorsal pigmentation of these small toads is generally not colorful. However, most of them possess a conspicuous color pattern on the belly (and sometimes on the hands and feet). The black, brown, or green background with red, yellow, white, green, or orange spots inspires their common name of red belly toads (Mebs *et al.* 2007; Caorsi *et al.* 2014). When threatened, *Melanophryniscus* individuals exhibit a defensive behavior, the *unken-reflex*, which includes displaying the bright coloration on their palms and belly (Toledo and Haddad 2009). Neither the color pattern nor the associated *unken-reflex* are apparent during the first days after metamorphosis in some species of this genus (Langone *et al.* 2008; Caorsi *et al.* 2014), or in toads belonging to the genus *Bombina* (Löhner 1919).

In Uruguay, there are six *Melanophryniscus* species, three are globally listed as threatened. One of them is *Melanophryniscus montevidensis* (IUCN 2017: *Vulnerable*). This is a small sized (adult snout-vent length [SVL]: 20–30 mm) coastal species, which exploits psammophilic vegetation (vegetation from sandy soils), and breeds in ephemeral ponds after heavy rains (Maneyro and Carreira 2012; Pereira and Maneyro 2016a). Zank *et al.* (2014) recommended population-level studies for this species, and non-invasive marking techniques like photo-identification are the most advisable approaches for threatened species.

Elgue *et al.* (2014) tested the accuracy of using photo-identification for adult individuals of *M. montevidensis* (mainly black with red and yellow spots) and it seems to perform well as an identification method. Using this method to identify individuals has no effect on the health and behavior of the animal, lasts for the duration of the study, and has minimal cost (Ferner 2007). As in other species of the genus, newly metamorphosed *M. montevidensis* may not possess the characteristic color pattern (Capítulo I). Furthermore, juveniles are difficult to find and there are no records of juvenile recaptures using photo-identification (Pereira and Maneyro 2016b). Some information about the appearance of the ventral patterns in young juvenile toads is available in the literature (Gollmann and Gollmann 2011; Caorsi *et al.* 2014), but our study is the first to quantify the development time of the color pattern and estimate the minimum body size at which the color pattern stabilizes and becomes useful for valid photo-identification. For *M. montevidensis* we predicted that the color patterns would develop progressively during the first year, which may violate the assumption of consistent appearance of the mark if researchers use photo-identification for early life stages.

## METHODOLOGY

On February 2014, during a breeding event, we captured nine amplectant pairs in a temporary pond located 1 km away from La Riviera, Rocha (34°32'42.40"S, 54°19'34.04"W; 3–10 m above sea level). This locality is within Paisaje Protegido Laguna de Rocha, part of the national system of protected areas (DINAMA 2010). To obtain spawns, we kept the amplectant pairs for 24 h in plastic cages containing water and vegetation from the pond. Later, we placed the spawns in separate *in situ* plastic enclosures and monitored them until tadpoles reached metamorphosis. To count the age of the larvae until front limb emergence, the day of oviposition was considered day 0.

From 19 to 25 March, we randomly selected four metamorphs (Gosner stage 42; Gosner 1960) from two different amplectant pairs. We reared these eight individuals inside individual 6-l plastic containers (30 cm × 15 cm × 13 cm) in an open room exposed to the natural photoperiod and temperatures (mean 15.7 ± [SD] 3.7° C). We periodically added soil and grass collected from the border of the pond as a source of invertebrate food and shelter. We supplied water from the pond daily to maintain humidity. When the pond dried up, we mixed water from a nearby water body with distilled water to emulate the original pond (mean conductivity = 64.3 ± 37.3 $\mu S\ cm^{-1}$, $n = 81$; mean pH = 6.2 ± 0.4, n = 81; pH and conductivity meter, Hanna Instruments, Woonsocket, Rhode Island, USA). During the sixth month, we offered ants (*Solenopsis* sp. and *Linepithema humile*; Formicidae: Myrmicinae and Dolichoderinae, respectively) to increase the size and variety of food items for the toads. However, some toads rejected the ants, especially *Solenopsis* sp., and died before the end of the first year (Table 1). We deposited all but one of the deceased toads in the scientific collection of Facultad de Ciencias, Universidad de la República (Montevideo, Uruguay); siblings from the spawn of couple 1: ZVCB 23365–23367; siblings from the spawn of couple 2: ZVCB 23368–23371.

We took photographs of the belly and pelvic patch every 2 d after front limb emergence during the first 70 d and then monthly for the lifespan of each animal (range, 200–400 d), using a Coolpix L810 camera (16.0 MP; Nikon Corporation, Tokyo, Japan) Because animals were small at the beginning (median = 5.8 mm SVL), we photographed them in a drop of water through a plastic dish. We included a ruler for scale in the picture frames and held the distance and lighting constant to standardize images and facilitate taking digital measurements. We used the software Micrometrics SE Premium (ACCU-SCOPE, Commack, New York, USA) to measure the SVL.

We predicted that the color pattern would stabilize at some point during the first year. Before that point, we suspected that the images would have low resemblance. However, when comparing the image at stabilization with the last picture of the animal, the level of resemblance should be similar to that reported for adults (Elgue *et al.* 2014). To test this, we created a database of 4,523 images documenting the developmental sequence of the color pattern for each animal. We chose 109 photos (one photo of each animal every 10 d until day 63 [from day 3 after front limb emergence] and one photo each month after day 63). We used the same procedure as Elgue *et al.* (2014). We cropped images to show only the ventral pattern, pelvic patch, and axillar marks, and modified the brightness and contrast to improve the visibility of the color pattern whenever necessary. Separately, we visually inspected the sequence of images of each animal to determine the image at which the color pattern stabilized. We defined the color pattern as stable when it stopped changing and retained its main contours and number of spots. One animal died before stabilization and we excluded it from analysis.

We used the software Wild-ID (Dartmouth College, Hanover, New Hampshire, USA; Bolger *et al.* 2011) to select the best images for visual confirmation of recapture. The software identified the top-ranked 20 photos that matched the analyzed one. Others have successfully used this software to identify individuals of species belonging to the *Melanophryniscus* genus (Abadie 2012; Caorsi *et al.* 2012; Elgue *et al.* 2014). The software computes a resemblance score for each pair of images (*i.e.*, qualifies the similarity among individuals) from zero to one; the higher the score, the higher the similarity between individuals.

To test our prediction, we contrasted four sets of similarity scores. Three sets were the scores derived by Wild-ID when we compared photographs of each juvenile (*n*

= 7) taken at the time of ventral color pattern stabilization with photographs of itself taken at a median of 100 d before, a median of 70 d before, and the last photograph of itself (taken at a median of 70 d after stabilization). The fourth set was the similarity scores of photographs of recaptured adults from the field (*n* = 18), taken at a maximum of 14 mo apart, from Elgue *et al.* (2014). This last set was included to decide which levels of similarity scores of juveniles were within the variation of adult scores, already considered valid for photo-identification by Elgue *et al.* (2014).

Because of the small sample sizes, we used the Kruskal-Wallis test in statistical software R (R Foundation for Statistical Computing, Vienna, Austria) to analyze the differences among these four sets of scores. We tested for significant differences among the four sets of scores using a multiple comparison Dunn *post hoc* test available in the R package PMCMR, which applies the Holm *P*-value adjustment method (Pohlert 2016). We considered the median SVL at which the color pattern stabilized as the minimum body size at which the photo-identification is a reliable marking method if two conditions were met: adult scores and the scores after the stabilization of the color pattern did not differ significantly, and adult scores and the scores before the stabilization of the color pattern did differ significantly.

## RESULTS

Our study subjects completed metamorphosis (*i.e.*, front-limb emergence; Gosner stage 42) after a median of 23 d after oviposition (range, 19–25 d). Unlike the black dorsum and colorful pattern of adults, the dorsal and ventral coloration of the newly metamorphosed toads was grayish-brown and slightly transparent in the ventral region (Figure 1). Dorsal darkening occurred gradually and reached the characteristic black coloration around the tenth day after front limb emergence (Figure 1). The ventral color pattern developed progressively by the accumulation of small spots (Figure 2). The age at which we considered the color pattern stable varied among juveniles (Table 1), median = 220 d after front limb emergence (range, 153–344 d). Although the hands, pelvic patch, and axillar spots developed the red coloration in early stages (range, 20–30 d), the color pattern in the belly developed first in yellow and the red coloration appeared later over the yellow spots (Figure 2). During manipulation for a photo session, one juvenile (ZVCB 23368) was the first to display *unken-reflex*, 34 d after front limb emergence.

When we made multiple comparisons of the sets of juvenile similarity scores and the set of adult scores (recaptures from Elgue *et al.* 2014), the four groups of Wild-ID scores differed significantly ($\chi^2 = 20.15$, df = 3, $P < 0.001$) (Figure 3). The adult scores and the scores of juveniles after the color pattern stabilized did not differ significantly ($P > 0.05$). There were significant differences among the adult scores and the scores obtained from comparisons at a median of 70 and 100 d before stabilization ($P \leq 0.05$). However, there were no differences between the scores obtained from comparisons at median = 70 d before and comparisons at median = 70 d after stabilization ($P > 0.05$). Once we verified that the requirements of the scores were fulfilled, we determined that the color pattern stabilized with a median SVL = 8.8 mm (range, 8.2–13.3 mm), which is the minimum body size at which the photo-identification is reliable in this species (Table 1).

## DISCUSSION

Our observations established a point at which the color patterns stabilize in young juveniles of *M. montevidensis*. The similarity scores before juveniles reach pattern stabilization do not resemble the adult re-capture scores. At earlier time intervals, the scores of toads compared to themselves were low and lacked statistical differences among age groups. This supports our prediction that the color patterns develop gradually. The low scores after stabilization and the lack of differences between the scores just before and just after the stabilization provide further evidence for gradual development of the pattern. However, the low scores and lack of differences also challenge our determination of the pattern stability of juveniles and photo-identification techniques should be applied judiciously taking into account the considerations below.

Changes in the color pattern may occur, especially in early juvenile stages (*e.g.*, Gollmann and Gollmann 2011). When slight variations in the pattern occur, photo-identification can still be applied, but visual methods combined with the use of visual-assisted software are more advisable than exclusive use of automated software (Gollmann and Gollmann 2011; Bendik *et al.* 2013). In addition, the image quality is important because high resolution, in-focus, and constant distance images are less prone to mismatches than low quality images (Gollmann and Gollmann 2011; Bendik *et al.* 2013). We expected later changes in juvenile coloration because we observed stable coloration patterns when only yellow spots were present whereas adults generally have both red and yellow spot coloration. We found that the red coloration appeared later and on top of the yellow spots (*i.e.*, not at the edges), and thus did not alter the main outline of the pattern. The software Wild-ID is based on an algorithm that recognizes and compares Key Points Independent from the Scale (Lowe 2004). It does not consider coloration and needs visual assistance to confirm matches (Bolger *et al.* 2011). Therefore, the later appearance of the red coloration would not affect the recognition using the Wild-ID software in subsequent recaptures.

Although juvenile similarity scores after pattern stabilization resembled adult scores, adult scores were low (generally < 0.2) and juvenile scores were even lower (generally < 0.05). Other studies reported low similarity scores from field photographed recaptures using Wild-ID, especially when the image quality was low and when animals experienced weight changes (i.e., allometric variations; *e.g.*, Bendik *et al.* 2013; Morrison *et al.* 2016). Differences between the adult and juvenile scores may be a result of the manner in which we photographed the juveniles versus adults. We photographed juveniles in a drop of water through a plastic dish, whereas Elgue *et al.* (2014) photographed adults in the field, dry, and upside down. Allometric variations (because juveniles were growing) and image quality differences were both influencing the similarity scores produced from our database. In spite of these difficulties, we consider the comparison of similarity scores a useful quantitative approach because the allometric variations are unavoidable in early stages, and the algorithm of the software still works at low scores (Bendik *et al.* 2013); thus, we strongly recommend this method. The manipulation of small animals without harming them is difficult and time consuming. If the SVL of the animal is < 10 mm, a drop of water reduces its movement and the animal can be photographed in a natural position from below with no handling.

We determined the minimum size at which researchers can reliably use the color pattern in *M. montevidensis* to be 8.8 mm SVL. However, because the age at which an individual attained pattern stability was highly variable and our sample size was small, we take caution in interpreting our results. The last individual developed a stable color pattern nearly one year after front limb emergence, which suggests that older animals would be at the point of stabilizing their pattern in the field. Therefore, we recommend the body size of this animal, 13.2 mm SVL, as the minimum size at which the color pattern is reliable for photo-identification.

Diet of the juveniles during captivity may have affected their growth and ontogeny of the color pattern. We expect that animals in nature might be larger at the same age as our study subjects and may therefore develop the color pattern earlier. Diet is also important because of its relationship with skin toxins (Darst *et al.* 2005; Mebs *et al.* 2005; Hantak *et al.* 2013). Some species sequester skin toxins from their food, so it is possible that a shift in coloration depends on the acquisition of these toxins (Caorsi *et al.* 2014). We found that the appearance of the *unken-reflex* and the development of the red coloration in hands, pelvic patch, and axillar spots were synchronous, and occurred earlier than the stabilization of color patterns. The body parts that develop a red coloration are most visible during the *unken-reflex* behavior of *M. montevidensis*. The belly pattern in some *Melanophryniscus* species is exhibited as part of the *unken-reflex* (Toledo and Haddad 2009; Caorsi *et al.* 2014), but it does not seem to be involved in the defensive behavior of *M. montevidensis*. The belly pattern would not be subjected to the same selective pressures as the hands, pelvic patch, or axillar spots and there may be no advantage to their early appearance. Further studies of early stages of the *Melanophryniscus* genus are needed to determine the role of diet in the development of coloration and the *unken-reflex* behavior.

Finally, there have been no reports of juvenile recaptures in *M. montevidensis* (Pereira and Maneyro 2016b). Therefore, information about the early juvenile stages of *Melanophryniscus* species is scarce (Caorsi *et al.* 2012, 2014). Young juveniles usually have high mortality rates, which means few are in the environment, and because young use different environments than the adults, they are intrinsically difficult to find (Semlitsch 2008; Walston and Mullin 2008; Pereira and Maneyro 2016a), but it is also plausible that photo-identification methods are not effective when applied to early stages. The determination of the minimum size at which the color pattern stabilizes improves the accuracy of this marking method for juveniles. We consider visual implant elastomers, already tested in 6 mm juvenile Blue Crabs (*Callinectes sapidus*; (Davis *et al.* 2004), as a complementary method to the photo-identification to identify younger and smaller individuals in population and life-history studies of *Melanophryniscus* species.

# TABLES III

**Table 1.** Ages (number of days after front limb emergence) and sizes of *Melanophryniscus montevidensis*. Age of image -100 d and Age of image -70 d refer to photographs from a median of 70 and 100 d before stabilization of the pattern, respectively. Age of stabilization refers to the first image with a stable pattern. Age of image +70 d refers to the final image (median time = 70 d after stabilization). Wild-ID stabilization scores are the similarity scores comparing the first image with a stable pattern to the final image. For Animal I.D., the first number indicates parental pair; the second is the catalog number in the scientific collection of Facultad de Ciencias, Universidad de la República (ZVCB). One catalog number was unavailable (noted as 1-n.a.).

| Animal I.D. | Age of image -100 d | Age of image -70 d | Age of stabilization | SVL at the age of stabilization (mm) | Age of image +70 d | Wild-ID stabilization scores |
|---|---|---|---|---|---|---|
| 1-23365 | 130 | 158 | 228 | 8.55 | 320 | 0.0395 |
| 1-23366 | 153 | 195 | 279 | 11.10 | 347 | 0.0088 |
| 1-n.a. | 220 | 276 | 344 | 13.24 | 400 | 0.0134 |
| 1-23367 | 122 | 150 | 220 | 8.95 | 312 | 0.0045 |
| 2-23368 | 53 | 102 | 158 | 8.48 | 228 | 0.0535 |
| 2-23369 | -- | -- | -- | -- | 200 | -- |
| 2-23370 | 53 | 97 | 153 | 8.18 | 223 | 0.0035 |
| 2-23371 | 97 | 125 | 195 | 8.86 | 315 | 0.0140 |

# FIGURES III

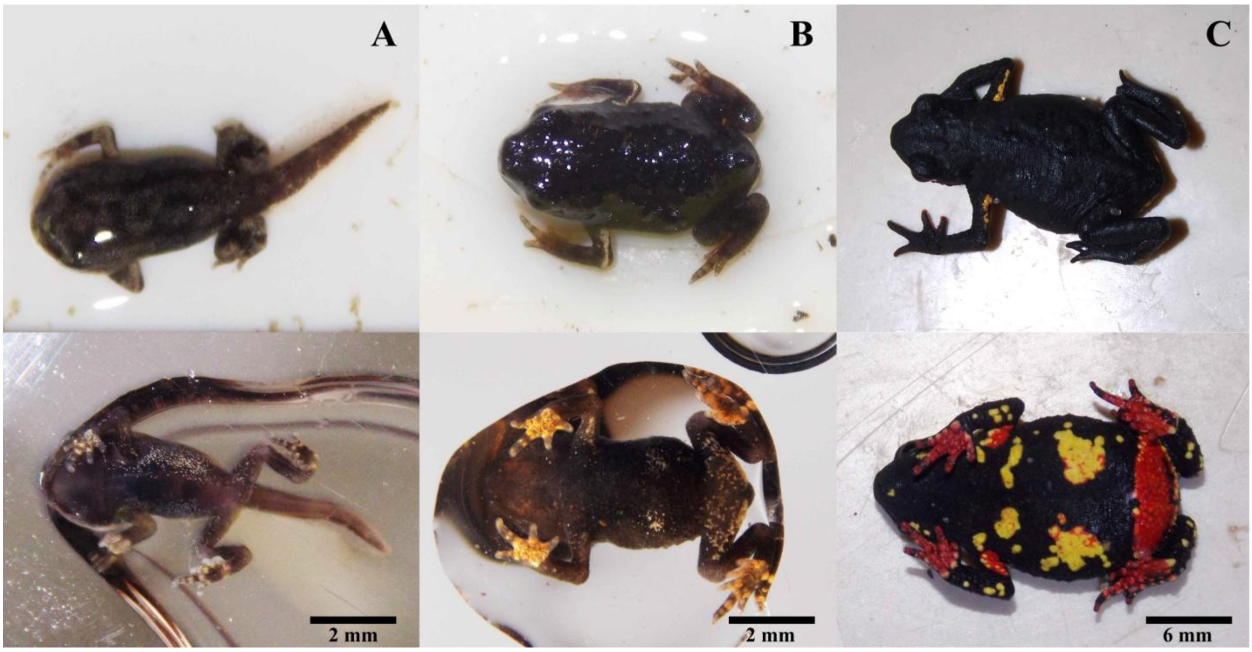

**FIGURE 1**. Dorsal (top) and ventral (bottom) views of *Melanophryniscus montevidensis* at different stages: A) newly metamorphosed individual (*i.e.*, front limb emergence; Gosner stage 42), B) 13 d after front limb emergence (ZVCB-23367), C) adult photographed in the field. Brightness and contrast of the images was adjusted (A and B photographed by Cecilia Bardier; C photographed by Nicolás Martínez).

# FIGURES III

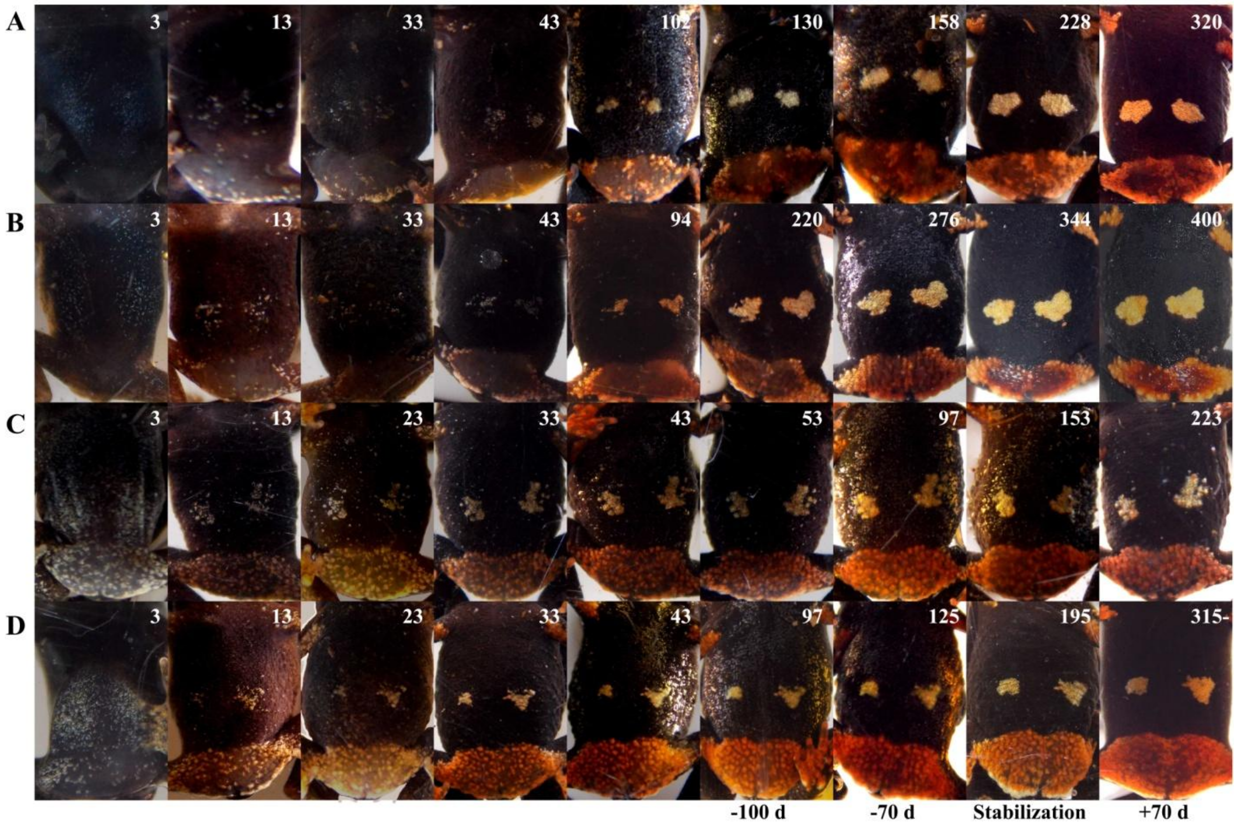

**Figure 2**. Developmental sequence of the color pattern of four juvenile toads (*Melanophryniscus montevidensis*) 1-23365 (A), 1-n.a. (B), 2-23370 (C), and 2-23371 (D). The first number of each animal indicates the amplectant pair, the second number is the catalog number in the scientific collection of Facultad de Ciencias, Universidad de la República (ZVCB). Top right numbers are the ages (d after front limb emergence. -100 d and -70 d are the images of the animals at a median of 70 and 100 d before the stabilization of the pattern, respectively. Stabilization represents the first images with stable pattern. +70 d are the last images of each animal (median = 70 d after stabilization of the pattern). Brightness and contrast of the images was adjusted to facilitate comparisons (Photographed by Cecilia Bardier).

