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Demographic and genetic parameters of two pond-breeding frogs in human-altered landscapes of the Brazilian Chaco

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Dedicated to my parents Marisa Paganini and Luiz Faggioni

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Table of contents

General abstract

Frogs are semiaquatic organisms that generally rely on water bodies for reproduction and tadpole development, but also depend on terrestrial habitats for multiple activities, such as sheltering, foraging, migration, and dispersal. Consequently, suitable terrestrial habitats surrounding water bodies are fundamental to fulfill the life requirements of many frog species. Conversely, the accelerated anthropogenic activities advancing over native vegetation are changing the composition and configuration of landscapes and splitting the aquatic and terrestrial environments. Throughout this dissertation I investigated the effects of human habitat modifications on the demographic and genetic parameters of two Neotropical pond-breeding frogs, *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc). Both species co-occur at the studied landscape in the Brazilian Chaco, municipality of Porto Murtinho, Mato Grosso do Sul state, but present distinct reproductive strategies and habitat preferences. In Chapter 1, I estimated the probabilities of habitat occupancy, detection, colonization, and local extinction through a multi-scale approach. My results showed that the conversion of native vegetation into pastures affected the probability of habitat occupancy by Lc, but had stronger negative effects on Lb parameters due to its terrestrial reproductive mode. In Chapter 2, in order to allow studies that investigate the effects of habitat modification on genetic parameters of Lb and Lc, I isolated and characterized microsatellite markers for both species. The new genetic markers developed were highly polymorphic and are among the few microsatellites available for studies of *Leptodactylus* species. In Chapter 3, I used these new genetic markers to look for evidences of bottlenecks and inbreeding, and also investigated the influence of the habitat modification on the functional connectivity among breeding ponds. I applied classic population genetic tests and also recent advances in theory and tools from landscape genetics, such as the quantitative parameterization

of resistance surfaces. Both species showed evidences of past (few generations) negative demographic impacts. My results indicated that the current landscape layout is narrowing the gene flow of Lb among breeding ponds, but is very permeable to Lc movements. In conclusion, the conversion of native vegetation in pastures imposes distinct effects on Lb and Lc populations. Lc uses any type of temporary ponds and moves across the pasture matrix. Lb is very dependent on forested habitats likely due to its reproductive mode. For this species, breeding ponds must be surrounded and connected by forested areas in order to ensure functional connectivity and reproduction. My dissertation highlights (1) the importance of the terrestrial habitat for semiaquatic frog species; (2) the links between species-specific characteristics and the consequences of landscape modifications; (3) the relevance of multi-species in landscape ecology studies; and (4) the usefulness of biological meaningful hypotheses based on species-specific characteristics.

Resumo geral

Rãs são organismos semiaquáticos dependentes de corpos d'água para reprodução e desenvolvimento dos girinos, mas que utilizam ambientes terrestres para procurar abrigo e alimento, assim como para migração e dispersão. Consequentemente, hábitats terrestres adequados devem estar distribuídos ao redor de corpos d'água para garantir o ciclo de vida completo da maioria das espécies de rãs. Na direção oposta, atividades antrópicas avançam sobre vegetações nativas, alterando a composição e configuração das paisagens e desconectando ambientes aquáticos e terrestres. Ao longo desta Tese, eu investiguei os efeitos da modificação antrópica da paisagem em parâmetros demográficos e genéticos de duas rãs Neotropicais que se reproduzem em poças temporárias, *Leptodactylus bufonius* (Lb) e *L. chaquensis* (Lc). As duas espécies coocorrem na paisagem de estudo, o Chaco brasileiro de Porto Murtinho, mas apresentam diferentes estratégias reprodutivas e preferências de hábitat. No Capítulo 1, eu estimei as probabilidades de ocupação de hábitat, detecção, colonização e extinção local através de análises em múltiplas escalas geográficas. Meus resultados mostraram que a substituição de vegetação nativa por pastos afetou a probabilidade de ocupação do hábitat por Lc, mas os efeitos mais fortes foram observados em Lb devido ao seu modo reprodutivo. No Capítulo 2, para possibilitar a investigação dos efeitos da modificação de hábitat em parâmetros genéticos de Lb e Lc, eu isolei e caracterizei marcadores microssatélites para as duas espécies. Os novos marcadores genéticos apresentaram alto polimorfismo e estão entre os poucos microssatélites disponíveis para o estudo de espécies do gênero *Leptodactylus*. No Capítulo 3, eu usei os novos marcadores genéticos para procurar por evidência de gargalos genéticos e endogamia, assim como para investigar a influência das modificações de hábitat na conectividade funcional entre as poças. Eu utilizei análises clássicas de genética de populações, assim como avanços recentes