## FIGURES III

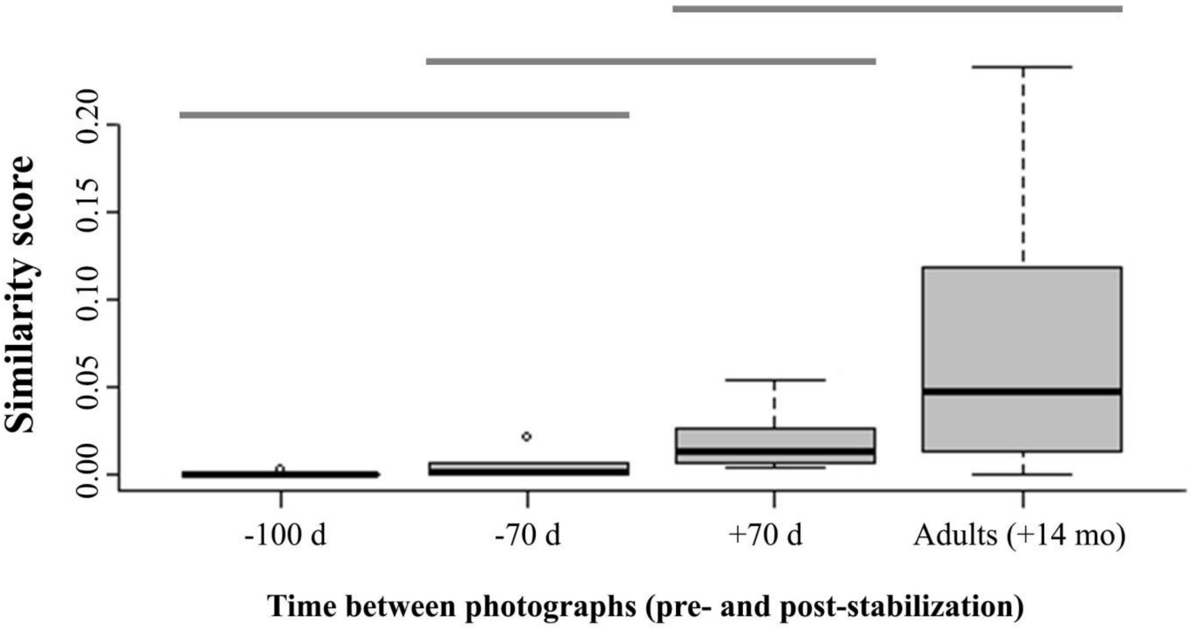

**Figure 3**. Boxplots of similarity scores comparing photographs of juvenile toads (*Melanophryniscus montevidensis, n* = 7) taken when the ventral color pattern stabilized with images taken at median = 100 d (-100 d) and 70 d (-70 d) prior to stabilization, and median = 70 d after stabilization (+70 d). Adults (+14 mo) refers to similarity scores comparing images of recaptured adult toads (*n* = 18) taken within a maximum of 14 mo apart and reported by Elgue *et al.* (2014). Similarity scores not joined by horizontal bars are significantly different from each other ($P \leq 0.05$; Dunn's *post hoc* comparisons).

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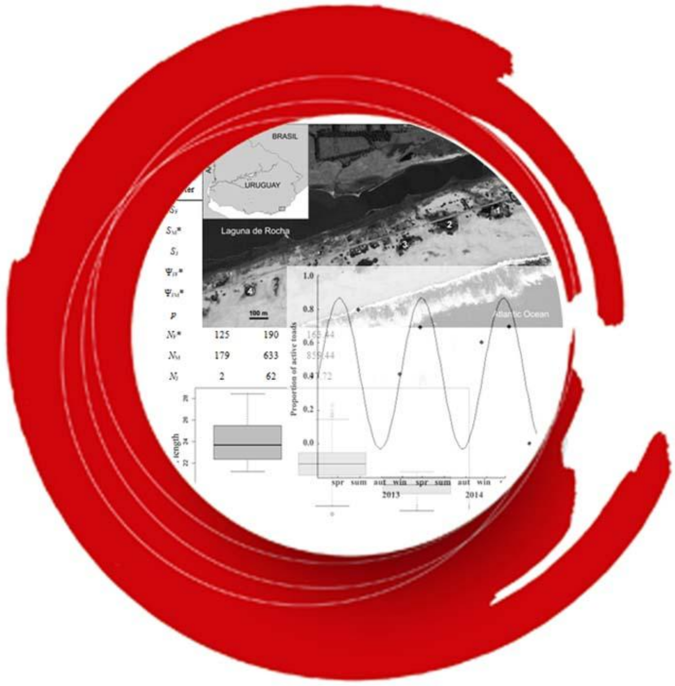
# Capítulo IV

## Seasonal estimations of survival rates and abundances of the threatened bufonid *Melanophryniscus montevidensis* in a protected area of Uruguay



## ABSTRACT-RESUMEN

**Abstract**. Studies aimed to estimate demographic parameters are few for Neotropical amphibian species, while this region has the highest proportion of threatened amphibians. We conducted a three-year study where we applied a robust design capture-mark-recapture (CMR) method to estimate seasonal survival rates of juveniles and adults, maturity rates, detection probabilities, and abundances of *Melanophryniscus montevidensis*, a threatened species, in a protected area of Uruguay. We also analyzed the breeding and non-breeding seasonal activity patterns to test different hypothesis for the demographic parameters of the species. State and seasonal dependence in survival, state dependence in transition probabilities and time dependence in detection probabilities was the best ranked multi state closed robust design model jfor our CMR data. Although median survivorships were high for adult and juvenile stages (> 80 %), slight decrease from autumn to winter and a drastic decrease from spring to summer were detected (< 50 %). As non-breeding activity patters seem to be shaped by seasonal cycles, and peaks of activity matched peaks of mortality, we suggest that some temporal cause, like touristic road-kills, is decreasing survival rates during the highest activity seasons. Transition rates were higher for juvenile to males than for juvenile to females, suggesting that the adult male-sex bias is already present in the juveniles. This sex bias did not respond to a sex biased detection probability. The long-term impact of a decreased survivorship of females, mainly in the context of the male-biased sex ratio, would be determinant for the extinction probabilities of this population, since female survivorship is generally the most important vital rate for population growth.

**Resumen**. Pocos estudios se han realizado para estimar los parámetros demográficos en especies neotropicales de anfibios, cuando la mayor proporción de anfibios amenazados se encuentra en esta región. A lo largo de tres años realizamos un estudio en el que aplicamos un método de diseño robusto de captura-marcaje-recaptura (CMR) para estimar las tasas de supervivencia estacional de juveniles y adultos, tasas de madurez, probabilidades de detección y abundancias de *Melanophryniscus montevidensis*, una especie amenazada, en un área protegida de Uruguay. También analizamos los patrones de actividad estacional para probar diferentes hipótesis sobre los parámetros demográficos de la especie. Aquel modelo con dependencia de los estados (hembra, macho, juvenil) y estaciones en la supervivencia, dependiente del estado en las probabilidades de transición y dependiente del tiempo en las probabilidades de detección, fue el modelo multiestado de diseño robusto mejor rankeado para nuestros datos de CMR. Aunque las medianas de supervivencia fueron altas para adultos y juveniles (> 80 %), se detectaron ligeras disminuciones de otoño a invierno y disminuciones drásticas de primavera a verano (< 50 %). Dado que los patrones de actividad no reproductiva fueron representados por ciclos estacionales, con picos de actividad que coincidieron con los picos de mortalidad, sugerimos que una causa temporal, como los atropellamientos, podría disminuir las tasas de supervivencia durante las temporadas de actividad más altas. Las tasas de transición fueron más altas de juveniles a machos que a hembras, lo que sugiere que el sesgo masculino en la proporción sexual ya estaría presente en los juveniles. Este sesgo sexual no fue explicado por una probabilidad de detección sesgada hacia los machos. El impacto a largo plazo de una disminución de la supervivencia de las hembras, principalmente en el contexto de la proporción sexual con sesgo masculino, sería determinante para las probabilidades de extinción de esta población, ya que la supervivencia de las hembras es generalmente la tasa vital más importante para el crecimiento de las poblaciones.

# INTRODUCTION

Accurate estimations of survival rates and population sizes are necessary for the study of population dynamics. For amphibians, the vertebrates with the highest proportion of threatened species (32 %, IUCN 2017), improving the knowledge of the population dynamics has been largely recommended to separate declining phenomena from natural fluctuations (Pechmann *et al.* 1991; Pechmann and Wilbur 1994) and for the proper design of conservation strategies (Schmidt 2003; Beebee and Griffiths 2005; Collins and Halliday 2005; Todd and Rothermel 2006; McCaffery and Lips 2013). However, few attempts to estimate population parameters have been accomplished for Neotropical species (Grafe *et al.* 2004; Cairo 2009; Vasconcellos and Colli 2009; Guimarães *et al.* 2011; Hiert *et al.* 2012; Lampo *et al.* 2012; Guimarães *et al.* 2014). This fact is concerning since the Neotropical realm is the richest in amphibian biodiversity and the region with the highest proportion of threatened amphibians (Stuart *et al.* 2008).

Capture-marking-recapture (CMR) methods are the most suitable for survival and abundance estimates of amphibians, as they adjust systematic count data to detection probabilities of the animals avoiding the bias of classical single-counting methods (Schmidt 2003, 2004). Some authors dismissed CMR approaches as effective methods for conservation purposes at large scales, because of their costs and the large time lapse they take to produce reliable results (*e.g.*, Cushman 2006). However, it has been recently proposed that local scales are more effective than broader geographical scales to approach conservation efforts for amphibians (Grant *et al.* 2016). Also, some examples in the literature indicate that short time scales, such as weekly or monthly time intervals between recaptures within a single year, can still produce robust estimations (Yang and Pal 2010; Cove and Spínola 2013). Additionally, an increasing number of studies have successfully applied non-invasive and low-cost marking techniques like photo-identification for CMR demographic estimations in amphibian populations (Cove and Spínola 2013; Lampo *et al.* 2012).

*Melanophryniscus montevidensis* (Philippi, 1902) is a Neotropical Bufonidae that formerly occupied the coastal region of the Río de la Plata and the Atlantic Ocean of Uruguay, and the Southern coast of Rio Grande do Sul (Brazil) (Tedros *et al.* 2001; Núñez *et al.* 2004). Currently this species is restricted to the northeastern part of its previous distribution, the species was categorized as *Vulnerable* in global lists (IUCN 2017), and it is threatened at country and state levels in both counties (Fundação Zoobotânica 2014; ICMBio 2014; Carreira and Maneyro 2015). Moreover, reductions of suitable habitats by climate change were predicted for several *Melanophryniscus* species (Toranza and Maneyro 2013; Zank *et al.* 2014; Oliveira *et al.* 2016). Particularly, *M. montevidensis* can potentially be one of the most affected by this phenomenon, with no suitable habitats projected by 2050 (Toranza and Maneyro 2013; Zank *et al.* 2014). For species of this genus, these studies strongly suggest improving the knowledge on population dynamics, and its relationship with climatic conditions.

Depending on the type of encounter data obtained from CMR methods, different suitable mathematical formulations are available for the estimation of vital rates (White and Burnham 1999; Cooch and White 2015). The software MARK allows the construction, evaluation and averaging of different candidate models (hypothesis) for the temporal structure of the data (White and Burnham 1999). Regarding to *M. montevidensis*, the only temporal dynamics known to occur to the populations are the increased activity patterns driven by local precipitation, which are related to the explosive breeding behaviour of the species (Maneyro and Carreira 2012; Pereira and Maneyro 2016a). However, the high probability of encounter during breeding activity can mask long term demographic patterns, especially in small-sized amphibians (Vasconcellos and Colli 2009). Pereira and Maneyro (2016) also reported a sheltering behaviour during rough winter and summer conditions, which was reported during cold seasons for other *Melanophryniscus* as well (Carvajalino-Fernández *et al.* 2013). Temporal patterns of survival, recruitment and abundance of amphibians, can be affected by breeding patterns of the species, as in other bufonids (Vasconcellos and Colli 2009), but they also can be affected by seasonal activity patterns independently form breeding activity (Guimarães *et al.* 2011), shift yearly or even have no clear pattern (Hiert *et al.* 2012).

We applied multistate closed robust design (MSCRD) models to analyze CMR data of *M. montevidensis* (Kendall 1999). This formulation allowed the estimation of survival rates of juveniles and adults, transition (maturity) rates from juvenile to adult, detection probabilities, and abundances of our target species. Also, we analyzed the breeding and non-breeding seasonal activity patterns to test different hypothesis for the demographic parameters of the species (*i.e.*, seasonality, annual shifts, or differential survival during breeding seasons). For this, we constructed a set of candidate models to analyze the CMR data, and determined the model with the best temporal structure for vital rate estimations.

## METHODOLOGY

### Study site and field methods

The study was conducted at the flood plain of four ephemeral ponds from the stable sand bar of Laguna de Rocha, a protected area of the National System of Protected Areas, Departamento de Rocha, Uruguay (34°39'51"S; 54°13'31" W; 3-10 m a.s.l.; Figure 1). This protected area is mainly composed by a microtidal coastal lagoon of 72 km$^2$, which is intermittently connected to the Atlantic Ocean, and a watershed area of 1312 km$^2$ (Conde *et al.* 1999). The climate is subtropical, and the mean annual air temperature is 16.4 °C (data from 1980 to 2009). Seasons are clearly differentiated in temperature (autumn, winter, spring and summer), and the rainfall is well distributed throughout the year: mean annual rainfall from 1980 to 2009 was 1277 mm (Fernández 2011). Ponds of this study, and their flood plain, were chosen because they were previously sampled to study population movements of *M. montevidensis*, confirming that a stable population occupies the area all year long (Pereira and Maneyro 2016a).

From January 2013 to November 2015 we conducted a MSCRD CMR method to monitor toads of *M. montevidensis* (Pollock 1982). For this method, we captured animals on two consecutive secondary sessions (days) within the primary sampling period (season), using photo-identification as a marking method (see Bardier *et al.* 2017). We conducted a total of 20 diurnal secondary sessions (from 7:00 to 20:00) within 10 primary samplings. Two seasons were not sampled, as we could not survey on autumn 2014 and winter 2015. During daily sessions, we manually collected active or sheltered toads using a standardized plot design. The plots within the ponds were three fixed rectangles of 50 x 4 m each (200 m$^2$) separated by 2-4 m inside the flood plain meadow of each pond. We surveyed each rectangle in groups of two searchers during 1 h of walking active search. The total effort per season was of 24 h over 4800 m$^2$.

We placed specimens in tagged individual plastic containers (with suitable humidity and temperature), and processed them at the end of each pond sampling. For each animal, we recorded the exact location of capture, assessed the presence-absence of vocal sac (as an indicator of sexual maturity in males), and recorded snout-vent length (SVL) using a digital caliper of 0.01 mm of precision. We recorded the behavior, considering “active” animals that were outside of their shelters, in breeding activity, or moving actively in their surroundings; and “sheltered” when animals were buried under *Eryngium paniculatum* (Apiales: Apiaceae) and reeds, or in caves (*sensu* Pereira and Maneyro 2016a). As we applied photo-identification as marking method, we took a ventral picture of each specimen in a standardized procedure, similar to that described for the validation of the technique for the species (Elgue *et al.* 2014; Bardier *et al.* 2017). The camera models used were Coolpix L810 (16.0 MP, Nikon Corporation, Tokyo, Japan) and Powershot A720 IS (8.0 MP; Canon Inc., Tokyo, Japan). To assess the recaptures using the ventral pictures, we used the software Wild-ID (Dartmouth College, Hanover, New Hampshire, USA; Bolger *et al.* 2011), which returns the top-ranked 20 pictures that match the analyzed one, for visual confirmation of recapture. This software has already been successfully applied to species belonging to the *Melanophryniscus* genus (Caorsi *et al.* 2012; Elgue *et al.* 2014; Bardier *et al.* 2017). As the minimum size for photo-identification purposes is 13.2 mm, but no animals smaller than 14 mm were captured, no alternative marking methods to photo-identification were applied (Bardier *et al.* 2017). We took two to five pictures of each individual, and immediately released them where they were collected.

Two seasonal samplings were completed during breeding events of the population: April 2013 and February 2014. *Melanophryniscus montevidensis* has an explosive breeding behavior, thus more animals than usual concur to the proximity of the ponds during breeding events. This information was considered in the latter recapture models. On these breeding events we collected six amplectant pairs within the plots during the standard samplings, and 29 more in ponds nearby (Capítulo I-II). We recorded SVL, sex and weight of 68 of these breeding animals. We only considered amplectant pairs collected within the plots for later population analysis.

### Sex determination, recapture rates and activity patterns

The presence of vocal sac is a good indicator of adulthood in males (da Silva and Rossa-Feres 2010) and it was used in previous studies of reproductive biology of this species (Pereira and Maneyro 2016b). However, it does not tell apart juvenile from adult females, because of their lack of vocal sac. To establish whether the animals collected in the plots were male, female or juvenile; we used SVL of amplectant females to determine a threshold size between juveniles and adult females. We calculated the average and standard deviation of their SVL and regarded as juveniles all those animals without vocal sac with an SVL smaller than 2SD below the female average; and as adult females all those animals without vocal sac with SVL above this threshold. Animals with vocal sac were considered adult males, regardless of their size. Once established the sample sizes of males, females and juveniles, we conducted exact binomial tests to determine the significance in the differences in sample sizes, and Wilcoxon rank sum tests to compare the number of recaptures and re-recaptures (animals recaptured twice or more times) within each group. We conducted these analyses using the statistical software R (R Foundation for Statistical Computing, Vienna, Austria).

The seasonal pattern in the active behavior of the species was tested fitting sinusoidal regressions to the proportion of active and sheltered animals detected in the field. These regressions with a 12 months period were previously applied to test the existence of seasonal cycles in anuran assemblies (Canavero *et al.* 2008; Canavero and Arim 2009). The model employed was: $P = Pmean + Pamp (\sin [2\pi (M + c) /12])$, where P is the proportion of animals (active or sheltered) at a given time M of the year, Pmean is the mean proportion of animals (active or sheltered) along the time series, Pamp is the maximum deviation from Pmean (amplitude of the function, seasonal oscillation of proportions), and c is a correction factor that synchronizes the sinusoidal function with proportion of (active or sheltered) animals. Model parameters were estimated by an iteration procedure minimizing the sum of squares (Canavero *et al.*, 2008). For this analysis, performed using Statistica 8.0 (StatSoft, Palo Alto, California, USA), we excluded data from the two samplings achieved during breeding events of the population, as we could not differentiate breeding activity from other movements within the home range of the animals.

### Model selection and demographic parameter estimations

We organized the recapture data outputted by the software Wild-ID in individual binary encounter histories (1 = “captured”, 0 = “not captured”) for each animal within 20 secondary sessions (*i.e.*, field surveys). We used these histories to build mark-recapture multistate closed robust design (MSCRD) models. For these models, closure of the population is assumed between secondary sessions, and intervals between primary periods are considered open to population additions and removals (deaths and migratory movements) (Kendall 1999). We considered valid these assumptions for the population during our sampling period for three reasons: (1) we achieved secondary sessions on two consecutive days, so we can assume no important movements in or out the study area within that short period of time; (2) the secondary sessions were on random days of the season, not on breeding events (aside of the two samplings on breeding events which were particularly modeled). Thus, no particular movements were expected within those sessions. And (3) *Melanophryniscus montevidensis* was considered a species with high fidelity to both breeding sites and land shelters in previous studies (Pereira and Maneyro 2016a).

We applied Huggins closed population formulations (Huggins 1989), *sensu* Cooch and White (2015), demographic parameters estimated by these type of MSCRD models over seasons (primary periods) were: (1) apparent survival probability, $S$, the probability that a marked animal in a given state survives between two primary periods; (2) transition probability, $\Psi$, the probability that an animal in given state at primary period, changes its state in the next primary period (given that it survives); (3) capture probability, $p$, the probability that an animal alive in a given state at secondary sampling occasion of a primary period is first captured; (4) recapture probability, $c$, the probability that an animal alive in given state is recaptured on the last day of a primary period. The “states” in our models were the observable life stages of the animals: juveniles (J), adult females (F) and adult males (M). Huggins closed models calculate the conditional population size $N$ as a derived parameter (number of individuals captured each primary period, divided by the probability of being captured at least once within the primary period), so we obtained the $N$ parameter for each state within each primary period of our study.

We constrained some parameters of the departure global model taking account the biology of our target species because models with full time-specificity in all parameters contain parameters that cannot be separately estimated (Gimenez *et al.* 2004). First, within our design for *Melanophryniscus montevidensis*, only two transitions between states were possible: $\Psi_{JF}$ and $\Psi_{JM}$, being also possible for a juvenile female to remain juvenile (calculated as $1- \Psi_{JF}$) and for a juvenile male to remain juvenile (calculated as $1- \Psi_{JM}$). We allowed these transitions to be estimated by the models, and the remaining transitions possible were set to zero. Second, within a primary period i, the final capture probability is confounded with recapture probability (session 2 in our design), being $p_i2$ and $c_i2$ not individually identifiable (Cooch and White 2015). Given that we conducted field samplings only on two consecutive days, and that secondary sessions were on random days of the season; we assumed capture probability was equal within a primary period ($p_i1 = p_i2$) and capture probability was equal to recapture probability ($p_i = c_i$) to make all parameters estimable. Constraining $p_i1 = p_i2$ also had the advantage that we avoided confounding survival and capture probability in the final sampling, a common problem in open live mark- recapture models (Lebreton *et al.* 1992).

The global model $S$(state*t)Ψ(state*t)$p$(state*t) included variation between the three states and over time (primary periods: seasons) in survival and capture probability, and variation over time for the two possible transition probabilities. Following Lebreton *et al.* (1992) we conducted a sequential modeling procedure where we first determined the best structure for the capture probability ($p_i$). For this, we held constant survival probability and transitions [$S$(.)Ψ(.)], and tested the following structures for $p_i$: (1) *p* was constant across seasons and states (.), (2) *p* was constant across seasons and differed between juvenile and adult states, but not within adult states (age), (3) *p* was constant across seasons but varied among states (state), (4) *p* varied across seasons but did not vary among states (t), (5) *p* varied across seasons and differed between juvenile and adult states, but not within adult states (age*t), (6) p varied across seasons and among all states (state*t), (7) *p* varied across the four seasons of the year and among all states (state*season), (8) *p* varied between years sampled and among all states (state*year), (9) *p* varied between hot and cold seasons of the year and among all states (state*hot-cold), and (10) *p* varied between breeding and non-breeding events and among all states (state*breeding). After obtaining the capture probability structure from the top ranked model, we used it to build a candidate model set of reduced models for *S* and Ψ. For the survival *S* probability we used the same suite of model structures applied to capture probability *p*. For the transitions Ψ, the structures modeled were the analogous to (1), (3), (4) and (6) of capture probability structure. Structures (7) to (10) of *S* were only modeled with structures (3) and (6) of Ψ. The final candidate model set of reduced models contained 32 models (Apéndice 8).

Selection of the most parsimonious model was based on Akaike's information criterion with correction for small sampling sizes (AICc). The best model is the one with the lowest AICc and the largest AICc weight (WAICc), which provides another measure of the strength of evidence for each model (Mazerolle 2006). We used software MARK (Colorado State University, Fort Collins, Colorado, USA; White and Burnham 1999) to run and rank our candidate models, and to obtain maximum likelihood estimates of model parameters. We used the Logit link function to estimate the parameters; we ran all models using the optimization routines based on simulated annealing, because they are more likely to converge to the true local minima for the parameter estimation (Cooch and White 2015). No goodness-of-fit test could be applied to our models as it currently does not exist for most robust design models (Cooch and White 2015). Model averaging weighted by AICc support of the parameters was applied whenever WAICc was <1, for this averaging we only included those models with valid estimation of all the required estimated and derived parameters. To obtain a unique seasonal-scale value for abundances and the probabilities of survival, transition, and capture, we calculated median, mean and standard error for the data estimated by primary period. As this is a small sample ($n = 10$), we tested the normality of each data set (using Shapiro-Wilk tests) to determine the best descriptive measure. We compared neighboring models (with valid estimation of the parameters) using likelihood ratio tests available in MARK (Lebreton *et al.* 1992).

## RESULTS

### Recaptures, sample size of the groups and activity patterns

We collected a total of 448 individuals, 47 of which (10.5 %) were recaptured. We determined the average and standard deviation of the SVL of adult (amplectant) females as 24.43 ± (SD) 1.61 mm, so the SVL threshold to separate females from juveniles was 21.21 mm (Figure 2). On this basis we recognized 114 females, 287 males and 47 juveniles. Regarding juveniles, 32 of them were captured only once (Table 1), two were recaptured as juveniles (one of which was later recaptured as a female), 10 were recaptured as males and three as females. This implies a significantly male-biased sex ratio of 2.52:1 (binomial test, $P < 0.001$, Table 1); as much as a low capture of juveniles, ratio adult-juvenile = 8.53:1 (binomial test, $P < 0.001$, Table 1). Animals initially captured as juvenile had significantly less recaptures (binomial test, $P < 0.05$, Table 1). However, animals initially captured as juvenile had significantly more re-recaptures than animals initially captured as adults (W = 337, $P < 0.05$, Table 1).

We detected a seasonal activity pattern as sinusoidal fit of the proportion of active and sheltered animals was significant (Pamp *t*-value = 2.92, $P < 0.05$), and explained 65 % of the variability of the data. This regression indicated that spring was the most active season and autumn was the season with highest proportion of sheltered animals (Figure 3).

## Model selection

From our pre-selection of capture probability models, the model in which $p$ varied across seasons but did not vary among states (t) carried 82 % of the AICc weight (AICc = 1430.60). All other capture probability structures had $\Delta$AICc > 3.67 and w < 0.13, indicating a high support for the top model. We used this capture probability structure in our candidate models (Table 2). Only six of them produced valid estimations of all the required estimated and derived parameters. No fully time dependent models in survival or transition probabilities were pre-selected.

We found a state and seasonal dependence in survival, and a state- but not time-dependence in transition probability, as the best ranked candidate model was $S$(state*season)$\Psi$(state)$p$(t). This model accounted for 91% of the AICc weight (Table 2). The second ranked model, $S$(state)$\Psi$(state)$p$(t) accounted for 6% of the AICc weight. Significant differences were detected between these two models ($\chi^2 = 11.77$, df = 3, $P < 0.05$). The most reduced model, $S(\cdot)\Psi(\cdot)p$(t) was ranked sixth and accounted for 0.04% of the AICc weight.

## Demographic parameter estimations

We obtained seasonal survival, transition probabilities, captures probabilities, and abundance estimates using averages from the entire model set weighted by AICc support. For most parameters, the averaged estimates by primary period did not have a normal distribution, so the median best summarized the information in those cases. Capture probability estimates varied widely by primary sampling period and without a consistent pattern, being always below 0.15 (Figure 4A, Table 3). Although median seasonal survival probabilities were higher for females and juveniles than for males, averaged values for the time series did no present important differences between sexes (Figure 4B, Table 3). As in most of the candidate models that produced valid estimations of the parameters, transition probabilities varied between sexes but were time-invariant. Transition probability from juveniles to males was higher than from juveniles to females.

Regarding to population sizes, females were the only state with a normal distribution of their abundance across the seasons. Median female abundance was lower than median male abundance, being both higher than juvenile abundances (Figure 4C, Table 3). Abundances of the three states had a seasonal pattern, consistent with the seasonal structure of the survival probability in our top ranked model (Figure 4).

## DISCUSSION

Aside from movements related to breeding activity, amphibians move within their home ranges in search for food or proper humidity and temperature conditions (Sinsch 1990; Lemckert 2004). Movements for amphibians involve energetic costs, exposure to predation, and exposure to drought or other unfavorable conditions (Wells 2007). Our results showed higher activity during spring and higher sheltering behavior during autumn. Though some non-breeding activity was previously recorded in autumn for this species (Pereira and Maneyro 2016a), this would indicate that *M. montevidensis* has a seasonal activity pattern. This pattern has already been observed for other bufonid species, even from the genus *Melanophryniscus* (Sinsch 1990; Carvajalino-Fernández *et al.* 2013).

Best model fitted supported a seasonal dependence in survival showing a slight decrease from autumn to winter and a remarkable decrease from spring to summer (especially for juveniles), as survival rates were calculated between seasons sampled (Cooch and White 2015). The cycle of activity-sheltering of this species would explain a higher exposure to mortality during spring. Seasonal survival patterns were reported for *Boana albopunctata* and were related to high predatory incidence during wet seasons (Guimarães *et al.* 2011). For *M. montevidensis*, considering predation as a potential mortality pressure would be arguably, as this species possesses typical features of an aposematic species: conspicuous coloration, defensive behavior (unkenreflex), and toxins in the skin (Kwet *et al.* 2005; Mebs *et al.* 2005). However, the incidence of road kills should be studied in this population, since the study area is located in a touristic part of the reserve, and the non-paved road (close to sampled ponds, Figure 1) is frequently transited by the cars of tourists and locals during warm seasons. Car kills were observed during our study and have been already reported as a problem for other *Melanophryniscus* species in the proximity of ponds (Cairo and Zalba 2007). Another potential threat for this species is the presence of the frog-killing fungus *Batrachochytrium dendrobatidis* in the study area (Borteiro *et al.* 2009). Although no reports are available for this species, a prevalence of 35.5 % was recently reported in terrestrial stages of *Melanophryniscus* sp. (aff. *montevidensis*; Agostini *et al.* 2015). Studies on the seasonality in the prevalence of this infection in amphibians indicate that cool seasons have the highest prevalence and mortality rates (Berger *et al.* 2004; Kriger and Hero 2007). The incidence of this disease would explain the cold-season decrease of the survival rates. However, studies on the prevalence and seasonality of Chytridiomycosis should be achieved on *M. montevidensis* to test this hypothesis.

In spite of the conspicuous breeding activity of this species, no differences due to immediate breeding activity were evident neither in survival probabilities, nor in detection probabilities or abundances, since models including breeding activity were not ranked top. We observed only two breeding events (April 2013 and February 2014), thus, a direct effect would not be evident in our models. Most reports of breeding events of the species were on warm seasons, and no breeding events were reported during winter (Pereira and Maneyro 2016a,b; current study). Although breeding activity of *M. montevidensis* is triggered by precipitation (Maneyro and Carreira 2012), it is possible that extreme cold (or physiological) conditions inhibits breeding activity during winter, as in another bufonids (Canavero *et al.* 2008; Pereyra *et al.* 2016). Considering this, the seasonal survival patterns reported would include the indirect effect of breeding activity over the survival probabilities.

Accounting for detection probabilities is important for amphibian studies to reduce bias in survival probabilities and abundances, because counting during punctual peaks of activity, such as breeding activity, may obscure the true number of animals in the study area (Schmidt 2003). Our results indicate that capture probabilities did not differ among age or sex groups, since the best temporal structure for this parameter was varied across seasons but not among states. Thus, using our field methods, no behavioral or microhabitat differential use was detected between age or sex groups. Low capture probabilities were possibly a result of the small body size of these toads, which reduces their detectability (Guimarães *et al.* 2011).

Abundance estimations followed a seasonal pattern, consistent with the seasonal structure of the survival probability of our top ranked model. Males were the most abundant group, but also the group with more drastic variations in abundance numbers (75 to 2632). These numbers should be taken cautiously, since the standard error of the estimations was high, which implies the sample size was small to make accurate abundance estimations (Cooch and White 2015). Despite, abundance levels of males and females agreed with the significantly male-biased sex ratio observed. Similarly to what Vasconcellos and Colli (2009) reported for other bufonids, there was no sexual variation in recaptures or survival estimates in our data, so the sex bias we reported was not related to these parameters. Considering the absence of sexual differences in recapture rates even during breeding events (which were particularly modeled), our results suggest that males in the population were more abundant than females, capture rates during field work were not sex-biased, and the sex-bias observed reflects the proportions in the population. This male-biased sex ratio has been repeatedly reported for bufonid populations (Zug and Zug 1979; Gittins 1983; Frétey *et al.* 2004; Vasconcellos and Colli 2009; Lampo *et al.* 2012). Given the sexual size dimorphism of *M. montevidensis*, one possible explanation to this sex-bias would be a delayed sexual maturity of females, a common phenomenon in species where females are the largest sex (Gittins 1983; Monnet and Cherry 2002). Though it has been observed that sex ratio in bufonids is not species-specific but determined by environmental variables encountered by each population (Zug and Zug 1979).

Low recapture rates of juveniles were according to low juvenile abundances, and high re-recapture rates to high survival rates of this stage. It must be considered that recently metamorphosed animals were not included in our analysis, as we did not captured juveniles smaller than 14 mm during our study, most juveniles were higher than 17 mm, while *M. montevidensis* toadlets have an average size of 5.7 mm in field conditions (Bardier *et al.* 2017; Capítulo I, III). Moreover, as the maximum body size reached by this species during the first year was 13.2 mm (Bardier *et al.*, 2017), we think that one-year animals were not present in the habitats sampled or were not detected using our methods. For this reason, we would not consider peaks in juvenile abundances (April 2013 and October 2013) as peaks of recruitment in the population. Populational dynamics of these juveniles would resemble those of adults, so they can be considered as non-breeder subadults.

Transition probabilities suggest that it was more likely for a juvenile maturate and become an adult male, than to maturate as an adult female. This indicates that male-sex bias is already present in the juvenile part of the population. Two explanations can be cited for this phenomenon. On one hand, it is possible for a sex-biased mortality to be occurring before sexual maturity (*i.e.*, at embryonic, larval or juvenile stages). Although no reports are available for amphibians, several examples can be cited for reptiles and birds (*e.g.*, Burger and Zappalorti 1988; Eiby *et al.* 2008). On the other hand, there is a possibility that phenotypic sex determination has a bias for males in *M. montevidensis*. Several reports account for sex reversal phenomena in amphibians (when epigenetic factors override genotypic sex determination), which can bias the sex ratio at metamorphosis, by altering water quality or temperature conditions (Eggert 2004). Though most of these reports come from experimental venues, studies on genotypic and phenotypic sex determination in *M. montevidensis* should be achieved, as the female component of the population is critical for the growth rate (Caswell 1989; Williams *et al.* 2002) and they would help detecting environmental factors that contribute to declines at a long term.

Finally, vital rates are useful to detect problems arising from habitat alteration because they specifically identify demographic processes responsible for changes in local populations (*i.e.*, survival, reproduction, or migration), providing planners with explicit targets for conservation management (Todd and Rothermel 2006). In our case, though no clear declining trend was observed along three years of study, decreased survivorship during warm seasons was concerning since many population studies documented the adult female survivorship as the most important vital rate for population growth (Homyack and Haas 2009; Unger *et al.* 2013; Kissel *et al.* 2014), even in a *Melanophryniscus* species (Cairo 2009). The long-term impact of a decreased survivorship of females, mainly in the context of the male-biased sex ratio, would be determinant for the extinction probabilities. Studies addressing the causes of this seasonal pattern (*i.e.*, road kills, Chytridiomycosis or others) should be prioritized. The design of conservation strategies focused on mitigating this temporal decreased survivorship should also be prioritized, although population studies assessing the relative importance of all vital rates (survivorship of aquatic and terrestrial stages and fecundities) over the population growth (such as matrix models) should be applied first to improve the accuracy of the conservation targets.

# TABLES IV

**Table 1**. General description of the data set on sex, age and recaptures during primary sampling periods (*i.e.*, seasons, daily recaptures are not shown). In the Captured group we included only the first capture of each individual, Recaptured are those animals that were captured twice at the end of the study, and Re-recaptured are the animals captured more than twice (recaptures within seasons, *i.e.*, on two consecutive days, were not considered).

| | Male | Female | Juvenile | Total |
|---|---|---|---|---|
| Captured | 267 | 102 | 32 | 401 |
| Recaptured | 19 | 11 | 9 | 39 |
| Re-recaptured | 1 | 1 | 6 | 8 |
| Total | 287 | 114 | 47 | 448 |

**Table 2**. Candidate models of MSCRD for seasonal apparent survival probability (*S*), transition probability (Ψ), and capture probability (*p*) of *Melanophryniscus montevidensis*. AICc = Akaike's information criterion for small samples (lower values meaning better model fit); WAICc = weight of fit for each model; *K* = number of estimated parameters. Subscripts: (state*season), state (females, male, juvenile) and seasonal (spring, summer, autumn, winter) variation; (t), time variation; (state), state variation only; (age), age variation (juvenile, adults [male and female together]); (state*breeding) state and breeding and non-breeding events variation; and (.), the parameter was held constant.

| Model | AICc | WAICc | *K* | Deviance |
|---|---|---|---|---|
| *S*(state*season)Ψ(state)*p*(t) | 1415.99 | 0.91 | 17 | 1379.79 |
| *S*(state)Ψ(state)*p*(t) | 1420.38 | 0.06 | 14 | 1391.56 |
| *S*(age)Ψ(state)*p*(t) | 1423.00 | 0.02 | 13 | 1396.29 |
| *S*(state*breeding)Ψ(state)*p*(t) | 1423.71 | 0.01 | 16 | 1390,65 |
| *S*(.)Ψ(state)*p*(t) | 1430.05 | 0.00 | 13 | 1403.34 |
| *S*(.)Ψ(.)*p*(t) | 1430.60 | 0.00 | 12 | 1406.00 |

# TABLES IV

**Table 3**. Median, mean and standard deviations (SD) of averaged seasonal estimations of apparent survival probability (*S*), transition probability (Ψ), capture probability (*p*), and abundances (*N*) of *Melanophryniscus montevidensis*. Sub indexes refer to states (life stages): juveniles (J), adult females (F) and adult males (M). Asterisks indicate normally distributed parameters.

| Parameter | Median | Mean | SD |
|---|---|---|---|
| $S_F$ | 0.98 | 0.80 | 0.23 |
| $S_M$* | 0.87 | 0.72 | 0.25 |
| $S_J$ | 0.99 | 0.80 | 0.40 |
| $\Psi_{JF}$* | 0.19 | 0.19 | 0.09 |
| $\Psi_{JM}$* | 0.55 | 0.55 | 0.13 |
| $p$ | 0.04 | 0.06 | 0.04 |
| $N_F$* | 125 | 190 | 163.44 |
| $N_M$ | 179 | 633 | 859.44 |
| $N_J$ | 2 | 62 | 97.72 |

# FIGURES IV

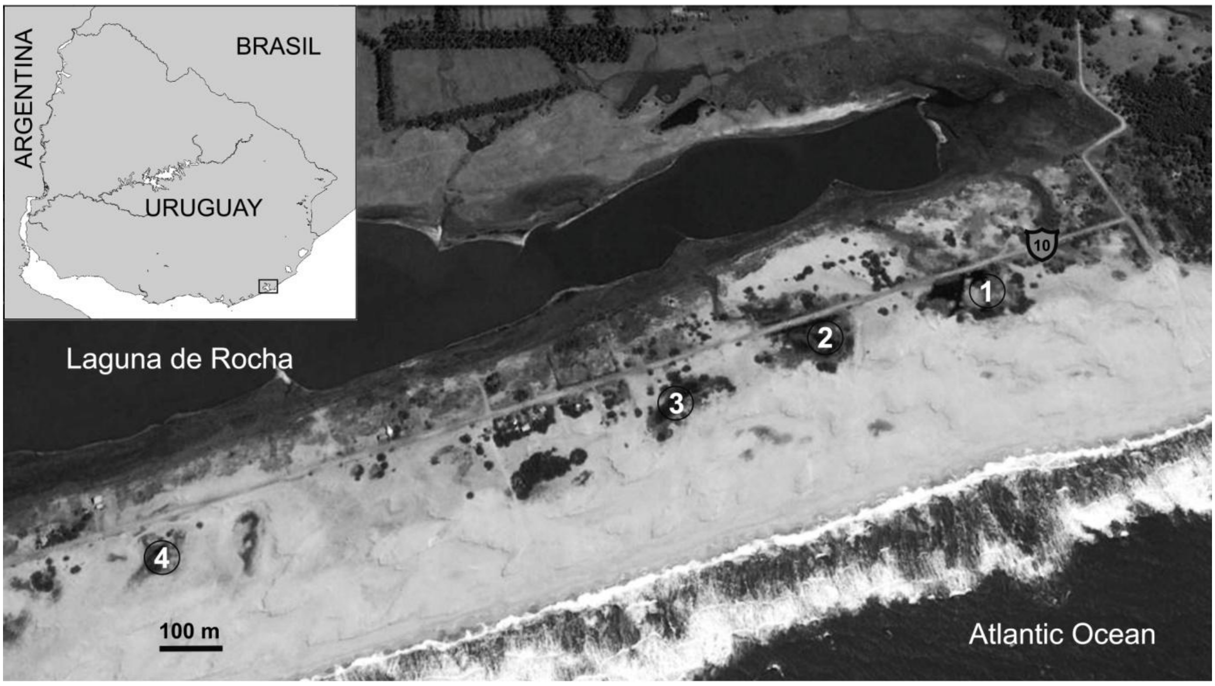


**Figure 1**. Location of sampled ponds (white numbers), route marker indicates the number of the non-paved road. Aerial photo obtained from Google Earth (34°39'51" S; 54°13'31" W) on 16 May 2017.

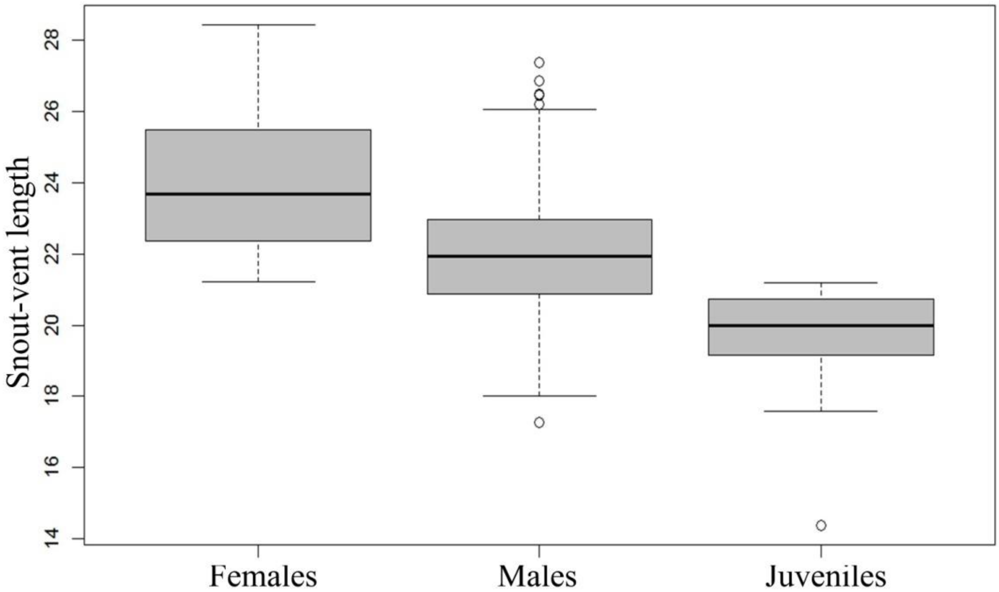


**Figure 2**. Snout-vent length (mm) box-plot of captured animals. Animals with vocal sac were regarded as adult males. Considering mean and standard deviation of adult (amplectant) females, 24.43 ± (SD) 1.61 mm, mean-2SD (21.21 mm) was the threshold size to separate juveniles from females.

# FIGURES IV

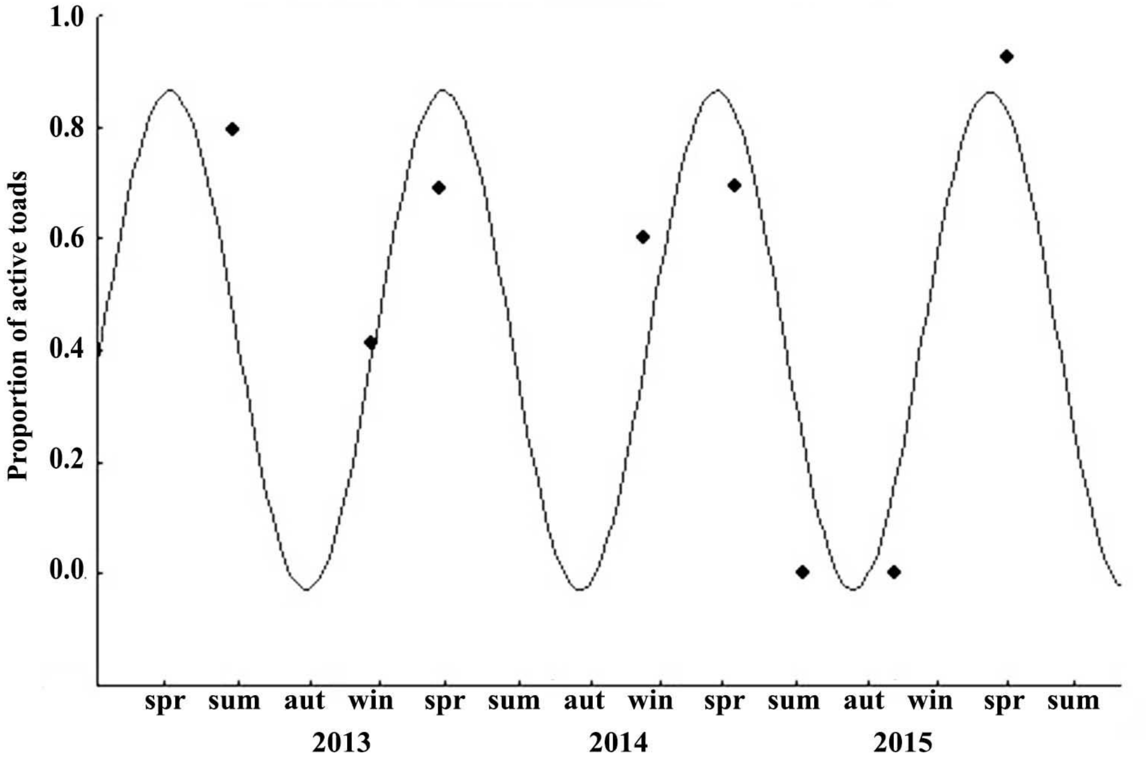


**Figure 3**. Sinusoidal fit of the proportion of active animals across seasons sampled. The formula fitted was P = Pmean + Pamp (sin [2π (M + c) /12]), where P is the proportion of animals (active or sheltered) at a given time M of the year, Pmean is the mean proportion of animals (active or sheltered) along the time series, Pamp is the maximum deviation from Pmean (amplitude of the function, seasonal oscillation of proportions), and c is a correction factor that synchronizes the sinusoidal function with proportion of (active or sheltered) animals. The function significantly explained 65 % of the variability of the data (Pamp *t*-value = 2.92, $P < 0.05$). Autumn 2014 and winter 2015 were not sampled; autumn 2013 and summer 2014 were not considered because breeding activity was reported on those months.

## FIGURES IV

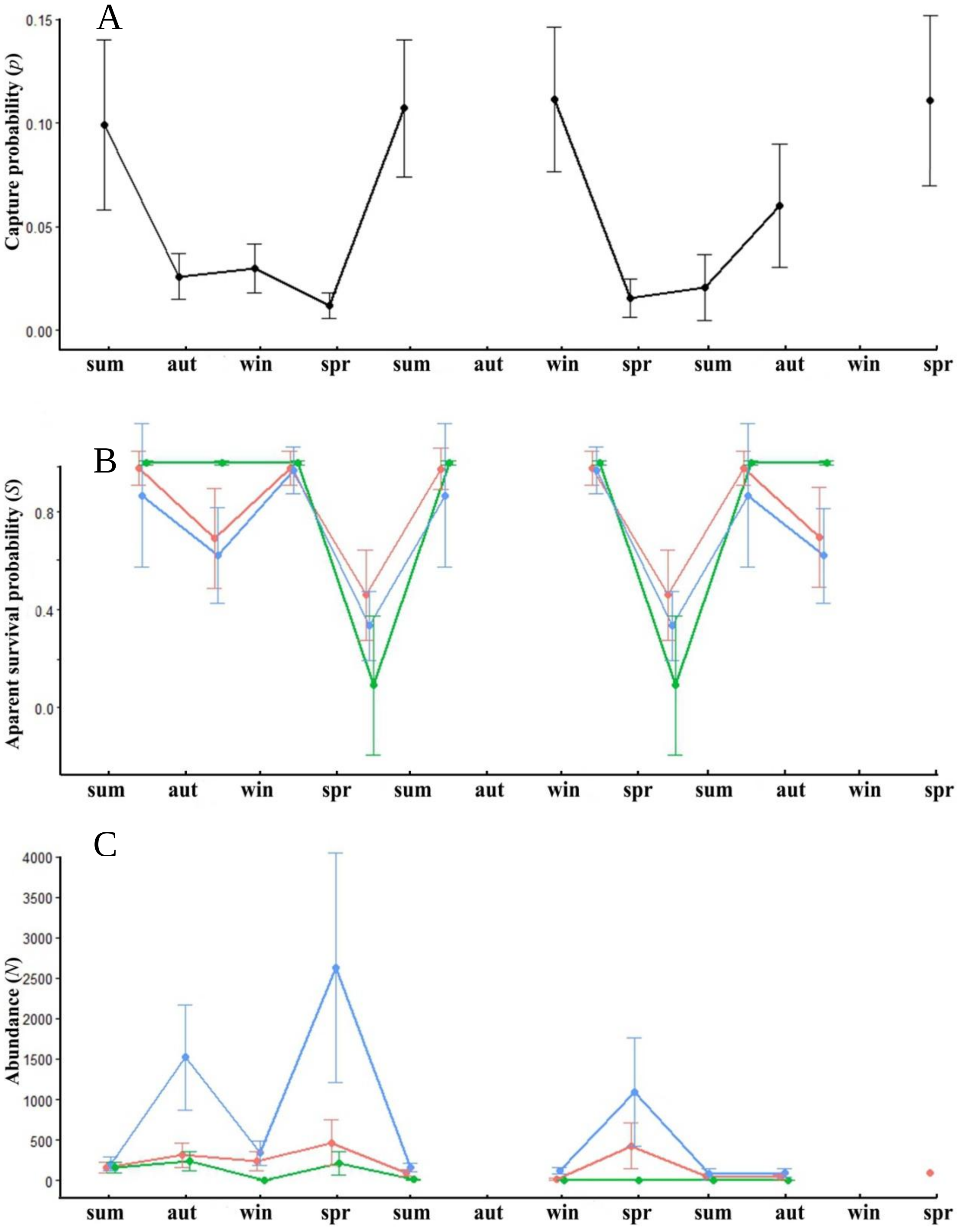


**Figure 4**. Seasonal capture probability (A), apparent survival rate (B), and abundance (C) estimates with standard errors of *Melanophryniscus montevidensis* across seasons sampled (from summer 2013 to spring 2015). Survival rates are calculated between primary periods (Cooch and White 2015). Red: females, blue: males, green: juveniles; capture probability (black) was not state-variant.