na teoria e ferramentas de genética de paisagem, como a parametrização de superfícies de resistência baseada em dados quantitativos. As duas espécies apresentaram evidências de impactos demográficos passados (poucas gerações). Meus resultados indicaram que a configuração atual da paisagem parece estar limitando o fluxo gênico de Lb, mas não de Lc entre as poças. Em conclusão, meus resultados indicaram que a conversão de matas nativas em pastos afetou diferentemente as populações de Lb e Lc. Indivíduos de Lc utilizaram todos os tipos de poças e foram capazes de se movimentar através da matriz de pastos. Lb foi dependente de hábitats florestados devido ao seu modo reprodutivo. Para esta espécie, poças temporárias devem estar cercadas e conectadas por áreas florestadas para que se garanta a conectividade funcional e a reprodução. A minha tese ressalta (1) a importância de hábitats terrestres para espécies de rãs semiaquáticas; (2) as conexões entre características espécie-específicas e as consequências da modificação de hábitat; (3) a relevância de estudos com múltiplas espécies em estudos de ecologia de paisagem; e (4) a utilidade de hipóteses biologicamente informativas baseadas em características espécies-específicas.

General introduction

As semiaquatic organisms, many amphibian species aggregate in water bodies to reproduce, but also rely on the terrestrial habitats for aestivation, sheltering, foraging, migration, and dispersion (Pope et al. 2000, Gibbons 2003, Semlitsch & Bodie 2003, Becker et al. 2007, Allentoft & O'Brien 2010). Besides than being essential for the protection of the water resources, suitable terrestrial habitats surrounding water bodies are thus fundamental to fulfill the life requirements of amphibians (Semlitsch & Bodie 2003, Becker et al. 2007). Such a statement is of great meaning in a scenario where habitat loss and fragmentation, imposed by human activities, are among the main drivers of the global declines of amphibian populations (Stuart et al. 2004, Young et al. 2004). Roads, settlements, agriculture, livestock, and urbanization inevitably change landscapes' composition and configuration, potentially leading to direct and indirect effects in the ecology and evolution of amphibian species (Becker et al. 2007, Eigenbrod et al. 2008, Cosentino et al. 2014). Given the current rates of habitat modification, studies investigating the relationships between population parameters (e.g. demographic and genetic) and landscape changes are of great value.

Significant advances in the study of demography and landscape ecology of amphibians came from the development of dynamic site-occupancy models and the inclusion of landscape elements in population modeling. First, site-occupancy models have the advantage of estimating probabilities of site occupancy while accounting for species-specific detectability, i.e., the chance of detecting at least one individual of a focal species when the species is present at a site (MacKenzie et al. 2002, 2003). Because the detection of a species is always indicative of its presence, but the nondetection can either indicate that the species is absent or present but not detected, accounting for imperfect detection is an important feature of a parameter estimator

(MacKenzie et al. 2002, 2003, Schmidt 2005). Second, the inclusion of landscape elements at multiple spatial scales allows researchers to explore the magnitude of the interaction among the focal species and their surrounding environments (Pope et al. 2000, Zanini et al. 2009, Jackson & Fahrig 2014, Lescano et al. 2015). As a result, it is now well established that the detectability of most amphibian species is below 100%, a finding with important implications for conservation and monitoring programs (Cosentino et al. 2014, Weir et al. 2014). Moreover, although it is clear that disturbed landscapes usually impose negative effects on the demography of amphibians (Cushman 2006, Eigenbrod et al. 2008), such effects are complex, vary among species, geographic region, and scales (Zanini et al. 2009).