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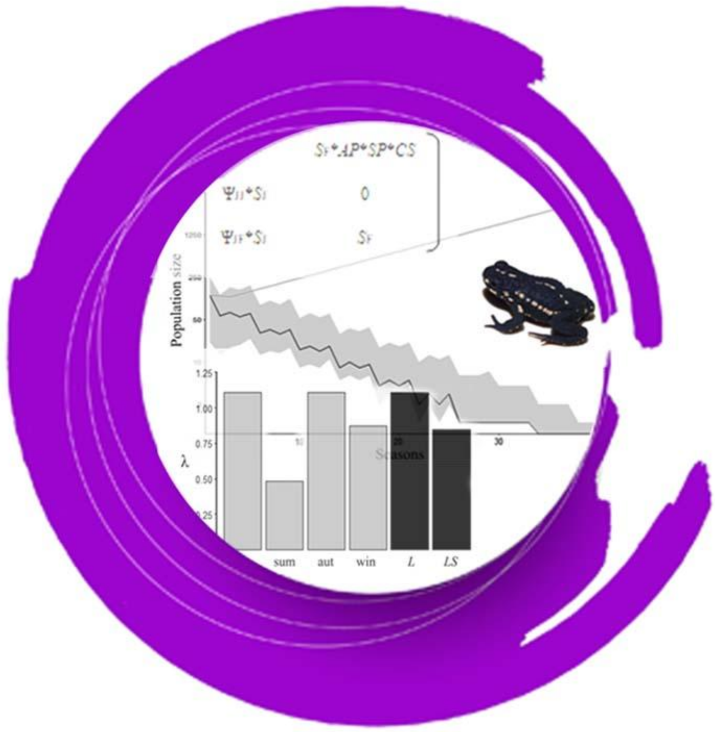
# Capítulo V

## Demographic projections and conservation strategies for the threatened bufonid *Melanophryniscus montevidensis* (Amphibia, Bufonidae)



# ABSTRACT-RESUMEN

**Abstract**. *Melanophryniscus montevidensis* is a Neotropical threatened amphibian. Quantitative information of life history aspects of this species is available from previous studies (presented in this work), allowing calculation of breeding rates of adults and survival rates of pre and post-metamorphic stages. We reviewed the information reported by these studies to construct female stage-structured Lefkovitch matrix models to determine population trends and probabilities of extinction in a protected area (Laguna de Rocha, Uruguay). We aimed to test if the seasonal decrease in adult female survival, reported in previous studies, increases the probability of extinction in the short term (10 years) by reducing season-specific population growth rates. Also, we applied these models to assess the effectiveness of habitat management and supplementation by captive breeding as conservation strategies. Our hypothesis was supported by the results as the population growth rate (λ) was mostly sensitive to female survival, λ declined on seasons where female survival declined, stochastic models with seasonal variation on female survival had a high probability of extinction in 10 years (> 90 %), and this high extinction probability was independent from the initial population size. Simulations of conservation strategies to revert this tendency indicated that habitat management during warm seasons would be the most effective practice. Habitat management reduced extinction probabilities to less than 50 % when population sizes ranged from 240 to 450 adult females and efforts were addressed to reduce at least 30% of adult female mortality. Given our results, conservation actions are needed in the short term to preserve the population of Laguna e Rocha, some practical recommendations are provided by this study.

**Resumen**. *Melanophryniscus montevidensis* es un anfibio Neotropical amenazado. La información cuantitativa de ciertos aspectos de la historia de vida de esta especie está disponible de estudios previos (expuestos en este trabajo), permitiendo el cálculo de las tasas de reproducción de adultos y las tasas de supervivencia en etapas pre y post-metamórficas. Se relevó la información reportada por estos estudios para construir modelos matriciales de Lefkovitch para determinar las tendencias poblacionales y las probabilidades de extinción en un área protegida (Laguna de Rocha, Uruguay). Nuestro objetivo fue comprobar si la disminución estacional en la supervivencia de las hembras adultas, reportada en estudios previos, aumenta la probabilidad de extinción a corto plazo (10 años) al reducir las tasas de crecimiento poblacionales durante estas estaciones. Además, se aplicaron estos modelos para evaluar la eficacia del manejo de hábitat y la suplementación por cría en cautividad como estrategias de conservación. Nuestra hipótesis fue apoyada por los resultados ya que la tasa de crecimiento de la población (λ) fue mayormente sensible a la supervivencia de las hembras, λ disminuyó en las estaciones donde la supervivencia de las hembras disminuyó, los modelos estocásticos con variación estacional en la supervivencia de las hembras tuvieron una alta probabilidad de extinción en 10 años (> 90%), y esta alta probabilidad de extinción fue independiente del tamaño inicial de la población. Las simulaciones de estrategias de conservación para revertir esta tendencia indicaron que el manejo de hábitat durante las estaciones cálidas sería la práctica más efectiva. El manejo de hábitat redujo las probabilidades de extinción a menos del 50% cuando el tamaño de la población estaba entre 240 y 450 hembras adultas y los esfuerzos fueron dirigidos a reducir al menos el 30% de la mortalidad de las hembras adultas. Teniendo en cuenta estos resultados, acciones de conservación son necesarias a corto plazo para preservar la población de Laguna e Rocha, algunas recomendaciones prácticas fueron proporcionadas por este estudio.

# INTRODUCTION

Anurans have complex life cycles, usually with aquatic larval stages and postmetamorphic terrestrial stages, which can be ecologically, physiologically, and morphologically as different as separated species (Werner 1986). This aspect of their life history makes them especially sensitive to habitat loss or alteration (Stuart *et al.* 2004; Bielby *et al.* 2008), and therefore a challenging group to develop effective conservation strategies (Dodd 2010). Studies in conservation biology tend to simplify this complexity to maximize the predictive and explanatory capacity from minimal information of threats and their effect over any given life stage (Biek *et al.* 2002; Conroy and Brook 2003; Todd *et al.* 2014). These simplifications are convenient since biological information for all vital rates (*i.e.*, survivorship of all life stages, maturity and fecundity rates of adults, *sensu* Caswell 1989) is only available for a few species (Werner 1988; Biek *et al.* 2002). Besides, conservation efforts in the current situation of contemporary amphibian declines must be achieved as soon as possible despite of incomplete biological information (Thorpe and Stanley 2011; Martin *et al.* 2012).

However, the quantitative link between reductions of any vital rate and reductions of the growth rate of a given amphibian population (which ultimately leads to declines), should not be neglected, because each threat may affect differently each life stage, and each stage has a different weight on population growth (Biek *et al.* 2002). The study of population ecology allows establishing this link through the application of quantitative models of population dynamics (Williams *et al.*, 2002). Particularly, stage-structured models, such as Lefkovich's matrices (Leftkovitch 1965; Caswell 1989), are best suited to integrally model population dynamics of amphibians given that the vital rates of aquatic and terrestrial stages can be modeled together. Studies in which matrix models were applied agree in that terrestrial phases are usually more important for population growth than aquatic stages (Biek *et al.* 2002). Among terrestrial vital rates, survival of reproductive females was repeatedly documented as the most important one for population growth (Cairo 2009; Homyack and Haas 2009; Unger *et al.* 2013; Kissel *et al.* 2014), with some exceptions when survival at juvenile stages is critical (Biek *et al.* 2002; Pickett *et al.* 2015). This could be related with a general pattern of life history strategies common to amphibians, as in mammals (Heppell *et al.* 2000).

Matrix models have relevant applications in species-specific conservation biology, such as determining the asymptotic population growth rate (λ), and estimating the relative importance of each vital rate over λ through sensitivity analysis (Caswell 1989; Heppell et al 2000). Whenever abundance records and standard deviations of vital rates are available, the population growth can be projected to estimate stochastic probabilities of extinction (Boyce 1992; McGowan *et al.* 2011; Morris and Doak 2002), or even simulate potential effects of conservation strategies over persistence of the population (Conroy and Brook 2003; Harper *et al.* 2008; Doak *et al.* 2014; Kissel *et al.* 2014). Few studies assessed the potential outcomes of different conservation strategies, such as captive breeding, head starting breeding (removal of individuals from the wild and rear them in captivity during life history stages with suspected low survival in the wild), translocations or habitat management (Dodd and Seigel 1991; Fischer and Lindenmayer 2000; Kissel *et al.* 2014). This is an important application of matrix models which can easily simulate different conservation scenarios to assist decision-makers about whether to invest conservation efforts in one strategy or another (Kissel *et al.* 2014).

*Melanophryniscus montevidensis* (Philippi, 1902) is a Neotropical bufonid that occupied the coastal region of the Río de la Plata and the Atlantic Ocean of Uruguay, and the Southern coast of Rio Grande do Sul (Brazil) in recent times (Bernardo-Silva 2012). Most southwestern populations have disappeared, reason why the population trend was established as decreasing and was categorized as *Vulnerable* in the global list of threatened species (IUCN 2017). It has been recently categorized as *Critically Endangered* in the Uruguayan redlist; and as *Near Threatened* and *Endangered* in Brazilian national and state-level lists, respectively (Fundação Zoobotânica, 2014; ICMBio, 2014; Carreira and Maneyro, 2015). At a large scale, habitat loss in coastal regions has been considered the main threat for this species (Langone *et al.* 2004; IUCN 2017) as for other species (Kolenc et al. 2009). Though, local scale threats remain unknown, which are more likely to explain declining trends than large scale threats (Grant *et al.* 2016). Additionally, it was predicted that future climate change may lead to a loss of proper environmental conditions for this species in the next half of the century (Toranza and Maneyro 2013; Zank *et al.* 2014). These studies suggest that the knowledge about the population dynamics of this species should be improved and *in situ* and *ex situ* conservation actions must be implemented.

Some quantitative life history aspects of this species were studied in Laguna de Rocha, a protected area of Uruguay (Pereira 2014; Bardier *et al.* 2017; Capítulo I, IV). These studies allow the calculation of breeding rates of adults and survival rates of pre and post-metamorphic stages. We used quantitative information reported by these studies to construct deterministic and stochastic simple stage-structured Lefkovitch matrix models to determine the population trend and probability of extinction of *M. montevidensis* at Laguna de Rocha (Uruguay). We used threshold criteria of an extinction probability of 50% over 10 years to determine the severity of the probability of extinction. This is the threshold criteria for taxa projected to experience a significant reduction in the near future (criterion A3 for species categorized as Endangered by IUCN, IUCN Standards and Petitions Subcommittee, 2016).

Survival of adults and juveniles showed drastic decrease mainly during spring-summer transitions, possibly as a result of local threats (Capítulo IV). Because terrestrial vital rates, especially adult female survival, are usually the most significant factors influencing the growth of amphibian populations, we hypothesized that the seasonal decrease in adult female survival increases the probability of extinction by reducing season-specific growth rates. To test this hypothesis, we predicted that 1) $\lambda$ is mostly sensitive to female survival; 2) population declines ($\lambda < 1$) in seasons where female survival declines; 3) stochastic models with seasonal variation of female survival have a high probability of extinction in the short term (10 years); and 4) for the range of population sizes observed in the field, this high probability of extinction is independent from the initial population size.

Vital rates are useful to detect problems arising from habitat alteration because they specifically identify demographic processes responsible for changes in local populations (*i.e.*, survival, reproduction, or migration), providing decision-makers with explicit targets for conservation management (Todd and Rothermel 2006). For amphibians, habitat management is usually recommended as a conservation strategy (Semlitsch 2002; Todd and Rothermel 2006; Dodd 2010), though few successful cases are documented (Rannap *et al.* 2009). Another approach for the conservation of imperiled species is supplementing wild populations with animals from a captive population (Dodd 2010). This *ex situ* approach is a high-technology-based option with few successful experiences as well (Griffiths and Pavajeau 2008). The effectiveness of captive breeding supplementation, however, may improve depending on the strategy adopted (*i.e.*, the vital stage supplemented or the number of animals supplemented) and population size (Kissel *et al.* 2014). We assessed the effectiveness of both types of management strategies: habitat management and supplementation by captive breeding, to determine which would draw the greatest reduction in decadal extinction probabilities with minimum effort.

## METHODOLOGY

### Wild population trends and hypothesis testing

*Deterministic matrix analysis*

We constructed an initial deterministic female-based, post-birth pulse Lefkovitch matrix *L* (Caswell 1989) seasonal scaled, representing a population with a life history of three stages: pre-juvenile (from egg to metamorph), juvenile (non-breeding terrestrial phase, from metamorph to adult), and reproductive adult (Figure 1). Parameters of this matrix were vital rates obtained in previous studies of the species for metamorphosing success and breeding rates (Pereira 2014; Bardier *et al.*, 2017; Capítulo I [Table 1]), and survival and transition rates of the terrestrial stages (Capítulo IV [Table 1]). As most part of these parameters were estimated for the population of the stable sand bar of Laguna de Rocha, a protected area of the national system of protected areas (Departamento de Rocha, Uruguay, 34°40'02"S, 54°14'00"W), we regarded this population as our wild modeled population. This population included the flooding plain of the most important and connected four ponds circumscribed to the narrow sand bar between the lagoon and the Atlantic coast (Capítulo IV).

We calculated the first eigenvalue of matrix *L*, which represents the asymptotic population growth $\lambda$ of the population, which grows at a stable rate when $\lambda > 1$, is stable when $\lambda = 1$ and decreases at a stable rate when $\lambda < 1$. We also calculated the intrinsic rate (%) of population increase as $r$ ($r = \ln(\lambda)$), population grows when $r > 0$, remains stable when $r = 0$ and decreases when $r < 0$ (Heppell *et al.* 2000). To determine if $\lambda$ is mostly sensitive to female survival (prediction 1), we calculated the standardized sensibilities (elasticities), which are the relative contribution of each vital rate over the growth rate $\lambda$ (Caswell 1989).

To test if the population declines in seasons where based on adult female survival declines (prediction 2), we assessed the effect of season-specific changes of survivorship over λ. For this, we modified parameters $S_F$ (female survival) and $S_J$ (female juvenile survival) of matrix *L* to include averaged survivals calculated for each season in Capítulo IV. Survival rates were estimated for the transitions between seasons (Capítulo IV), but were considered as the survival at the end of the transition (*e.g.*, autumn-winter survival was considered winter survival), to simplify matrix parameterization. In this sense, $S_F$ took the alternative values: autumn = 0.98, winter = 0.69, spring = 0.98, summer = 0.46, and $S_J$ took the values: autumn = 0.50, winter = 0.50, spring = 0.50, summer = 0.05 (Capítulo IV). We calculated λ and elasticities for each of these season-specific matrices, and compared our results.

A population vector *N* was also constructed, containing three abundances: pre-juvenile, juvenile and adult. The parameters of this vector were median abundances of the population obtained from the post-metamorphic survival study (Capítulo IV), and from previous studies on the species for the pre-juvenile state from Capítulo I (Table 1). We used *N* as initial abundances (calculated on a seasonal scale), to project matrix *L* to the future, using the $N_{t+1}=L\times N_t$ iteration formula, being *N* the abundances in the $t = 0$ season (Caswell 1989; Williams *et al.* 2002). All projections were set to 10 years (40 seasons), in order to follow IUCN criteria (IUCN Standards and Petitions Subcommittee 2016).

*Stochastic matrix analysis*

We constructed a stochastic version of matrix *L*, matrix *LS*, which incorporated uncertainty functions for some of the parameters of matrix *L* (McGowan *et al.* 2011) for short term projections (10 years = 40 seasons). For the wild population matrix *LS* we used a normal distribution *norm*(*m,s*) to randomly generate values of *CS* (clutch size), using ((157 + 135)/2)/2 as *m* (mean clutch size of females divided by two assuming 1:1 sex ratio, see Table 1) and ((73.3 + 50.5)/2)2 as *s* (standard deviations from Pereira 2014 and Capítulo I) (Legendre and Clobert 1995; McGowan *et al.* 2011). For survival and transition rates $S_P$, $S_F$, $S_J$, $\Psi_{JJ}$ and $\Psi_{JF}$, we used a beta distribution *beta*(*a,b*) to randomly generate values of these parameters. This distribution is bounded between zero and one and the shape of the distribution can be modified to meet the specific needs of the problem at hand (McGowan *et al.* 2011). For this distribution, parameters *a* and *b* were calculated using mean (*m*) and standard deviation (*s*) following Kruschke (2014), where $m = a/(a + b)$ and $s = m$ (1- mean)/($a + b$ +1). For pre-juvenile survival $S_P$ (survival from egg to metamorph), we considered the deterministic value of $S_P$ (0.024, Table1) as the mean (*m*) value of a beta distribution, and a 75% of this value as the standard deviation (*s*), to represent extreme values (as one of the ponds had survival = 0, Capítulo I-II). For adult and juvenile survival $S_F$, $S_J$, we simulated the seasonal shift of survival rates for 10-year-stochastic projections. We constructed a beta distribution for each season, parameters *a* and *b* were calculated using averaged survivals calculated for each season in Capítulo IV as mean (*m*), and fixed 0.05 as standard deviation (*s*), because of the small variation among same season values. We constructed a vector using the sequence of seasonal survivorships beta-distributed, repeating this sequence 10 times. For each seasonal iteration, those 40 values were taken sequentially. In the case of transition rates $\Psi_{JJ}$ and $\Psi_{JF}$, parameters *a* and *b* were calculated using the mean (*m*) and standard deviation (*s*) of transition rates from Capítulo IV (Table 1).

To estimate stochastic λ and sensitivities, applying a Monte Carlo procedure to stochastic-variant parameters in *LS* matrix (Morris and Doak 2002; Stubben *et al.* 2016), we run 1000 simulations of 10 years (40 seasons) projections departing from *N*. In order to test if there is a high extinction probability of the wild population in the short term, and also its independence from population size (predictions 3 and 4), we run 100 times 1000 simulations of 10 year projections of matrix *LS* for different population sizes. For this, we departed from a vector *N* with different values of adult female abundances $N_F$ (range = 30 – 450, following average values estimated in Capítulo IV). Juvenile ($N_J$) and pre-juvenile ($N_P$) abundances were fixed to 0 to standardize results, as in other studies (Kissel *et al.* 2014). All simulations started on autumn-winter transition. To calculate extinction probabilities, we used a quasi-extinction threshold of 10 adult females, as already applied for other bufonid species (Willson *et al.* 2012). Matrix analyses were conducted using built-in functions of the statistical software R (R Foundation for Statistical Computing, Vienna, Austria), stochastic functions of the R package popbio (Stubben *et al.* 2016), and graphic functions of the R package ggplot2 (Wickham and Chang 2016).

### Assessment of conservation strategies

Based on matrix *LS*, we simulated two management scenarios for *Melanophryniscus montevidensis*: 1) habitat management, and 2) supplementation by captive breeding. For scenario 1, we simulated the effects of habitat management strategies on the increase of survival rates of the wild population during spring-summer periods. Two different scenarios were assessed for habitat management: survival increments of female survival (1A) and survival increments in both female and juvenile survival (1B). Three effort levels were tested for both subset scenarios: survival increments of 0.15 (low), 0.3 (medium) and 0.45 (high) above the reported 0.46 and 0.05 spring-summer survival rate of adult and juvenile females respectively (Table 2).

For scenario 2, we simulated the results of supplementing animals from a captive breeding population of adults on the wild population (Kissel *et al.* 2014). Subset scenarios were: supplementation of newly metamorphosed individuals (2A), and supplementation of two-year old juveniles (2B). Similarly to the effort levels applied in previous supplementation assessments (Kissel *et al.* 2014), we tested for effort levels of: 10 (low), 30 (medium) and 60 (high) females from an *ex situ* breeding population (Table 2). These effort levels were previously applied for other amphibian supplementation assessments (Kissel *et al.* 2014). To model scenario 2A, as captive female population and breeding would be controlled, we assumed captive female survival probability ($CS_F$), captive amplectant probability (*CAP*) and captive spawning probability (*CSP*) to be all 100%, being clutch size (*CS*) the only stochastic variable with a normal distribution *norm*(*m*,*s*) as described for matrix *LS*. We assumed the survival probability from egg to metamorph ($CS_P$) of 0.11, survivorship reported in captivity by Cairo (2009) for *Melanophryniscus* sp. (aff. *montevidensis*; divided by two assuming 1:1 sex ratio). The number of female metamorphs to be supplemented by captive female breeder was calculated as $CS_F*CAP*CSP*CS*CS_P$. In the case of scenario 2B, we made analogous assumptions to scenario 2A, plus assuming annual survival probability of juvenile females in captivity ($ACS_J$) to be at least 0.13, survival calculated by Biek *et al.* (2002) for wild populations of *Anaxyrus boreas* and applied in population models of *Melanophryniscus* sp. (aff. *montevidensis*) by Cairo (2009), divided by two assuming 1:1 sex ratio. Thus, the number of female juveniles to be supplemented by captive female breeder was calculated as $CS_F*CAP*CSP*CS*CS_P*ACS_J$. Using these supplementation formulas we constructed stochastic vectors of the number of animals to be supplemented under scenarios 2A and

2B (Table 2), which were added annually only on summer (the period with lowest terrestrial survival rate).

For all scenarios we estimated the 10-year extinction probability by stochastic simulations (using methods and software above mentioned). All scenarios were projected for three levels of initial female abundances: 30, 240 and 450, to compare the extinction probability under management scenarios with the extinction probabilities of the wild population (simulations of matrix *LS* projections). Effectiveness across scenarios, effort and abundances, was calculated as the decrease in extinction probability when compared to the not supplemented wild population *LS* (E = E*LS* – E*scenario*, herein effectiveness) (Kissel *et al.* 2014). We only considered effective those reductions of extinction probability above 50%.

## RESULTS

### Wild population trends and hypothesis testing

We estimated a population growth rate of λ = 1.11 when we analyzed the deterministic matrix *L*. This would represent an intrinsic rate of seasonal increase of 10% (Figure 2-3). However, we found a high vulnerability of the population during autumn-winter and spring-summer transitions when λ was calculated for each season-specific matrix, as the analysis showed λ < 1 in both cases (Figure 2). For all season-specific matrices, standardized sensibilities of each vital rate suggest that adult female survival has the heaviest effect over λ (50-88%, Table 3). Sensitivities of other parameters were below 10% for season-specific and *L* matrices, except for winter matrix, in which female fertility, female pre-juvenile survival rate and the probability of surviving and becoming adult female reached 13% each. These results agree with predictions 1 and 2 of our hypothesis.

Stochastic seasonal growth rate was λ = 0.85 when we run 1000 simulations of the wild population matrix *LS*. This represents a 16% of seasonal decrease of the population (Figure 3). Stochastic standardized sensibilities also support giving main importance to adult female survivorship (68%), followed by female fertility ($S_F$**AP***SP***CS*; 10%, Table 3). When the extinction probability was calculated departing from different initial adult female abundances, though it diminished, it was always above 90% (Figure 4). These results agree with predictions 3 and 4 of our hypothesis.

### Assessment of conservation strategies

Regardless the initial population size, management scenarios showed contrasting results (Figure 5). No effort level of supplementation by captive breeding showed effective reductions of the extinction probability. In regard to habitat management scenarios, only high efforts conducted to increase adult and juvenile female survival (1B) were effective for small sized populations (effectiveness = 84%). For medium sized populations, medium efforts in both scenarios 1A and 1B effectively reduced extinction probabilities (effectiveness 1A= 63%, 1B = 91%).  High efforts on both scenarios conducted to effective reductions (>90%) when population sizes were medium to high. Low efforts did not conduce to effective reductions of the extinction probabilities in any habitat management scenario.

## DISCUSSION

Our results supported that seasonal decrease in adult female survival increase the extinction probability of the wild population of *Melanophryniscus montevidensis* from Laguna de Rocha by reducing season-specific growth rates, which was our initial hypothesis. Although deterministic projections depicted a growing population, when we incorporated stochasticity into our model (*i.e.*, incorporation of seasonal variations of survival rates and other reported deviations of matrix parameters) the results were exactly the opposite, with a high extinction probability predicted for the next 10 years. Although stochastic models provide more realistic results and allow estimations of extinction probabilities (Boyce 1992), the use of an adequate unique value for survival rate of juvenile and adult females respectively is arguably for this population, because of the non-normal highly season-variant estimations of these parameters in previous studies (Capítulo IV).

Growth rates of deterministic and stochastic matrices were mostly sensitive to adult female survival. This result suggests that slight reductions of adult female survival are likely to reduce population growth rate, which was particularly true on spring-summer and autumn-winter periods, when adult female survival rate decreased and population growth rate declined concomitantly. High standardized sensitivities of matrices to adult female survival rates have been largely reported in other amphibians (Homyack and Haas 2009; Unger *et al.* 2013; Kissel *et al.* 2014), including species of *Melanophryniscus* (Cairo 2009). As observed by Unger *et al.* (2013), the importance of adult female presence in the populations is consistent with other studies in long-lived species with high reliance on longevity rather than reproduction, contrary to the usual conception of amphibians as r-strategists (Pianka 1970; Jeschke *et al.* 2008).

In spite of the limitations of working with predictive models based only on the female part of the population (Rankin and Kokko 2007), we considered valid this approach given the highly male biased sex ratio reported in our wild population (Capítulo IV), which places the female fraction of this population as the limiting factor for reproduction. Male biased sex ratio was suggested to be present in early life stages, such as pre-juvenile or embryonic stages (Capítulo IV). However, to simplify estimations, we did not incorporate this unbalance into our matrix models (we assumed all juvenile and pre-juvenile sex ratios to be 1:1). Another caveat in our models is that juvenile survival was assumed to be equal from metamorphosis up to sexual maturity. As juveniles smaller than 14 mm were not collected, and most juveniles were higher than 17 mm, it is possible that survival of one-year-old juveniles was not assessed (Bardier *et al.* 2017; Capítulo IV). Survival rate of this early post-metamorphic stage (*e.g.*, one-year-old juveniles) is likely to be lower than older subadult juvenile stages (Zug and Zug 1979; Semlitsch 2008), as already estimated by other models (Cairo 2009; McCaffery and Maxell 2010). Given both the male-biased sex ratio (*i.e.*, less of 50% of metamorphs or juveniles are females) and the low survivorship of early juvenile stages, it would be expected that the incorporation of this information to further matrix models leads to even higher adult female survival sensitivities (Caswell 1989). Conversely, Cairo (2009) noticed that it is possible for the clutch size to be underestimated in the field, which would lead to higher growth rates (Cairo 2009) and maybe to heavier sensitivities of fertility rates (Caswell 1989). Pairs were already in amplexus when they were found (Pereira 2014; Capítulo I), so they would have spawned before and would still have mature eggs after spawning in a given sample (Prigioni and Garrido 1989). Studies towards a more precise estimation of these life history traits should be achieved to improve population studies on this species.

Declining trends with high extinction probabilities in matrix analysis were already reported for amphibian wild populations (Unger *et al.* 2013; Kissel *et al.* 2014), even in theoretic deterministic models constructed for *Melanophryniscus* sp. (aff. *montevidensis*; Cairo 2009). For our population, simulations incorporating seasonal variability of adult female survival predicted a high probability of extinction (>90%) over the next 10 years. Although the extinction probability for a wild population is a function of population size and rate of decline (Morris and Doak 2002), for the range of adult female abundances reported in Capítulo IV, the high extinction probability of *M. montevidensis* in Laguna de Rocha was not reverted by high initial population sizes. Again, the theoretic approach of our models is simplistic and depends on the accuracy of population parameters, more data on survival and fertility rates are needed to improve the estimations of extinction probabilities. Even so, these results are based on the current knowledge on the species and reinforce the concern about its conservation status. The need for management strategies is imperative since these populations may suffer demographic reductions before the loss of proper environmental conditions by climate change in the next half of the century (Toranza and Maneyro 2013; Zank *et al.* 2014).

The first conservation strategy proposed for *M. montevidensis* was the creation of protected areas (Langone *et al.* 2004). The National System of Protected Areas recently incorporated three areas where this species occurs: Laguna de Rocha, Cabo Polonio, and Cerro Verde (DINAMA 2010). However, as we showed, the simple introduction of a species into a protected area does not guarantee its medium-term survivorship. Therefore, constant intervention measures would be necessary to ensure the stability of threatened amphibian populations (Eagles *et al.*, 2002). Toranza and Maneyro (2013) and Zank *et al.* (2014) noticed the necessity for *in situ* and *ex situ* conservation to be implemented on these populations, given the imminent loss of suitable environmental conditions for this toad by climate change. Our assessments from matrix model simulations addressed the question of which type of conservation strategy would result in a greatest reduction of the extinction probability across different effort levels.

In this sense, for different initial population sizes, *in situ* habitat management strategies would lead to effective reductions of the extinction probability. In spite that no low-effort scenario leaded to effective results, medium to high-efforts addressed only to improve adult female survival (increments of 30%) during the spring-summer period effectively reduced the extinction probability of the current population. This was true at medium to high population sizes (as stated in methodology). In the case of small population sizes, only high efforts conducted to increase adult and juvenile female survival resulted effective. These results corroborate our initial hypothesis, indicating that it is possible the reliance only on adult female survival on the spring-summer period for effective conservation efforts. Population sizes should be estimated before deciding which conservation actions to accomplish (*e.g.*, by using quick capture-marking-recapture surveys to build Jolly-Seber POPAN formulations in MARK; Schwarz and Arnason 2015). Though, all efforts addressed to reduce at least 30% of adult female mortality on the spring-summer period would lead to successful prevention of the extinction probabilities.

To translate these results into practical conservation actions, we should determine the causes of population declines (*i.e.*, decreased adult female survival) during the spring-summer period. In Capítulo IV it was discussed the incidence of road kills in the nearby of the ponds as a potential threat. Touristic traffic during warm seasons is a geographically and temporally well-defined threat that would be prevented by building fences and tunnels in the road sides (see examples in Dodd 2010). Fences or walls prevent trespassing while they guide toad movements through a series of underpasses connecting to the other side of the road. This solution was also simulated for *Melanophryniscus* sp. (aff. *montevidensis*) with positive results on the growth rate (Cairo 2009). Funneling road crossings would reduce road kills not only for adult females in the population, but for moving subadult juveniles (scenario 1B) and even for other amphibians, reptiles, small mammals and terrestrial invertebrates. Additionally, as this species has high site fidelity during breeding and non breeding seasons (Pereira and Maneyro 2016), the protection of the breeding ponds, and the surrounding areas, must be prioritized. Protection of ponds while they are filled after heavy rains would reduce the mortality rates of practically all life stages: breeding adults, eggs, larvae, and early juveniles (Semlitsch 2008). However, even though they have no water, floodplains where these ponds are formed are the natural habitats of this species and where most part of the juvenile and adult movements occur (Pereira and Maneyro 2016; Capítulo IV). As they are more active during warm seasons (Pereira and Maneyro 2016; Capítulo

IV), efforts focused on the protection of these habitats at least during this part of the year would lead to significant reductions in the extinction probabilities.

Supplementation efforts by *ex situ* breeding predicted positive results for other species (Cairo *et al.* 2009; Kissel *et al.* 2014), though few successful cases were actually documented for amphibians (Griffiths and Pavajeau 2008). In our case, no scenario built using this approach reduced the extinction probability of *M. montevidensis*. On one hand, this is possibly because we simulated the supplementation of metamorphs and juveniles, while the growth rate of our population was more sensitive to adult female survival. *Ex situ* breeding programs are usually expensive options, which require adequate infrastructure, implementation of rigorous protocols that embrace acclimation, pre- and post-release training, health screening, genetic management, long-term monitoring, and involvement of local stakeholders (Griffiths and Pavajeau 2008 and references therein). Considering these costs, we regarded as highly striven the maintenance of an adult population to supplement metamorphs and juveniles. Therefore, their maintenance until sexual maturity for the supplementation of adult females would be an even more costly solution, as they possibly attain sexual maturity on the third year (Jeckel *et al.* 2015). On the other hand, underestimation of clutch sizes would have also played a role on the ineffectiveness of supplementation essays, since the amount of metamorphs and juveniles to be supplemented in nature may be greater and so, have a greater impact on the population growth rate.

Finally, it must be highlighted that most part of the vital rates employed in this study were estimated in previous *in situ* studies achieved in the same area for the target population, which supports the applicability of our results to the population from Barra de Laguna de Rocha. These results strongly suggested that conservation measures are needed for the persistence of this population in particular, and provided some guidelines with explicit targets for conservation management. Moreover, matrix parameters calculated for our target population can be easily adapted to matrix models of other populations of *M. montevidensis*, even for other congeneric or ecologically similar species (Heppell *et al.* 2000). To our knowledge, previous to our study only Cairo (2009) modeled a South American amphibian species. Therefore, we encourage herpetologists to assess the conservation status and the effectiveness of different conservation strategies using matrix models, to improve the knowledge of local populations and help establishing conservation priorities.

# TABLES V

**Table 1**. Seasonal-scale vital rates of *Melanophryniscus montevidensis* used to parameterize deterministic projection matrix *L* and population vector *N*. $S_F$, female survival rate; *AP*, female amplecting probability; *SP*, spawning probability of an amplectant female; *CS*, female clutch size; $S_P$, female pre-juvenile survival rate (survival rate from egg to metamorph); $\Psi_{JF}$, probability of a juvenile female become adult; $\Psi_{JJ}$, probability of a juvenile female remain juvenile; $S_J$, female juvenile survival rate; $N_P$, female pre-juvenile abundance; $N_J$, female juvenile abundance; $N_F$, adult female abundance.

| Vital rate | Source | Value |
|---|---|---|
| $S_F$ | Median of averaged values calculated using software MARK (Capítulo IV) | 0.976 |
| *AP* | Based on 59 amplectant females of 75 found in breeding activity (Pereira, 2014), and six amplectant females of eight found in breeding activity in the same area (Capítulo IV). Calculated as (59+6)/(75+8) | 0.783 |
| *SP* | Based on 44 spawning females of 59 amplexus (Pereira, 2014), and 25 spawning females of 28 amplexus (Capítulo I). Calculated as (44+25)/(59+28) | 0.793 |
| *CS* | Averaged results from Pereira (2014) and Capítulo I. Calculated as (157+135)/2, divided by two assuming 1:1 sex ratio | 73.00 |
| $S_P$ | 100 metamorphs obtained from 2088 eggs (Capítulo I), divided by two assuming 1:1 sex ratio | 0.024 |
| $\Psi_{JF}$ | Median of averaged values calculated using software MARK (Capítulo IV) | 0.193 |
| $\Psi_{JJ}$ | Calculated as 1- $\Psi_{JF}$ (Cooch and White 2015) | 0.807 |
| $S_J$ | Median of averaged values calculated using software MARK (Capítulo IV). Divided by two assuming 1:1 sex ratio | 0.500 |
| $N_P$ | Assuming 1:1 sex ratio: 50 female metamorphs (Capítulo I), divided by two, to express results per pond, and multiplied by four, to adjust the results to the sampling area of four ponds | 100 |
| $N_J$ | Median of averaged juvenile abundance values calculated using software MARK (Capítulo IV). Divided by two assuming 1:1 sex ratio | 1 |
| $N_F$ | Median of adult female averaged abundance values calculated using software MARK (Capítulo IV) | 125 |

# TABLES V

**Table 2**. Comparison of effort levels for survival increase by habitat management (scenario 1) and supplementation by captive breeding (scenario 2), with respective subset scenarios A and B.

| | Survival increase by habitat management | | Number of captive breeding females, simulated supplementation output (SD) | |
|---|---|---|---|---|
| Effort level | 1A | 1B | 2A | 2B |
| Low | 0.15 summer $S_F$ | 0.15 summer $S_F$ and $S_J$ | 10 females, 78 (11) female metamorphs | 10 females, 10 (1) female juveniles |
| Medium | 0.30 summer $S_F$ | 0.30 summer $S_F$ and $S_J$ | 30 females, 238 (17) female metamorphs | 30 females, 31 (2) female juveniles |
| High | 0.45 summer $S_F$ | 0.45 summer $S_F$ and $S_J$ | 60 females, 473 (27) female metamorphs | 60 females, 62(3) female juveniles |

**Table 3**. Standardized sensitivities (elasticities) of λ to different vital rates. Comparisons among season-specific deterministic matrices (estimated using corresponding season-specific values of $S_F$ and $S_J$ from Capítulo IV), deterministic matrix *L* (estimated using median values of $S_F$ and $S_J$), and stochastic matrix *LS* (estimated by simulations using a vector with the sequence of seasonal survivorships $S_F$ and $S_J$ beta-distributed). $S_F*AP*SP*CS$ represents female fertility (actual reproductive performance *sensu* Caswell 1989), $S_P$ is female pre-juvenile survival rate; $\Psi_{JJ}*S_J$ represents the probability of surviving and remaining as juvenile; and $\Psi_{JF}*S_J$ represents the probability of surviving and becoming adult female. Highest elasticities for each matrix are in bold.

| Parameter | Spring | Summer | Autumn | Winter | *L* | *LS* |
|---|---|---|---|---|---|---|
| $S_F*AP*SP*CS$ | 0.091 | 0.039 | 0.091 | 0.129 | 0.091 | 0.100 |
| $S_P$ | 0.091 | 0.039 | 0.091 | 0.129 | 0.091 | 0.092 |
| $\Psi_{JJ}*S_J$ | 0.052 | 0.003 | 0.052 | 0.111 | 0.052 | 0.039 |
| $\Psi_{JF}*S_J$ | 0.091 | 0.039 | 0.091 | 0.129 | 0.091 | 0.092 |
| $S_F$ | **0.677** | **0.880** | **0.676** | **0.502** | **0.675** | **0.677** |

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$$\boldsymbol{L} = \begin{pmatrix} 0 & 0 & S_F{*}AP{*}SP{*}CS \\ S_P & \Psi_{JJ}{*}S_J & 0 \\ 0 & \Psi_{JF}{*}S_J & S_F \end{pmatrix}$$

**Figure 1**. Female-based, seasonal projection matrix *L* for *Melanophryniscus montevidensis*. $S_F$**AP***SP***CS* represents female fertility (actual reproductive performance *sensu* Caswell 1989), where $S_F$ is female survival rate; *AP* is female amplecting probability; *SP* is spawning probability of an amplectant female; and *CS* is female clutch size. $S_P$ is female pre-juvenile survival rate (survival rate from egg to metamorph). For juvenile females, $\Psi_{JJ}{*}S_J$ represents the probability of surviving and remaining as juvenile, where $\Psi_{JJ}$ is the probability of a juvenile female remain juvenile and $S_J$ is female juvenile survival rate. $\Psi_{JF}{*}S_J$ represents the probability of surviving and becoming adult female, where $\Psi_{JF}$ is the probability of a juvenile female to become adult.

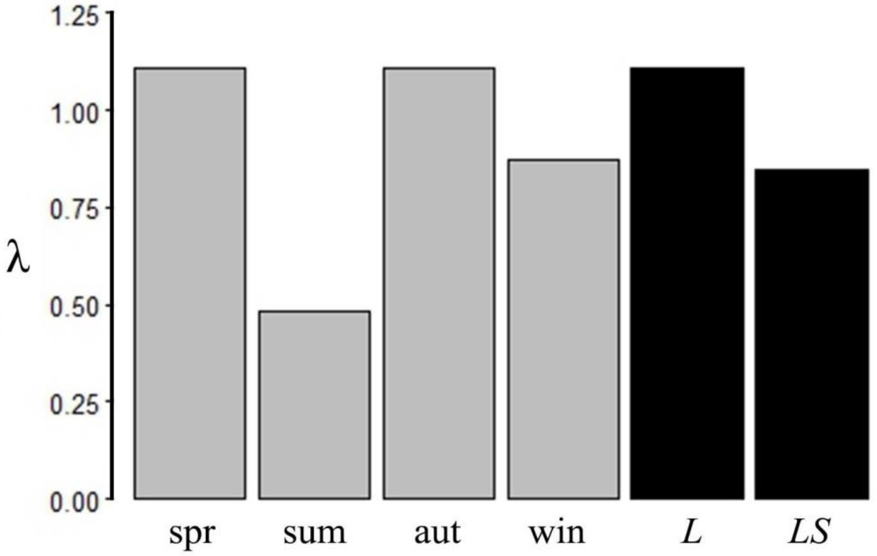

**Figure 2**. Growth rate (λ) of season-specific deterministic matrices (gray, estimated using corresponding season-specific values of $S_F$ and $S_J$ from Capítulo IV), deterministic matrix *L* (black, estimated using median values of $S_F$ and $S_J$), and stochastic matrix *LS* (estimated by simulations using a vector with the sequence of seasonal survivorships $S_F$ and $S_J$ beta-distributed).

# FIGURES V

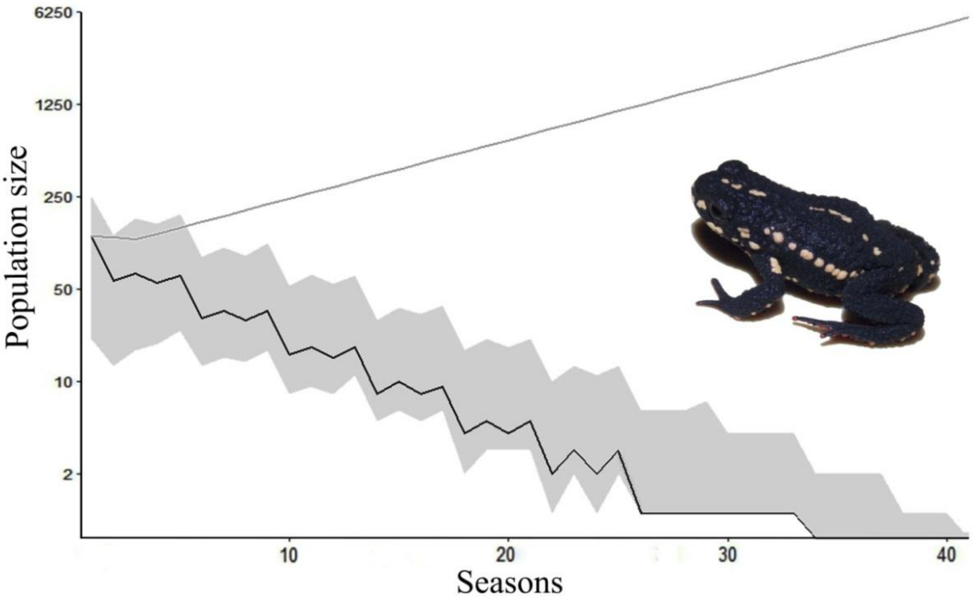

**Figure 3**. Ten-year (40 seasons) projections of population size (adult female abundance only) of deterministic matrix *L* (gray line) and stochastic matrix *LS* (black line). Shaded area represents percentiles 2.5 and 97.5 of stochastic simulations. All projections departed from population vector *N* ($N_P$ = 100, $N_J$ = 1, $N_F$ = 125). Population size was transformed to logarithmic scale to show low and high values in projections of both matrices.

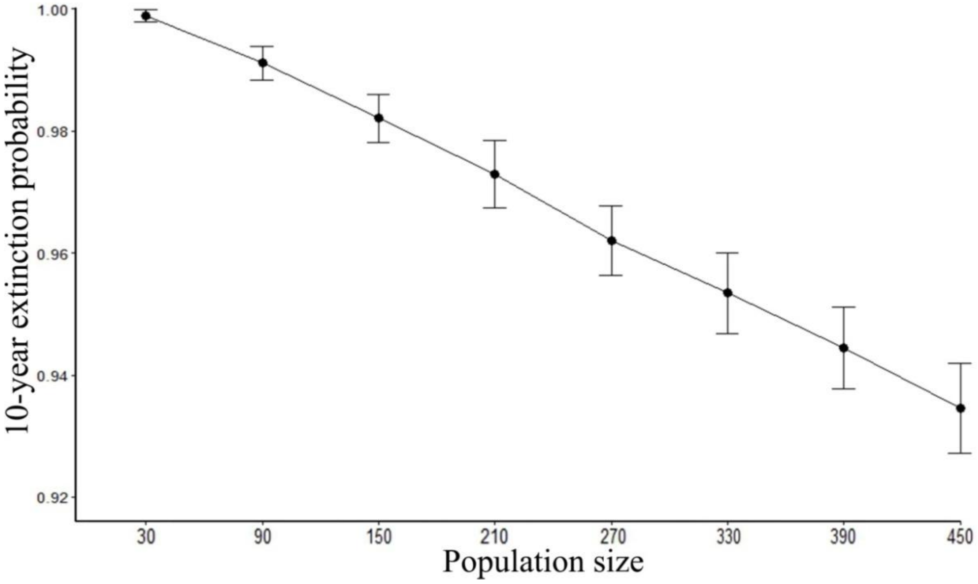

**Figure 4**. Stochastic extinction probability versus population size (adult female abundance only) for matrix *LS*. Bars represent standard deviations for 100 runs of 1000 simulations for each population size.

**FIGURES V**

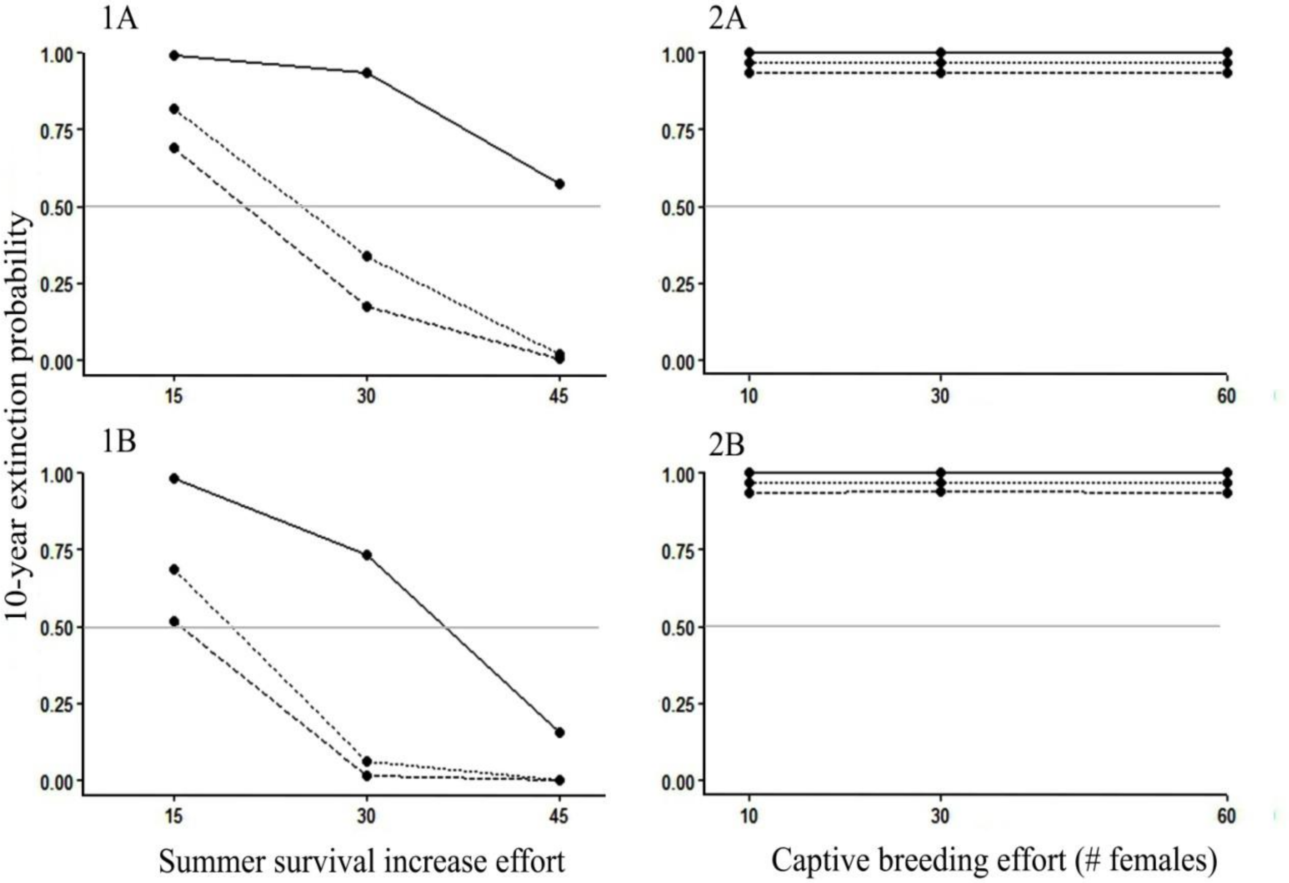

Figure 5. The 10-year extinction probability across different effort levels for scenarios of 1) habitat management addressed to increase summer survival rates and 2) captive breeding of females. 1A) increase in survival rates of adult females only; 1B) increase in survival rates of juvenile and adult females; 2A) captive breeding of females to annually supplement female metamorphs on summer; 2B) captive breeding of females to annually supplement female juveniles on summer. Lines depict initial wild population sizes (adult female abundances only, solid = 30, dotted = 240, dashed = 450). The gray line shows 50% extinction risk.