In addition to the direct effects in demographic parameters, landscape modifications will often affect the movement of individuals across the landscape (Fischer & Lindenmayer 2007, Holderegger & Wagner 2008). Thus, the maintenance or restoration of migration, dispersal, and gene flow across the landscape is high relevant for the long-term persistence of populations, especially for patchily distributed species, such as many pond-breeding amphibians (Holderegger & Wagner 2008, Zanini et al. 2009). However, due to financial costs and time associated with tracking animals in the field, estimating rates of exchange of individuals among populations is labor intensive (Bowne & Bowers 2004, Storfer et al. 2010). Therefore, genetic markers such as neutral microsatellite became widely used in population genetic studies and offer an alternative way to look for movement patterns across the landscape (Manel et al. 2003, Storfer et al. 2010). Due to the high mutation rate at neutral regions of the DNA, recent landscape modifications may be detected in the genetic profile of populations (Piry et al. 1999, Freeland 2005). For example, habitat alteration can decrease population sizes and accelerate the negative effects of genetic drift and inbreeding (Frankham 1995, 2005, Vos et al. 2001, Halverson et al. 2006). These processes

will eliminate some of the population's alleles and increase the fixation of others, leading to a reduced genetic diversity and heterozygosity (Andersen et al. 2004, Frankham 2005). Ultimately, it will result in patterns of reduced genetic diversity, strong genetic structure, and increased relatedness (Andersen et al. 2004, Frankham 2005, Halverson et al. 2006, Dixo et al. 2009, Allentoft & O'Brien 2010). However, microsatellite markers are generally species-specific and the development of new markers often precedes their application in population studies (Freeland 2005). Consequently, the isolation and characterization of new species-specific microsatellite markers are essential to conduct conservation genetic studies.

Technological advances and improvements in the genetic and spatial analytical tools have been expanding our ability to link habitat modification, demography, and genetic parameters. Noteworthy was the establishment of landscape genetics, an integrative discipline combining theories and tools from landscape ecology, population genetics, and spatial statistics (Manel et al. 2003, Habel et al. 2015). Landscape genetics aims to identify specific landscape and/or environmental features that facilitate or restrict the movement of individuals among populations, highlighting the links between structural and functional connectivity, usually assessed by analysis of microsatellite loci (Manel et al. 2003, Storfer et al. 2010, Manel & Holderegger 2013). For example, recent studies showed that the gene flow among populations of patchily-distributed amphibians can be predicted by species' movement abilities, ecological strategies, and physiological limitations across different habitat types (Richardson 2012, Peterman et al. 2014, Mims et al 2015, Nowakowski et al. 2015a). However, similarly to landscape ecology, landscape genetics studies have been found that population responses may vary among species, landscapes, and scales, precluding generalizations and models´ transferability (e.g. Mims et al. 2015).

Despite the great diversity of species and the alarming population declines in tropical regions (Stuart et al. 2004, Ricketts et al. 2005, Pyron & Wiens 2013, Newbold et al. 2014), we still have limited information about the landscape ecology and landscape genetics of tropical amphibians (Storfer et al. 2010, Zancolli et al. 2014). Moreover, the recognition that the influences of the landscape features are usually species- and landscape-specific does not make the task of conservation any easier (Zanini et al. 2009, Richardson 2012, Scherer et al. 2012). Therefore, studies including wide-ranging species, to which the effects of anthropogenic disturbance can be assessed at many different landscapes and scales, as well as multiple species studies, in which the species-specific ecological strategies are explicit considered in alternative hypotheses, can provide meaningful information for amphibian conservation (see references in Krug & Pröhl 2013 on *Hyla arborea* and Cosentino et al. 2015 on *Lithobates silvaticus*), leading to important generalizations and insights about the processes underlying the distribution, richness, abundance, and occupancy of amphibian species.