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# Discusión general

Las tasas de supervivencia de los estadios larvarios, juveniles y adultos, las tasas de transición de juveniles a adultos (maduración sexual), y las tasas reproductivas de los adultos de *Melanophryniscus montevidensis* fueron exitosamente estimadas utilizando datos obtenidos *in situ* (capítulos I y IV) en conjunción con datos disponibles en bibliografía sobre reproducción (Capítulo V). En cuanto a los estadios larvarios, las estimaciones utilizando encierros en los cuerpos de agua fueron: 1.2 % cuando se calculó la mediana por amplexo en cada encierro, 3 % cuando se calculó en base al número de huevos que concluyeron exitosamente la metamorfosis en todos los cuerpos de agua, y 4.8 % en base al número de huevos que concluyeron la metamorfosis considerando sólo aquellos cuerpos de agua con metamorfos (Capítulo I).

Si bien algunos parámetros de calidad de agua presentaron niveles no adecuados para los anfibios en particular, y para la vida acuática en general (bajos niveles de oxígeno disuelto, altos niveles de amoníaco y turbidez, *sensu* Allen Burton Jr. y Pitt, 2001; Jofre y Karasov, 1999; Mann y Bidwell, 2001; USEPA 2000; NYCRR 2017), especialmente donde registramos una mortalidad total de huevos, no se encontraron evidencias significativas de procesos vinculados con actividades antrópicas (ganadería, escorrentía urbana y agrícola, o aguas residuales) tales como eutrofización o acidificación (Capítulo II). A pesar de que no se analizó la presencia de otros contaminantes tales como metales pesados, pesticidas o detergentes, el perfil fisicoquímico de los cuerpos de agua analizados fue similar al esperable como consecuencia de procesos naturales de descomposición y sedimentación en charcos temporales (Chapman, 1996). Las lluvias intensas que comenzaron antes del estudio podrían estar involucradas con tales procesos (Capítulo II). Los niveles de conductividad y turbidez, pero también de amoníaco y oxígeno disuelto, tuvieron un efecto negativo sobre las tasas de supervivencia, especialmente durante las etapas embrionarias y también sobre el crecimiento en las etapas larvarias. No obstante, las bajas tasas de supervivencia fueron explicadas en mayor proporción por la densidad larvaria actuando directamente sobre la supervivencia, e indirectamente sobre el crecimiento larvario (capítulos I y II). Este efecto pudo verse acentuado artificialmente por el tamaño de los encierros, ya que, debido al bajo nivel de agua inicial de los cuerpos de agua, el nivel de agua de los encierros fue generalmente inferior a 13 cm (la mitad de la altura de los mismos, apéndices I y III). Estudios en zonas de ocurrencia de la especie que se encuentren más antropizadas dentro de Laguna de Rocha, controlando la densidad larvaria y analizando la concentración de otros contaminantes (*e.g.* pesticidas: Peltzer *et al.* 2008), deberían ser realizados para poner a prueba la hipótesis de que la baja calidad del agua, asociada con otro tipo de factores, reduce el éxito de la metamorfosis al reducir el crecimiento y la supervivencia de las larvas de *M. montevidensis*.

En base a estos resultados, consideramos que los efectos del cambio climático predicho para la especie (Toranza y Maneyro 2013; Zank *et al.* 2014) podrían ser potencialmente más nocivos en el área de estudio que las actividades antrópicas para el adecuado desarrollo larvario de *M. montevidensis*, ya que la temporalidad de los charcos se vería alterada por la intensificación de la desecación bajo escenarios de calentamiento global. Esta acelerada desecación disminuiría la duración de los cuerpos de agua y aumentaría las densidades larvarias y la conductividad en cuerpos de agua efímeros, tales como los utilizados por *M. montevidensis* para la reproducción. La alta densidad larvaria, y la elevada concentración de solutos que aumenta la conductividad cuando los cuerpos de agua se están secando (Chapman 1996), a su vez, dificultarían la capacidad de los individuos de responder a la desecación, enlenteciendo el crecimiento y resultando en muertes masivas cuando el agua se seca por completo (Capítulos I y II). Por otra parte, los procesos de descomposición son esperables luego de lluvias intensas y retención de agua por tiempo prolongado. Si bien esto forma parte de las fluctuaciones naturales de estos sitios, el aumento de la frecuencia de las floctuaciones (i.e. aumento en el régimen hídrico), en un contexto de cambio climático, también podría tener consecuencias negativas sobre el reclutamiento (Capítulo II).

Las tasas de supervivencia de estadios terrestres fueron mayores que las de estadios larvarios (40-100% en adultos y 10-100% en juveniles, estimaciones a escala estacional, Capítulo IV). A pesar de ello, los modelos de diseño robusto que mejor se ajustaron a los datos de captura-marcaje-recaptura (CMR) mostraron un patrón estacional con una leve disminución en la transición otoño-invierno, y una gran disminución en la transición primavera-verano, especialmente para los juveniles en el último caso (Capítulo IV). Tanto adultos como juveniles mostraron un patrón estacional de actividad, con picos de actividad durante las estaciones cálidas, por lo que es posible que exista una amenaza temporal en el área de estudio relacionada con el patrón de actividad, tal como la mortalidad por atropellamiento durante la temporada de mayor ingreso de turistas al área (Capítulo IV). No obstante, este fenómeno no explicaría la mortalidad durante las estaciones frías en las que la mayoría de los animales se encuentran inactivos. Dado que en el área de estudio ya se ha reportado la infección por el hongo quítrido *Batrachochytrium dendrobatidis* (quitridiomicosis) en otras especies de anfibios (Borteiro *et al.* 2009), y que estudios sobre la estacionalidad en la prevalencia de esta infección indican que las estaciones frías poseen las tasas de prevalencia y mortalidad más altas (Berger *et al.* 2004; Kriger y Hero 2007), la infección por este hongo podría contribuir a la mortalidad de *M. montevidensis* durante las épocas frías aunque poco se sabe sobre los efectos deletéreos de este parásito en especies nativas (Borteiro 2016). Estudios procurando estimar tanto la incidencia de atropellamientos como la prevalencia de la quitridiomicosis, analizando la distribución espacial y temporal (a lo largo del año) de ambos tipos de amenazas, permitirían corroborar su vinculación con el patrón estacional de mortalidad reportado en este estudio.

Las larvas de *M. montevidensis* culminan la metamorfosis con un largo hocico-cloaca (LHC) de 5.7 mm (Capítulo I). El estudio realizado en post-metamorfos de esta especie, con el fin de monitorear el desarrollo de la coloración y el crecimiento durante el primer año de vida, mostró que estos animales alcanzaron a los 220 días (post-metamorfosis) un patrón de coloración estable y confiable para su uso como método de marcaje (por fotoidentificación), con un LHC de 13,24 mm (Capítulo III; Bardier *et al.* 2017). No obstante, durante los muestreos de campo para la estimación de supervivencia de adultos y juveniles no se encontraron juveniles con un LHC menor a 17 mm. Por consiguiente, es posible que los juveniles de un año de edad no fueran considerados en tales estimaciones. Probablemente, las tasas de supervivencia de esta etapa juvenil post-metamórfica sean menores que las de los estadios juveniles subadultos (Zug y Zug, 1979, Semlitsch 2008), como ya ha sido reportado en otros estudios (Cairo 2009; McCaffery y Maxell 2010). Además, los juveniles tempranos suelen tener una movilidad restringida y son más susceptibles a la deshidratación que los estadios más avanzados, por ello muchos suelen permanecer en la pradera inundable o en el margen del cuerpo de agua del que emergieron (Semlitsch 2008).

A efectos de mejorar las estimaciones de supervivencia de las etapas juveniles tempranas, sería útil conocer los ambientes utilizados por los estadios juveniles post-metamórficos, y conducir estudios de CMR dirigidos especialmente a estos estadios. Dado que el patrón de coloración característico de *M. montevidensis* no está presente en estos juveniles, el uso de métodos de marcaje como los implantes de elastómeros, ya probado en crustáceos de pequeña talla (*Callinectes sapidus*, Davis *et al.* 2004), sería recomendable. Paralelamente, estudiar la estructura etaria de esta población (utilizando métodos esqueletocronológicos, *e.g.*, Castanet y Smirina 1990), y la relación de cada franja etaria con el tamaño corporal, aportaría mayor resolución dentro de los estadios que identificamos globalmente como "juveniles" y "adultos".

Otro aspecto a destacar de la demografía de *M. montevidensis*, surgido de los datos de CMR en estadios juveniles y adultos, es el marcado sesgo masculino en la proporción de sexos, existiendo 2.52 machos por cada hembra capturada (Capítulo IV). Considerando que 1) el mejor modelo para las probabilidades de captura no fue el que diferenciaba entre sexos (sino el que presentaba diferencias exclusivamente temporales independientemente del sexo o estado), 2) que las tasas de supervivencia de machos y hembras no fueron significativamente diferentes, y 3) que la actividad reproductiva no fue la hipótesis mejor sustentada por los datos de recaptura para la estimación de las tasas de supervivencia (Capítulo IV), el sesgo masculino en la proporción de sexos reportado estaría reflejando las proporciones reales en la población, y no las diferencias circunstanciales que hacen más conspicuos a machos que a hembras durante los muestreos. Adicionalmente, las probabilidades de transición de juveniles a adultos reportadas sugieren que fue más probable para un juvenil madurar sexualmente como macho adulto que como hembra adulta (Capítulo IV). Esto indica que, al menos parcialmente, el sesgo masculino en la proporción de sexos ya está presente en la fracción juvenil de la población. Dado que el componente femenino es crítico para la tasa de crecimiento poblacional (Caswell 1989), conocer los factores que sesgan la proporción de sexos en esta población podría contribuir con su conservación a largo plazo. Por ejemplo, la reversión del sexo genotípico por factores epigenéticos, tales como alteraciones en la calidad de agua o temperaturas extremas, puede sesgar hacia los machos la proporción sexual al momento de la metamorfosis en anfibios (Eggert 2004).

La estimación de los parámetros demográficos mencionados permitió la construcción de modelos matriciales de Lefkovitch para determinar las tendencias de poblacionales y probabilidades de extinción para la población de *M. montevidensis* estudiada. Aplicando modelos matriciales, la supervivencia de las hembras adultas ha sido reiteradamente documentada como la tasa vital con mayor importancia relativa para el crecimiento poblacional de los anfibios (Cairo 2009; Homyack y Haas 2009; Unger *et al.* 2013; Kissel *et al.* 2014). Dado que la supervivencia de adultos (y juveniles) mostró una drástica disminución principalmente durante las transiciones primavera-verano (Capítulo IV), pusimos a prueba la hipótesis de que esta disminución estacional en la supervivencia de las hembras adultas durante las estaciones cálidas, aumenta la probabilidad de extinción de la población a corto plazo al reducir las tasas de crecimiento poblacionales durante tales estaciones. Dicha hipótesis fue apoyada por los resultados, ya que la tasa de crecimiento de la población (λ) fue mayormente sensible a las tasas de supervivencia de las hembras adultas y λ alcanzó valores de una población en declive en aquellas estaciones en que la supervivencia de las hembras disminuyó (Capítulo V). A su vez, los modelos en los que la variabilidad aleatoria (estocasticidad) de los parámetros demográficos, y la variabilidad estacional en la supervivencia de las hembras, fueron incorporadas, las probabilidades de extinción de la población en 10 años fueron altas (> 90%, Capítulo V). Dentro de los rangos de abundancia reportados para las hembras (Capítulo IV), esta alta probabilidad de extinción fue independiente del tamaño inicial de la población. Si bien estos son los resultados de un modelaje teórico y dependen de la precisión de los parámetros demográficos, están basados en el conocimiento más actualizado de la biología de la especie y refuerzan la preocupación sobre su estado de conservación en el área protegida.

De manera similar a lo reportado en otros estudios realizados en anfibios, las hembras adultas fueron el estadio más importante para la tasa de crecimiento de la población en nuestros modelos. Esto refuerza la importancia de estudiar aquellos factores locales que disminuyen estacionalmente la supervivencia de los estadios terrestres, principalmente de los adultos, ya que estos serían los que aumentan las probabilidades de extinción de la población. En este sentido, la evaluación del manejo de hábitat y la suplementación de animales criados en cautiverio, como estrategias de conservación, indicó que la primera es la única medida que reduciría efectivamente las probabilidades de extinción de la población en 10 años (Capítulo V). Mientras no fue posible plantear escenarios de suplementación con resultados favorables para la población, un manejo del hábitat dirigido a reducir en un 30% la mortalidad de las hembras adultas, cuando el tamaño poblacional se encuentra entre 240 y 450 hembras adultas, reduciría las probabilidades de extinción de la población a menos del 50% (Capítulo V). Resultado que también apunta hacia un manejo preferentemente *in situ* de la población de *M. montevidensis* de Laguna de Rocha.

Nuestros resultados resaltan la utilidad de la aplicación de modelos estructurados por estadios para la conservación de una especie amenazada como *M. montevidensis*, ya que fue posible tanto conocer la tendencia poblacional, como cuáles aspectos de la historia de vida de la especie son los más influyentes sobre dicha tendencia, cuáles amenazas podrían tener más impacto sobre dicha tendencia (por su efecto sobre cada parámetro poblacional), y las posibles medidas que podrían revertir dicha tendencia. No obstante, estos resultados pueden ser mejorables, ya que existe la posibilidad de que los tamaños de las desovas hayan sido subestimados en el campo, lo que implicaría que los parámetros de fertilidad fueran más altos, aumentando los valores de sensibilidad de la fertilidad sobre la tasa de crecimiento (Cairo 2009; Capítulo V). Además, como ya fuera mencionado, las tasas de sobrevivencia de los juveniles tempranos (de un año de edad luego de la metamorfosis) posiblemente sean bajas y hayan sido sobreestimadas al ingresar (en los modelos matriciales) como un único grupo de “juveniles” a todos los animales desde la metamorfosis hasta la madurez sexual, cuando no fue posible estimar las tasas de supervivencia de los juveniles (capítulos IV y V). Recomendamos realizar estudios para una estimación más precisa de estas tasas vitales, a fin de mejorar el modelaje poblacional de esta especie y, por consiguiente, contribuir a su conservación.

La estimación de las tasas vitales permitió determinar que la tendencia de *M. montevidensis* de Laguna de Rocha es decreciente con altas probabilidades de extinción a corto plazo. La protección de aquellos cuerpos de agua donde esta especie amenazada normalmente habita y se reproduce es importante para la conservación de la misma, especialmente recomendamos un monitoreo sistemático de las condiciones hidrológicas y los parámetros de calidad de agua de estos sitios. No obstante, dada la sensibilidad reportada de las tasas de crecimiento hacia los estadios adultos de la población, observándose principalmente que las hembras fueron el grupo menos representado, sugerimos con mayor énfasis profundizar en el conocimiento de aquellos factores que inciden estacionalmente sobre la supervivencia de los estadios terrestres, para la generación de medidas dirigidas a mitigar los efectos de estas amenazas. Acciones tales como el mantenimiento de la calidad de los cuerpos de agua, o la canalización, guiando el movimiento de los animales por debajo de la calle (lo que durante períodos de actividad proporcionaría un pasaje seguro), contribuirían a mantener al menos un nivel basal estable en el tamaño poblacional de esta especie. Estas medidas no beneficiarían únicamente a *M. montevidensis*, sino también a otros anfibios, reptiles y mamíferos (Semlitsch 2000; Semlitsch y Bodie 2003; Dodd 2010), favoreciendo particularmente aquellas especies del área que se asemejen morfológicamente y ecológicamente a nuestra especie objetivo (*e.g.*: *Odontophrynus maisuma*, Rosset 2008). Finalmente, la disponibilización de los parámetros demográficos estimados permitiría el modelaje de otras poblaciones de la misma especie, así como también de poblaciones amenazadas de especies biológicamente similares a *M. montevidensis* (Heppell *et al.* 2000). Como ensayamos en este estudio, mediante la construcción de modelos matriciales es posible aplicar los parámetros demográficos para simular diferentes estrategias que facilitarían la conservación de otras especies de *Melanophryniscus*. Este enfoque sería altamente útil para especies expuestas a amenazas ya reportadas y cuyas poblaciones requerirían medidas urgentes de conservación, tales como aquellas especies que, al igual que nuestra especie objetivo, podrían ser afectadas por el cambio climático (*M. tumifrons* o *M. cambaraensis*, Zank *et al.* 2014); o aquellas en las que la quitridiomicosis ya ha sido detectada (*Melanophryniscus* sp. [aff. *montevidensis*], Agostini *et al.* 2015).

# Medidas de manejo específicas

## Monitoreo

Para avanzar en el entendimiento de las posibles amenazas que afectan a *Melanophryniscus montevidensis* en Laguna de Rocha y mejorar la precisión de los parámetros demográficos se recomienda:

- **Monitorear el hidroperíodo de los cuerpos de agua estudiados y otros cercanos con similares características**. Esto implica registrar, luego de lluvias intensas, el momento en que cada cuerpo de agua se llena y el tiempo transcurrido hasta que se seca. También la frecuencia con que cada uno recibe y retiene agua. Aquellos cuerpos de agua que no retienen agua por más de 22 días no tendrían reclutamiento exitoso de *M. montevidensis*.
- **Registrar la actividad reproductiva**. Por tratarse de una especie de reproducción explosiva, la actividad de vocalización comienza poco después de aquellas lluvias intensas que logren llenar los cuerpos de agua, y dura un máximo de dos días. El registro de la actividad de vocalización en los cuerpos de agua estudiados (y similares) permitiría saber con qué frecuencia ocurre la reproducción, si ocurre en épocas frías y cuáles cuerpos de agua son los más frecuentados.
- **Monitorear la calidad de agua**. Sería recomendable hacerlo al menos anualmente en los cuerpos de agua estudiados (y similares), particularmente midiendo los niveles de: amoníaco, nitratos, fosfatos, oxígeno disuelto, pH, conductividad y turbidez. También la presencia de pesticidas de ser posible. Dependiendo de los valores resultantes de este conjunto mínimo de parámetros, podrían inferirse procesos de descomposición, contaminación por aguas residuales domésticas y/o por actividades agrícolas.
- **Registrar la presencia de juveniles**. Específicamente registrar animales de hasta 21,21 mm en el margen de los cuerpos de agua y ambientes adyacentes, a efectos de conocer los ambientes utilizados por los mismos.
- **Establecer un plan de monitoreo ciudadano de los estadíos terrestres**. Este plan requeriría que cada participante contara simplemente con un celular con cámara

fotográfica. Sería necesario delimitar ciertas parcelas en las que guardaparaques, habitantes locales y turistas pudieran hacer (una vez por semana como frecuencia máxima) una búsqueda activa de individuos de la especie durante un tiempo determinado (*e.g.* 15 minutos), obtuvieran una imagen ventral de cada individuo y lo enviaran a una base de datos general. Esta actividad debería ser supervisada por personal del área protegida o investigadores, en el caso de ser realizada por voluntarios, para controlar tiempos y asegurar la mínima manipulación de los animales, la cual sólo podría ser realizada con guantes. También podría desarrollarse una aplicación de celular o una página web. La información recabada permitiría construir nuevos modelos poblacionales para la estimación de tasas de supervivencia por Captura-Marcaje-Recaptura (por fotoidentificación) a diferentes escalas temporales, permitiendo comparaciones con estimaciones previas (Capítulo IV). Una experiencia de monitoreo ciudadano similar se viene llevando a cabo en Chile para *Rhinoderma darwinii*: https://www.ranitadedarwin.org/observa-ranas.

- **Colectar animales muertos**. Todos aquellos individuos que sean encontrados muertos deberían ser colectados para establecer las causas y la frecuencia de tales muertes. Aquellos animales que se encuentren secos se pueden colectar en una bolsa en seco, en el caso de conservar humedad, colocar inmediatamente en formol al 4% o alcohol 70%.

### Medidas en los cuerpos de agua

Los cuerpos de agua muestreados en esta tesis deben ser particularmente protegidos ya que el completo desarrollo larvario de la especie ha sido confirmado en los mismos (Capítulos I y II). No obstante, dado que los adultos de *M. montevidensis* son muy fieles a los cuerpos de agua, habitan en la planicie inundable cuando están secos, y se reproducen en masa cuando se llenan de agua, el cuidado de los mismos no sólo contribuye con la supervivencia larvaria, sino también de juveniles y adultos. Se recomienda:

- **Mantener la higiene.** Evitar disponer de residuos orgánicos, plásticos o de cualquier otra naturaleza en los cuerpos de agua. Evitar el uso de estos ambientes y sus cercanías como baños públicos. Esto ayudaría a mantener la calidad de agua necesaria para el desarrollo larvario de *M. montevidensis*.

- **Evitar el ingreso de personas y vehículos.** Los vehículos pueden atropellar individuos de la especie objetivo y destruír ambientes. La exclusión de personas reduciría el riesgo de ingreso de todo tipo de desechos o la colecta indebida de los animales. A su vez, reduciría el riesgo de ingreso de *Batrachochytrium dendrobatidis*, presente en otras localidades de Uruguay. Las esporas de este hongo pueden viajar en ropa y calzado, causando infecciones cruzadas entre localidades. La desinfección del calzado del personal del área protegida puede realizarse con hipoclorito de sodio.
- **Excluír animales domésticos (gatos, perros, vacas y caballos principalmente) de los cuerpos de agua**. Estos animales deberían mantenerse fuera de un radio de 290 m desde margen de los cuerpos de agua (Semlitsch y Bodie 2003). En el caso de los pequeños animales, para evitar la caza y residuos. En el caso de los animales grandes, para evitar residuos, la turbidificación del agua al caminar, y también evitar muertes accidentales por pisadas. Cercar los cuerpos de agua sería una medida ideal, si bien debería ser un cerco selectivo y bien diseñado para no obstaculizar los movimientos de la fauna local.

### Medidas en áreas adyacentes a los cuerpos de agua

Los adultos de *M. montevidensis* realizan movimientos en el entorno de los cuerpos de agua a distancias generalmente no mayores a 100 m del centro del cuerpo de agua (Pereira y Maneyro 2016). Estos movimientos son más frecuentes y masivos durante los eventos de reproducción, pero también existe actividad locomotora durante las épocas cálidas (Capítulo IV). Por ello se recomienda:

- **Evitar el tránsito de vehículos fuera de la caminería establecida.** Además de destruír los ambientes, los vehículos pueden atropellar individuos de la especie objetivo, así como también de otras especies.
- **Restringir el tránsito dentro del área y/o reducir velocidades.** Un menor ingreso de vehículos disminuiría los riesgos relacionados con los mismos, al igual que la imposición de una velocidad máxima. Esto sería particularmente útil durante las épocas cálidas, especialmente luego de lluvias intensas, cuando hay más riesgo de atropellamiento para la especie.
- **Construír un sistema de cercos y túneles en la calle principal**. En cada porción de la calle que tiene cuerpos de agua a un lado o ambos (*e.g.* charcos 1 y 2, Capítulo

IV) se debería construir un sistema de cercos, o una elevación abrupta del terreno, que impida a los anfibios cruzar la calle. Este cercado, a su vez, debería orientar el movimiento de los animales hacia túneles que crucen la calle de lado a lado, a efectos de evitar atropellamientos cuando los animales están en movimiento, que generalmente ocurre en la época de mayor tránsito turístico del área (primavera-verano). Dependiendo del diámetro de los túneles, esta madida también evitaría el atropellamiento de otros anfibios, reptiles e inclusive mamíferos medianos. Existen módulos prefabricados (ver ejemplo en https://www.legacy-habitat.co.uk/amphibian-climate-tunnel.html), pero también pueden construírse vallas con materiales simples (troncos verticales, bloques, etc.) y excavar canales que crucen la calle y sean cubiertos con rejillas para no dificultar el tránsito. Cualquiera sean los materiales o diseño a emplear, debería reducirse al mínimo la modificación del hábitat circundante para la construcción de este sistema, así como evitar la aplicación de mezclas de portland, ya que podrían contaminar los cuerpos de agua.

- **Evitar la construcción de pozos negros en las cercanías**. Esto prevendría que los cuerpos de agua entraran en contacto con desechos domésticos (principalmente materia orgánica y detergentes), ya que la contaminación con los mismos podría tener consecuencias negativas sobre la supervivencia larvaria de la especie. Se recomienda enfáticamente avanzar en la construcción de una red de saneamiento en esta zona para prevenir, además de los problemas sanitarios de los habitantes locales, riesgos de infiltración y contaminación de los cuerpos de agua.
- **Evitar el uso de productos agrícolas**. No deberían ser aplicados plaguicidas o fertilizantes en las cercanías de las poblaciones de *M. montevidensis*, para evitar su ingreso en los cuerpos de agua que la especie utiliza para la reproducción.

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# Apéndices

# Apéndice 1

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# QUANTITATIVE DETERMINATION OF THE MINIMUM BODY SIZE FOR PHOTO-IDENTIFICATION OF *MELANOPHRYNISCUS MONTEVIDENSIS* (BUFONIDAE)

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***Abstract*.—Toads of the genus *Melanophryniscus* possess unique color patterns on the belly, which allows for individual recognition. Photo-identification has proven to be an efficient non-invasive technique to identify individuals for this genus. However, such color patterns are absent in newly metamorphosed individuals. We studied the development of the ventral coloration pattern and evaluated its persistence in *Melanophryniscus montevidensis* to determine the minimum size and age at which use of the color pattern is a trustworthy (i.e., as stable as in adults) identification method for this species. From spawns raised in the field, we obtained eight metamorphs and maintained them in semi-natural conditions to photograph their bellies. We visually analyzed the images to establish the stabilization point of the color pattern. Using the software Wild-ID, we calculated the similarity score between the images from the stabilization point with sets of images before and after stabilization. Similarity scores of adults from previous studies did not differ significantly from the scores of juveniles after the pattern stabilized, but they did differ significantly from the scores of juveniles compared to themselves at least 70 d before stabilization. The color pattern developed progressively and stabilized at a median of 220 d after metamorphosis, with a maximum snout-vent length of 13.2 mm, which we considered the minimum size for photo-identification purposes. Although we observed ontogenetic and individual variation, the pattern remained unchanged since just before the first year of age. Taking into account the threshold size we determined, photo-identification is a suitable method for ecological studies of this species.**

*Key Words.*—age; color pattern; non-invasive methods; ontogeny

## INTRODUCTION

The Neotropical genus *Melanophryniscus* comprises 29 species distributed from central-southern Brazil to central-eastern Argentina, including Bolivia, Paraguay, and Uruguay (Amphibian Species of the World: an Online Reference. 2016. Version 6.0. American Museum of Natural History, New York, USA. Available from http://research.amnh.org/herpetology/amphibia/index.html. [Accessed 25 January 2016]). The dorsal pigmentation of these small toads is generally not colorful. However, most of them possess a conspicuous color pattern on the belly (and sometimes on the hands and feet). The black, brown, or green background with red, yellow, white, green, or orange spots inspires their common name of red belly toads (Mebs et al. 2007; Caorsi et al. 2014). When threatened, *Melanophryniscus* individuals exhibit a defensive behavior, the unken reflex, which includes displaying the bright coloration on their palms and belly (Toledo and Haddad 2009). Neither the color pattern nor the associated unken reflex are apparent during the first days after metamorphosis in some species of this genus (Langone et al. 2008; Caorsi et al. 2014), or in toads belonging to the genus *Bombina* (Löhner 1919).

In Uruguay, there are six species of *Melanophryniscus*, and three are globally listed as threatened. One of them is *Melanophryniscus montevidensis* (International Union for the Conservation of Nature [IUCN] global: Vulnerable; IUCN national: Critically Endangered; Carreira and Maneyro 2015; IUCN, Red List of Threatened Species. 2016. Available from http://www.iucnredlist.org/ [Accessed 15 April 2016]). This is a small sized (adult snout-vent length [SVL]: 20–30 mm) coastal species, which exploits psammophilic vegetation (vegetation from sandy soils), and breeds in ephemeral ponds after heavy rains (Maneyro and Carreira 2012; Pereira and Maneyro 2016a). Zank et al. (2014) recommended population-level studies for this species, and non-invasive marking techniques like photo-identification are the most advisable approaches for threatened species.

# Apéndice I

Elgue et al. (2014) tested the accuracy of using photo-identification for adult individuals of *M. montevidensis* (mainly black with red and yellow spots) and it seems to perform well as an identification method. Using this method to identify individuals has no effect on the health and behavior of the animal, lasts for the duration of the study, and has minimal cost (Ferner 2007). As in other species of the genus, newly metamorphosed *M. montevidensis* may not possess the characteristic color pattern. Furthermore, juveniles are difficult to find and there are no records of juvenile recaptures using photo-identification (Pereira and Maneyro 2016b). Some information about the appearance of the ventral patterns in young juvenile toads is available in the literature (Gollman and Gollman 2011; Caorsi et al. 2014), but our study is the first to quantify the development time of the color pattern and estimate the minimum body size at which the color pattern stabilizes and becomes useful for valid photo-identification. For *M. montevidensis* we predicted that the color patterns would develop progressively during the first year, which may violate the assumption of consistent appearance of the mark if researchers use photo-identification for early life stages.

## MATERIALS AND METHODS

On February 2014, during a breeding event, we captured nine amplectant pairs in a temporary pond located 1 km away from La Riviera, Rocha (34°32'42.40"S, 54°19'34.04"W; 3–10 m above sea level). This locality is within Paisaje Protegido Laguna de Rocha, part of the national system of protected areas (Dirección Nacional de Medio Ambiente [DINAMA] 2010). To obtain spawns, we kept the amplectant pairs for 24 h in plastic cages containing water and vegetation from the pond. Later, we placed the spawns in separate *in situ* plastic enclosures and monitored them until tadpoles reached metamorphosis. To count the age of the larvae until front limb emergence, the day of oviposition was considered day 0.

From 19 to 25 March, we randomly selected four metamorphs (Gosner stage 42; Gosner 1960) from two different amplectant pairs. We reared these eight individuals inside individual 6-l plastic containers (30 cm × 15 cm × 13 cm) in an open room exposed to the natural photoperiod and temperatures (mean 15.7 ± [SD] 3.7° C). We periodically added soil and grass collected from the border of the pond as a source of invertebrate food and shelter. We supplied water from the pond daily to maintain humidity. When the pond dried up, we mixed water from a nearby water body with distilled water to emulate the original pond (mean conductivity = 64.3 ± 37.3 µS cm-1, n = 81; mean pH = 6.2 ± 0.4, n = 81; pH and conductivity meter, Hanna® Instruments, Limena, Padua, Italy). During the sixth month, we offered ants (*Solenopsis* sp. and *Linepithema humile*; Formicidae: Myrmicinae and Dolichoderinae, respectively) to increase the size and variety of food items for the toads. However, some toads rejected the ants, especially *Solenopsis* sp., and died before the end of the first year. We deposited all but one of the deceased toads in the scientific collection of Facultad de Ciencias, Universidad de la República (Montevideo, Uruguay); siblings from the spawn of couple 1: ZVCB 23365–23367; siblings from the spawn of couple 2: ZVCB 23368–23371.

We took photographs of the belly and pelvic patch every 2 d after front limb emergence during the first 70 d and then monthly for the lifespan of each animal (range, 200–400 d), using a Coolpix L810 camera (16.0 MP; Nikon, Tokyo, Kantō, Japan). Because animals were small at the beginning (median = 5.8 mm SVL), we photographed them in a drop of water through a plastic dish. We included a ruler for scale in the picture frames and held the distance and lighting constant to standardize images and facilitate taking digital measurements. We used the software Micrometrics® SE Premium (ACCU-SCOPE, Commack, New York, USA) to measure the SVL.

We predicted that the color patterns would stabilize at some point during the first year. Before that point, we suspected that the images would have low resemblance. However, when comparing the image at stabilization with the last picture of the animal, the level of resemblance should be similar to that reported for adults (see Elgue et al. 2014). To test this, we created a database of 4,523 images documenting the developmental sequence of the color pattern of each animal. We chose 109 photos (one photo of each animal every 10 d until day 63 [from day 3 after front limb emergence] and one photo each month after day 63). We used the same procedure as Elgue et al. (2014). We cropped images to show only the ventral pattern, pelvic patch, and axillar marks, and modified the brightness and contrast to improve the visibility of the color patterns whenever necessary. Separately, we visually inspected the sequence of images of each animal to determine the image at which the color pattern stabilized. We defined a color pattern as stable when it stopped changing and retained its main contours and number of spots. One animal died before stabilization and we excluded it from analysis.

We used the software Wild-ID (Dartmouth College, Hanover, New Hampshire, USA) to select the best images for visual confirmation of recapture. The software identified the top-ranked 20 photos that matched the analyzed one (Bolger, D.T., B. Vance, T.A. Morrison, and H. Farid. 2011. Wild ID user guide: pattern extraction and matching software for computer-assisted photographic mark. Available from http://envs.dartmouth.edu/people/douglas-thomas-bolger

# Apéndice 1

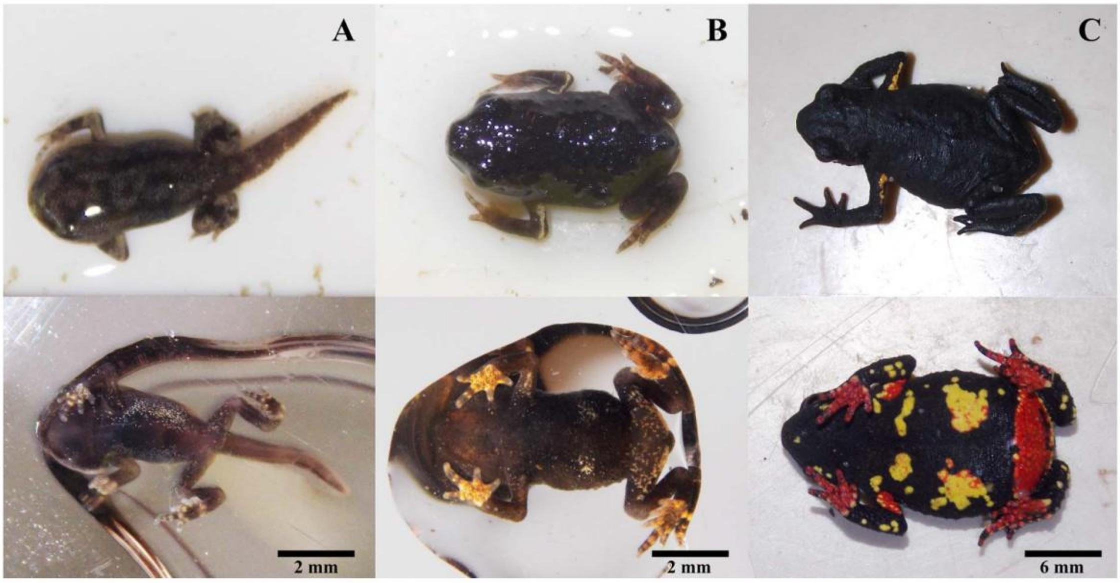

**Figure 1**. Dorsal (top) and ventral (bottom) views of *Melanophryniscus montevidensis* at different stages: A) newly metamorphosed individual (i.e., front limb emergence; Gosner stage 42), B) 13 d after front limb emergence (ZVCB-23367), C) adult photographed in the field. Image brightness and contrast were adjusted. (A and B photographed by Cecilia Bardier; C photographed by Nicolás Martínez).

[Accessed 20 March 2016]). Others have successfully used this software to identify individuals of species belonging to the *Melanophryniscus* genus (Abadie 2012; Caorsi et al. 2012; Elgue et al. 2014). Wild-ID computes a resemblance score for each pair of images (i.e., qualifies the similarity among individuals) from zero to one; the higher the score, the higher the similarity between individuals (Bolger, D.T., B. Vance, T.A. Morrison, and H. Farid. 2011. *op. cit*.).

To test our prediction, we contrasted four sets of similarity scores. Three sets were the scores derived by Wild-ID when we compared photographs of each juvenile (n = 7) taken at the time of ventral color pattern stabilization with photographs of itself taken at a median of 100 d before, a median of 70 d before, and the last photograph of itself (taken at a median of 70 d after stabilization). The fourth set was the similarity scores of photographs of recaptured adults from field data (n = 18) taken at a maximum of 14 mo apart, from Elgue et al. (2014). This last set was included to decide which levels of similarity scores of juveniles were within the variation of adult scores, already considered valid for photo-identification by Elgue et al. (2014).

Because of the small sample sizes, we used the Kruskal-Wallis test in statistical software R (The R Foundation for Statistical Computing. 2002. R. Available from https://www.r-project.org/foundation/ [Accessed 10 February 2015]) to analyze the differences among these four sets of scores. We tested for significant differences among sets of scores using a multiple comparison Dunn post hoc test available in the R package PMCMR, which applies the Holm P-value adjustment method (Pohlert, T. 2016. Calculate Pairwise Multiple Comparisons of Mean Rank Sums. Version 4.1. Available from https://cran.r-project.org/web/packages/PMCMR/index.html [Accessed 12 April 2016]). We considered the median SVL at which the color pattern stabilized as the minimum body size at which the photo-identification is a reliable marking method if two conditions were met: adult scores and the scores after the stabilization of the color pattern did not differ significantly, and adult scores and the scores before the stabilization of the color pattern did differ significantly.

## Results

Our study subjects completed metamorphosis (i.e., front-limb emergence; Gosner stage 42) after a median of 23 d after oviposition (range, 19–25 d). Unlike the black dorsum and colorful ventral pattern of adults, the dorsal and ventral coloration of the newly metamorphosed toads was grayish-brown and slightly transparent in the ventral region (Fig. 1). Dorsal darkening occurred gradually and reached the characteristic black coloration around the tenth day after front limb emergence (Fig. 1). The ventral color patterns developed progressively by the accumulation of small spots (Fig. 2). The age at which we considered the color pattern stable varied among juveniles (Table 1), median = 220 d after front limb emergence (range, 153–344 d). Although the

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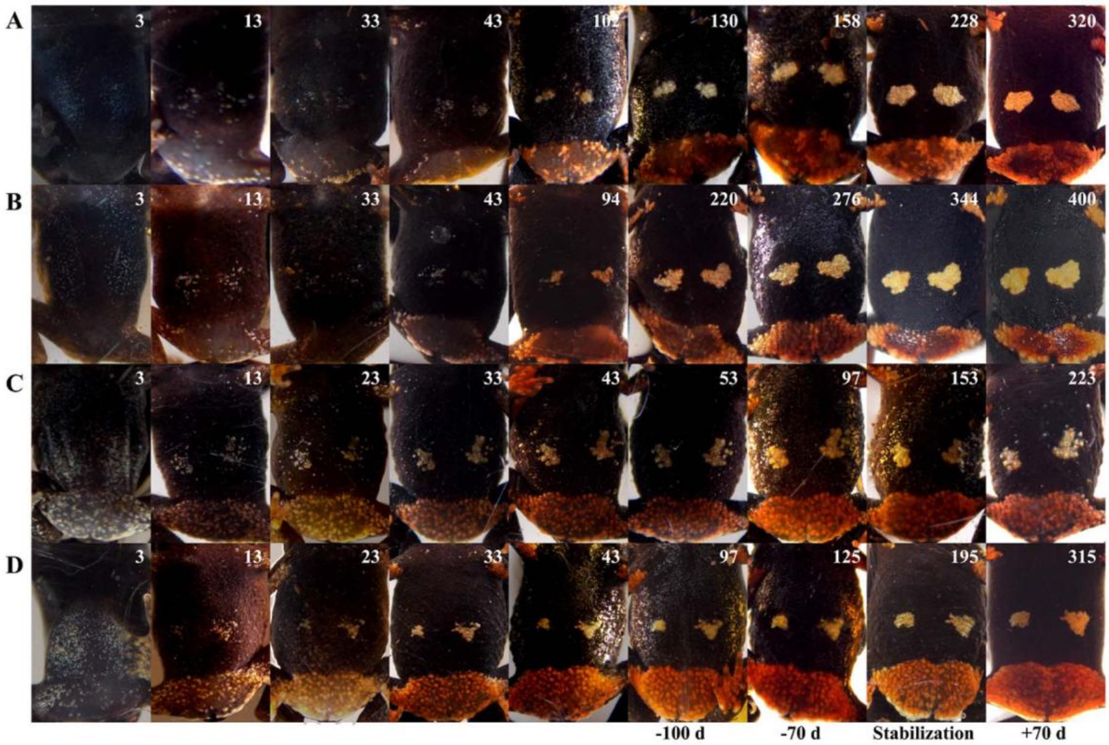

**Figure 2**. Developmental sequence of the color pattern of four juvenile toads (*Melanophryniscus montevidensis*) 1-23365 (A), 1-n.a. (B), 2-23370 (C), and 2-23371 (D). The first number of each animal indicates the amplectant pair, the second number is the catalog number in the scientific collection of Facultad de Ciencias, Universidad de la República (ZVCB). Top right numbers are the ages (d after front limb emergence. -100 d and -70 d are the images of the animals at a median of 70 and 100 d before the stabilization of the pattern, respectively. Stabilization represents the first images with stable patterns. +70 d are the last images of each animal (median = 70 d after stabilization of the pattern). Image brightness and contrast were adjusted to facilitate comparisons. (Photographed by Cecilia Bardier).

hands, pelvic patch, and axillar spots developed the red coloration in early stages (range, 20–30 d), the color pattern in the belly developed first in yellow, and the red coloration appeared later over the yellow spots (Fig. 2). During manipulation for a photo session, one juvenile (ZVCB 23368) was the first to display unken reflex, 34 d after front limb emergence.

When we made multiple comparisons of the sets of juvenile similarity scores and the set of adult scores (recaptures from Elgue et al. 2014), the four groups of Wild-ID scores differed significantly ($\chi^2 = 20.15$, df = 3, $P < 0.001$; Fig. 3). The adult scores and the scores of juveniles after the color pattern stabilized did not differ significantly ($P > 0.05$). There were significant differences among the adult scores and the scores obtained from comparisons at a median of 70 and 100 d before stabilization ($P \leq 0.05$). However, there were no differences between the scores obtained from comparisons at median = 70 d before and comparisons at median = 70 d after stabilization ($P > 0.05$). Once we verified that the requirements of the scores were fulfilled, we determined that the color pattern stabilized with a median SVL = 8.8 mm (range, 8.2–13.3 mm), which is the minimum body size at which the photo-identification is reliable in this species (Table 1).

## Discussion

Our observations established a point at which the color patterns stabilize in young juveniles of *M. montevidensis*. The similarity scores before juveniles reach pattern stabilization do not resemble the adult re-capture scores. At earlier time intervals, the scores of toads compared to themselves were low and lacked statistical differences among age groups. This supports our prediction that the color patterns develop gradually. The low scores after stabilization and the lack of differences between the scores just before and just after the stabilization provide further evidence for gradual development of the pattern. However, the low scores and lack of differences also challenge our determination of the pattern stability of juveniles, and photo-identification techniques should be applied judiciously taking into account the considerations below.

Changes in the color pattern may occur, especially in early juvenile stages (e.g., Gollmann and Gollmann

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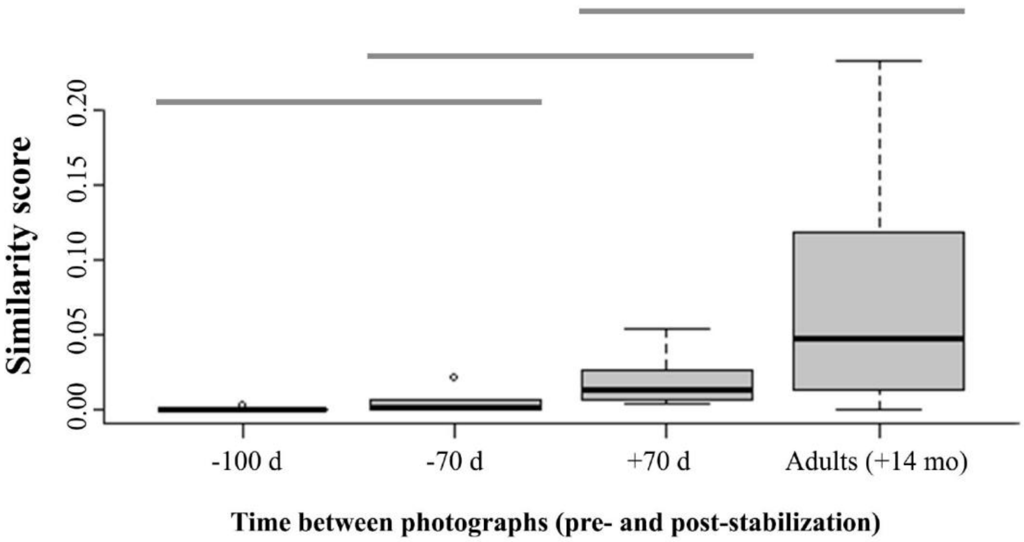

**Figure 3**. Boxplots of similarity scores comparing photographs of juvenile toads (Melanophryniscus montevidensis, n = 7) taken when the ventral color pattern stabilized with images taken at median = 100 d (-100 d) and 70 d (-70 d) prior to stabilization, and median = 70 d after stabilization (+70 d). Adults (+14 mo) refers to similarity scores comparing images of recaptured adult toads (n = 18) taken within a maximum of 14 mo apart and reported by Elgue et al. (2014). Similarity scores not joined by horizontal bars are significantly different from each other ($P \leq 0.05$; Dunn's *post hoc* comparisons).

2011). When slight variations in the pattern occur, photo-identification can still be applied, but visual methods combined with the use of visual-assisted software are more advisable than exclusive use of automated software (Gollmann and Gollmann 2011; Bendik et al. 2013). In addition, the image quality is important because high resolution, in-focus, and constant distance images are less prone to mismatches than low quality images (Gollmann and Gollmann 2011; Bendik et al. 2013).

We expected later changes in juvenile coloration because we observed stable coloration patterns when only yellow spots were present whereas adults generally have both red and yellow spot coloration. We found that the red coloration appeared later and on top of the yellow spots (i.e., not at the edges), and thus did not alter the main outline of the pattern. The software Wild-ID is based on an algorithm that recognizes and compares Key Points Independent from the Scale (Lowe 2004). It does not consider coloration and needs visual assistance to confirm matches (Bolger, D.T., B. Vance, T.A. Morrison, and H. Farid. 2011. *op. cit.*). Therefore, the later appearance of the red coloration would not affect the recognition using the Wild-ID software in subsequent recaptures.

**Table 1**. Ages (number of days after front limb emergence) and sizes of *Melanophryniscus montevidensis*. Age of image -100 d and Age of image -70 d refer to photographs from a median of 70 and 100 d before stabilization of the pattern, respectively. Age of stabilization refers to the first image with a stable pattern. Age of image +70 d refers to the final image (median time = 70 d after stabilization). Wild-ID stabilization scores are the similarity scores comparing the first image with a stable pattern to the final image. For Animal I.D., the first number indicates parental pair; the second is the catalog number in the scientific collection of Facultad de Ciencias, Universidad de la República (ZVCB). One catalog number was unavailable (noted as 1-n.a.).

| Animal I.D. | Age of image -100 d | Age of image -70 d | Age of stabilization | SVL at the age of stabilization (mm) | Age of image +70 d | Wild-ID stabilization scores |
|---|---|---|---|---|---|---|
| 1-23365 | 130 | 158 | 228 | 8.55 | 320 | 0.0395 |
| 1-23366 | 153 | 195 | 279 | 11.10 | 347 | 0.0088 |
| 1-n.a. | 220 | 276 | 344 | 13.24 | 400 | 0.0134 |
| 1-23367 | 122 | 150 | 220 | 8.95 | 312 | 0.0045 |
| 2-23368 | 53 | 102 | 158 | 8.48 | 228 | 0.0535 |
| 2-23369 | — | — | — | — | 200 | — |
| 2-23370 | 53 | 97 | 153 | 8.18 | 223 | 0.0035 |
| 2-23371 | 97 | 125 | 195 | 8.86 | 315 | 0.0140 |

# Apéndice 1

Although juvenile similarity scores after pattern stabilization resembled adult scores, adult scores were low (generally < 0.2) and juvenile scores were even lower (generally < 0.05). Other studies reported low similarity scores from field photographed recaptures using Wild-ID, especially when the image quality was low and when animals experienced weight changes (i.e., allometric variations; Bendik et al. 2013; Morrison et al. 2016). Differences between the adult and juvenile scores may be a result of the manner in which we photographed the juveniles versus adults. We photographed juveniles in a drop of water through a plastic dish, whereas Elgue et al. (2014) photographed adults in the field, dry, and upside down. Allometric variations (because juveniles were growing) and image quality differences were both influencing the similarity scores produced from our database. In spite of these difficulties, we consider the comparison of similarity scores a useful quantitative approach because the allometric variations are unavoidable in early stages, and the algorithm of the software still works at low scores (Bendik et al. 2013); thus, we strongly recommend this method. The manipulation of small animals without harming them is difficult and time consuming. If the SVL of the animal is < 10 mm, a drop of water reduces its movement and the animal can be photographed in a natural position from below with no handling.

We determined the minimum size at which researchers can reliably use the color pattern in *M. montevidensis* to be 8.8 mm SVL. However, because the age at which an individual attained pattern stability was highly variable and our sample size was small, we take caution in interpreting our results. The last individual developed a stable color pattern nearly one year after front limb emergence, which suggests that older animals would be at the point of stabilizing their pattern in the field. Therefore, we recommend the body size of this animal, 13.2 mm SVL, as the minimum size at which the color pattern is reliable for photo-identification.

Diet of the juveniles during captivity may have affected their growth and ontogeny of the color patterns. We expect that animals in nature might be larger at the same age as our study subjects and may therefore develop the color pattern earlier. Diet is also important because of its relationship with skin toxins (Darst et al. 2005; Mebs et al. 2005; Hantak et al. 2013). Some species sequester skin toxins from their food, so it is possible that a shift in coloration depends on the acquisition of these toxins (Caorsi et al. 2014). We found that the appearance of the unken reflex and the development of the red coloration in hands, pelvic patch, and axillar spots were synchronous, and occurred earlier than the stabilization of the color patterns. The body parts that developed a red coloration are most visible during the unken reflex behavior of *M. montevidensis*. The belly pattern in some *Melanophryniscus* species is exhibited as part of the unken reflex (Toledo and Haddad 2009; Caorsi et al. 2014), but it does not seem to be involved in the defensive behavior of *M. montevidensis*. The belly pattern would not be subjected to the same selective pressures as the coloration, pelvic patch, or axillar spots and there may be no advantage to their early appearance. Further studies of early stages of the *Melanophryniscus* genus are needed to determine the role of diet in the development of coloration and the unken reflex behavior.

Finally, there have been no reports of juvenile recaptures in *M. montevidensis* (Pereira and Maneyro 2016b). Therefore, information about the early juvenile stages of *Melanophryniscus* species is scarce (Caorsi et al. 2012; Caorsi et al. 2014). Young juveniles usually have high mortality rates, which means few are in the environment, and because young use different environments than the adults, they are intrinsically difficult to find (Semlitsch 2008; Walston and Mullin 2008; Pereira and Maneyro 2016b), but it is also plausible that photo-identification methods are not effective when applied to early stages. The determination of the minimum size at which the color pattern stabilizes improves the accuracy of this marking method for juveniles. We consider visual implant elastomers, already tested in 6 mm juvenile Blue Crabs (*Callinectes sapidus*; Davis et al. 2004), as a complementary method to the photo-identification to identify younger and smaller individuals in population and life-history studies of *Melanophryniscus* species.

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to manipulate animals, provided by the Comisión Honoraria de Experimentación Animal (CHEA: http://chea.edu.uy/node/21).

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## Apéndice 2

Main features of the water bodies surveyed and additional biotic information of the experimental design. Average depth was measured the first day of experiment as the average of depths inside the enclosures within in each pond. Average water temperature, averaging temperature inside the enclosures of each pond, was presented for three time intervals of the development. Vegetation in the margins and inside the pond refers to the dominant families present in those parts of the ponds. Matrix refers to the dominant landscape of the water body. All sample sizes (number of enclosures) are available for biotic information. Predators were counted on days six-seven of the development. "Initial larval density" is the median number of larvae/liter per enclosure on day seven.

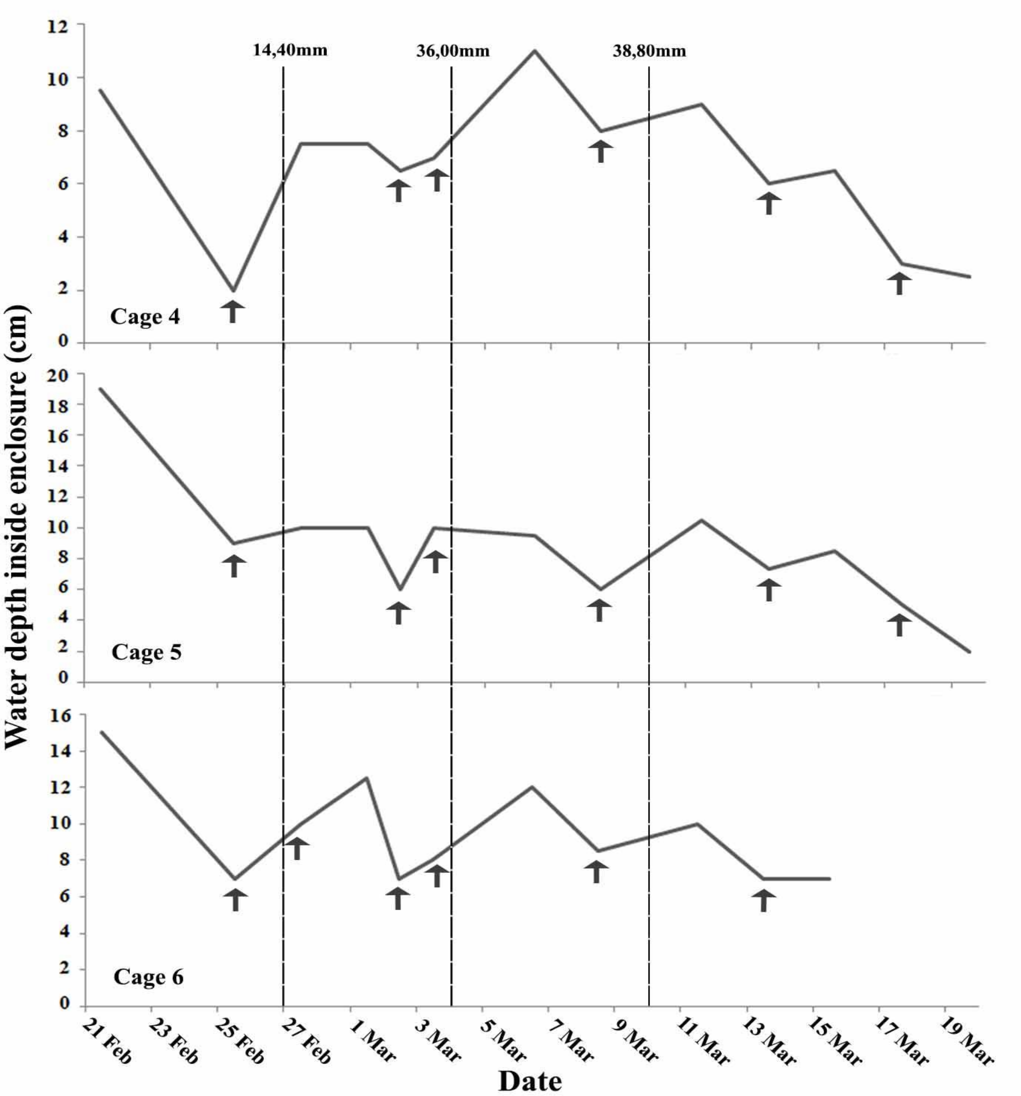
| | | Pond 1 | Pond 2 | Pond 3 |
|---|---|---|---|---|
| Location | | 34°40'0.8"S<br>054°13'57.1"W | 34°39'37.4"S<br>054°13'3.5"W | 34°32'39.4"S<br>054°19'20.6"W |
| Area ($m^2$) | | 3010.0 | 278.0 | 287.0 |
| Average depth (cm) | | 14.5 | 5.5 | 6.5 |
| Average temperature ± SD (°C) | Days 1–7 | 25.6 ± 3.9 | 26.4 ± 4.1 | 26.4 ± 3.8 |
| | Days 8–19 | 23.2 ± 3.0 | - | 23.6 ± 2.9 |
| | Days 20–29 | 23.0 ± 4.1 | - | 23.2 ± 2.5 |
| Vegetation in the margins | | - | Cyperaceae,<br>Juncaceae,<br>Poaceae | Poaceae |
| Vegetation inside the pond | | Cyperaceae,<br>Juncaceae | Cyperaceae,<br>Juncaceae | - |
| Matrix | | dune | dune | grassland |
| Median predators inside the enclosures (*n*) | | 19 (6) | 0 | 4 (9) |
| Total eggs laid during breeding events (*n*) | | 839 (6) | 1281 (10) | 1249 (9) |
| Initial larval density (*n*; min. – max.) | | 2.1 (4; 0.9 – 5.2) | - | 5.3 (9; 1.5 – 30.1) |
| Total of survivors until Gosner stage 42 (*n*) | | 5 (1) | 0 | 95 (7) |

# Apéndice 3

Weather conditions before and during the experiment for the three breeding events reported. Temperature in °C and rainfall in mm.

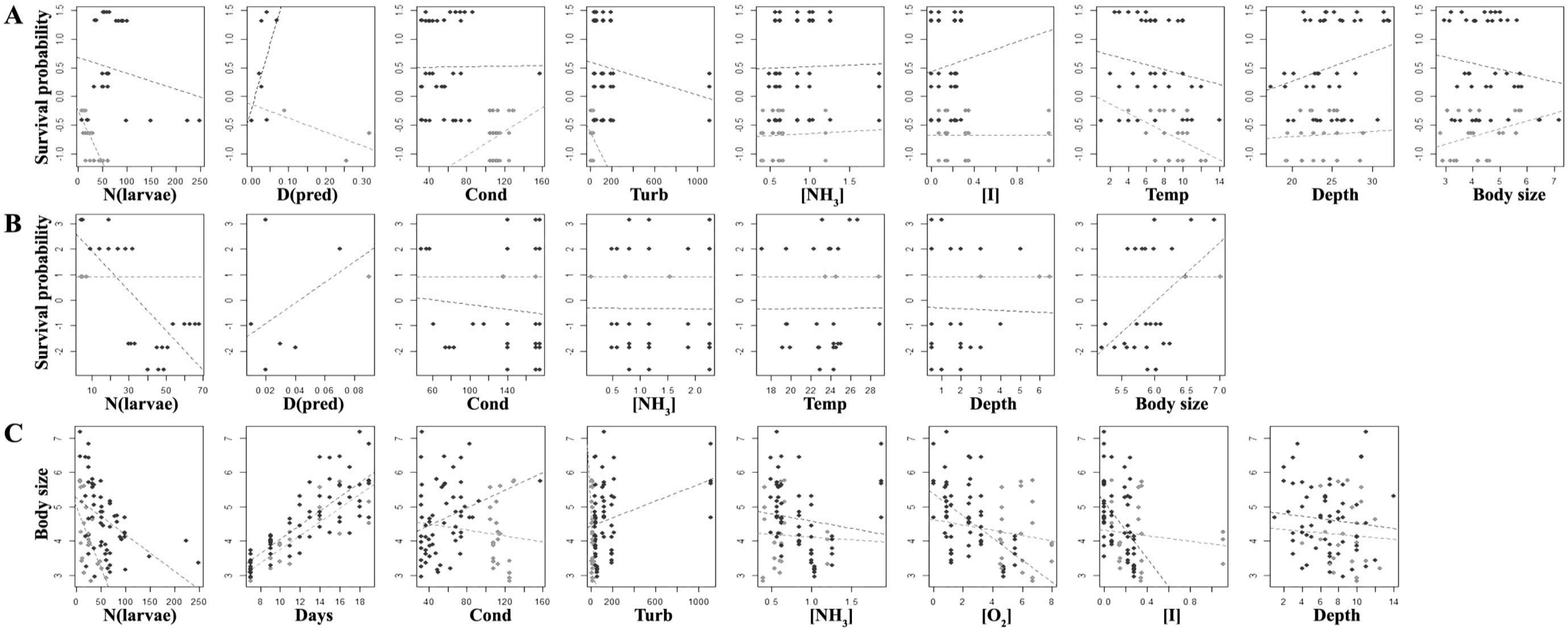
| | | First event | Second event | Third event |
|---|---|---|---|---|
| First day of activity reported | Date | 20 Feb 2014 | 27 Feb 2014 | 4 Mar 2014 |
| | Pond | 1 and 2 | 3 | 3 |
| | Mean day temperature | 18.8 | 18.7 | 21.3 |
| | Cumulative rain | 4.1 | 14.4 | 36.0 |
| 20 Days before (including activity day) | Mean temperature ± SD | 22.2 ± 2.1 | 21.5 ± 2.2 | 20.8 ± 2.0 |
| | Mean rain ± SD | 11.7 ± 16.6 | 13.6 ± 22.8 | 9.4 ± 19.6 |
| | Maximum rain | 68.6 | 83.1 | 83.1 |
| Days of experiment | Total days | 27 | 34 | 29 |
| | Mean temperature ± SD | 20.0 ± 1.2 | 19.7 ± 1.2 | 19.6 ± 2.0 |
| | Mean rain ± SD | 8.3 (18.5) | 3.6 ± 19.6 | 3.7 ± 9.6 |
| | Maximum rain | 83.1 | 38.8 | 22.7 |

# Apéndice 4



Water depth inside three enclosure cages from pond 1 along the study period. Solid line indicates the water level before moving each cage into a deeper part of the pond; small arrows indicate that the cage was moved on that day. The three most important rainfall events and their millimetres were included using dashed lines.

# Apéndice 5



Interactions with the different levels of site for predictors retained using the "select07" procedure. Pond 1 is gray, Pond 3 is black. N(larvae) is number of larvae inside the enclosures; D(pred) is predator density inside the enclosures; Cond are the conductivity levels; Turb are the turbidity levels; [NH$_3$] is the ammonia concentration; [I] is the iodine concentration; Temp is water temperature inside the enclosures; Depth is water depth inside the enclosures; Body size is the body length of larvae and metamorphs (mm); Days are the number of days since oviposition; [O$_2$] is the dissolved oxygen concentration. A) Survival probability *vs.* predictors in Period 2. Interactions (crossed lines of ponds) were only detected in the plot D(pred). B) Survival probability *vs.* predictors in Period 3. Crossed lines were observed in plots N(larvae) and Body size. C) Body size *vs*. predictors in Period 2. Interactions were observed in Cond, [O$_2$] and [I]. No interactions were detected for the GLMs of Period 1, and the interactions of the MLMs for Period 3 were not tested because only one pond was modeled (Pond 3); so graphs are not shown for these two analyses.

## Apéndice 6

Normal range of water quality parameters for sustainability of aquatic life obtained from bibliography.

| Parameter | Normal range | Source |
|---|---|---|
| pH | 6.5 – 8 | USEPA 1997 |
| Conductivity | 150 – 500 µS cm $^{-1}$ | USEPA 2005 |
| Turbidity | 0.25 – 5.25 FAU/NTU (maximum) | USEPA 2000; NYCRR 2017 |
| Ammonia | 0.03 mg $L^{-1}$ (maximum for salmonids) | USEPA 1986 |
|  | 0.6 mg $L^{-1}$ (maximum for amphibians) | Jofre and Karasov 1999 |
| Dissolved oxygen | 5 mg $L^{-1}$ (minimum for aquatic life) | USEPA 1997; Allen Burton Jr. and Pitt 2001 |
|  | 2 mg $L^{-1}$ (minimum for amphibians) | Mann and Bidwell 2001 |
| Nitrites | 5 mg $L^{-1}$ (maximum) | USEPA 1986 |
| Nitrates | 3 mg $L^{-1}$ (maximum to prevent eutrophication) | USEPA 1997 |
| Phosphates | 25 mg $L^{-1}$ (maximum to prevent eutrophication) | Allen Burton Jr. and Pitt 2001 |

## Apéndice 7

Descriptive statistics of predators and physicochemical parameters measured in each pond. Predators is the number of predators inside the enclosures; Temp is water temperature inside the enclosures; Cond and Turb are the conductivity and turbidity levels respectively; $[NH_3]$ is the ammonia concentration; $[O_2]$ is the dissolved oxygen concentration; [I] is the iodine concentration; $[NO_3^-]$ is the nitrate concentration; $[NO_2^-]$ is the nitrite concentration; $[PO_4^{3-}]$ is the phosphate concentration.

| | Predators (N) | Temp (°C) | pH | Cond ($\mu$S cm$^{-1}$) | Turb (FAU) | $[NH_3]$ (mgL$^{-1}$) | $[O_2]$ (mgL$^{-1}$) | [I] (mgL$^{-1}$) | $[NO_3^-]$ (mgL$^{-1}$) | $[NO_2^-]$ (mgL$^{-1}$) | $[PO_4^{3-}]$ (mgL$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pond 1 | | | | | | | | | | | |
| N data | 6 | 40 | 22 | 22 | 16 | 16 | 15 | 16 | 16 | 16 | 16 |
| min | 11 | 18.00 | 6.86 | 105.00 | 12.43 | 0.10 | 4.50 | 0.07 | 0.00 | 0.00 | 0.00 |
| max | 46 | 28.90 | 9.33 | 234.00 | 29.90 | 1.85 | 9.00 | 1.24 | 0.02 | 0.04 | 0.00 |
| median | 19 | 22.70 | 7.47 | 117.50 | 16.55 | 0.64 | 5.90 | 0.33 | 0.00 | 0.01 | 0.00 |
| mean | 24.17 | 23.10 | 7.56 | 126.27 | 17.64 | 0.67 | 5.94 | 0.38 | 0.00 | 0.01 | 0.00 |
| std.dev | 14.09 | 2.78 | 0.51 | 28.03 | 4.61 | 0.45 | 1.33 | 0.33 | 0.01 | 0.01 | 0.00 |
| Pond 2 | | | | | | | | | | | |
| N data | 10 | 15 | 7 | 7 | 5 | 5 | 3 | 3 | 4 | 4 | 3 |
| min | 0 | 21.90 | 5.18 | 215.00 | 119.80 | 4.08 | 0.00 | 0.14 | 0.00 | 0.00 | 0.00 |
| max | 0 | 25.90 | 6.17 | 302.00 | 495.15 | 7.79 | 0.00 | 0.96 | 0.00 | 0.00 | 0.00 |
| median | 0 | 24.30 | 6.10 | 224.00 | 380.12 | 7.20 | 0.00 | 0.14 | 0.00 | 0.00 | 0.00 |
| mean | 0 | 24.31 | 5.79 | 252.71 | 349.25 | 6.14 | 0.00 | 0.41 | 0.00 | 0.00 | 0.00 |
| std.dev | 0 | 1.04 | 0.45 | 40.93 | 138.13 | 1.74 | 0.00 | 0.47 | 0.00 | 0.00 | 0.00 |
| Pond 3 | | | | | | | | | | | |
| N data | 9 | 117 | 81 | 81 | 28 | 27 | 27 | 28 | 27 | 27 | 27 |
| min | 0.00 | 17.00 | 4.70 | 28.00 | 32.00 | 0.59 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| max | 10.00 | 32.10 | 7.18 | 174.00 | 1113.8 | 2.71 | 5.50 | 0.33 | 1.00 | 0.02 | 0.00 |
| median | 4.00 | 23.90 | 6.05 | 54.00 | 109.73 | 1.02 | 1.90 | 0.14 | 0.00 | 0.00 | 0.00 |
| mean | 4.11 | 23.97 | 6.15 | 64.26 | 227.80 | 1.26 | 2.09 | 0.14 | 0.04 | 0.00 | 0.00 |
| std.dev | 2.98 | 3.58 | 0.42 | 37.27 | 311.47 | 0.68 | 1.85 | 0.11 | 0.19 | 0.00 | 0.00 |

# Apéndice 8

Complete set of candidate models tested using Multi State Closed Robust Design for seasonal apparent survival probability (*S*), transition probability (Ψ), and capture probability (*p*) of *Melanophryniscus montevidensis*. Departing from the saturated model *S*(state*t)Ψ(state*t)*p*(state*t), simplified models were ordered by decreasing complexity. Subscripts: (state*season), state (females, males, juveniles) and seasonal (spring, summer, autumn, winter) variation; (t), time variation; (state), state variation only; (age), age variation (juvenile, adults [male and female together]); (state*breeding) state and breeding and non-breeding events variation; and (.), the parameter was held constant. All 32 models were built using the best *p* structure: *p*(t). Except for model 32, candidate models for the simplification of *p* are not shown (all possible structures of *p* holding [*S*(.)Ψ(.)], see Methodology in Capítulo IV). Only models 6, 12, 18, 22, 30, 32 provided valid estimations of all parameters, thus only those were ranked and averaged.

| Num. | Model description | Num. | Model description |
|---|---|---|---|
| 1 | *S*(state*t)Ψ(state*t)*p*(t) | 17 | *S*(state)Ψ(state*t)*p*(t) |
| 2 | *S*(state*t)Ψ(state)*p*(t) | 18 | *S*(state)Ψ(state)*p*(t) |
| 3 | *S*(state*t)Ψ(t)*p*(t) | 19 | *S*(state)Ψ(t)*p*(t) |
| 4 | *S*(state*t)Ψ(.)*p*(t) | 20 | *S*(state)Ψ(.)*p*(t) |
| 5 | *S*(state*season)Ψ(state*t)*p*(t) | 21 | *S*(age)Ψ(state*t)*p*(t) |
| 6 | *S*(state*season)Ψ(state)*p*(t) | 22 | *S*(age)Ψ(state)*p*(t) |
| 7 | *S*(state*year)Ψ(state*t)*p*(t) | 23 | *S*(age)Ψ(t)*p*(t) |
| 8 | *S*(state*year)Ψ(state)*p*(t) | 24 | *S*(age)Ψ(.)*p*(t) |
| 9 | *S*(state*hot-cold)Ψ(state*t)*p*(t) | 25 | *S*(t)Ψ(state*t)*p*(t) |
| 10 | *S*(state*hot-cold )Ψ(state)*p*(t) | 26 | *S*(t)Ψ(state)*p*(t) |
| 11 | *S*(state*breeding)Ψ(state*t)*p*(t) | 27 | *S*(t)Ψ(t)*p*(t) |
| 12 | *S*(state*breeding)Ψ(state)*p*(t) | 28 | *S*(t)Ψ(.)*p*(t) |
| 13 | *S*(age*t)Ψ(state*t)*p*(t) | 29 | *S*(.)Ψ(state*t)*p*(t) |
| 14 | *S*(age*t)Ψ(state)*p*(t) | 30 | *S*(.)Ψ(state)*p*(t) |
| 15 | *S*(age*t)Ψ(t)*p*(t) | 31 | *S*(.)Ψ(t)*p*(t) |
| 16 | *S*(age*t)Ψ(.)*p*(t) | 32 | *S*(.)Ψ(.)*p*(t) |