Therefore, the overall goal of my dissertation was to investigate the effects of human habitat modification on demography and genetic parameters of semiaquatic frog species. I present a case study of the impacts caused by the substitution of native vegetation by pastures on the probability of habitat occupancy, genetic diversity, and functional connectivity of two pond-breeding Neotropical frogs, *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc). Both species are sympatric at the studied landscape in the Brazilian Chaco, municipality of Porto Murtinho, Mato Grosso do Sul state, Brazil, and also co-occur in a large area of South America. However, Lb and Lc exhibit different reproductive strategies and habitat preferences, which allowed the formulation of biological informative hypotheses.

Specifically, in Chapter 1, I used site-occupancy models together with covariates measured at multiple scales to investigate the effects of habitat modification on the probability of habitat occupancy, colonization, and local extinction of Lb and Lc in temporary breeding ponds. Using multi-modeling inference, I identified key habitat features affecting the probability of pond occupancy by Lb and Lc and the scale of these effects. I discuss the importance of the terrestrial habitat for semiaquatic amphibians and the distinct effects imposed by human activities on frogs with contrasting ecological strategies. In chapter 2, to be able to assess the genetic diversity of Lb and Lc populations, I isolated and characterized several microsatellites markers for both species. The characterization included optimization of polymerase chain reactions and exploratory tests of polymorphism, heterozygosity, and genetic structure. I discuss the importance of the new genetic markers for population studies and the lack of microsatellite marker for species in the highly diverse genus *Leptodactylus*. In Chapter 3, I applied the new microsatellite markers and tools from population genetics, landscape genetics, and spatial statistics to look for signatures of bottlenecks and inbreeding. I also evaluated the effects of human habitat modifications on the functional connectivity among breeding ponds. I discuss the convergent and contrasting results found for Lb and Lc based on their ecological characteristics, with special attention for the processes shaping the genetic distribution of the specialist species Lb.

1. Multiscale impacts of landscape modifications on population parameters of two frog species in the Brazilian Chaco

Abstract

As semiaquatic organisms, frogs need aquatic and terrestrial parts of the landscapes to fulfill requirements of their contrasting life stages. Habitat loss and landscape modification caused by human activities are among the main causes of amphibian population declines worldwide, influencing population parameters of frogs from distances as far as 3,000m from the target water bodies. Here we investigated the scale of interaction of the frog species *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc) with the landscape surrounding wetlands (temporary ponds) and the consequences of the anthropogenic modifications of the landscape on probabilities of habitat occupancy, colonization, and local extinction by both species. We predicted that because of the specific habitat requirements for Lb reproduction and its association with more forested areas, landscape modifications will impose stronger negative effects on Lb than on Lc, considered to be a habitat generalist species. We used dynamic site-occupancy models and information-theory to estimate probabilities of detection, occupancy, colonization, and extinction of Lb and Lc in 50 temporary ponds of the Brazilian Chaco during two reproductive seasons. While Lb occupancy was dependent on pond and landscape characteristics, Lc occupancy was only affected by landscape characteristics. Scales of interactions between the studied species and the landscape indicated that the replacement of native vegetation by pastures is negatively impacting reproduction, habitat quality, migration, and dispersal of Lb. Colonization and local extinction between two consecutive reproductive seasons were not strongly affected by any of the habitat

covariates. Modification of the habitat caused a stronger negative effect on Lb populations due to specific habitat requirement for reproduction. We highlight that as observed for temperate amphibians, tropical frogs are not automatically protected by laws regarding the size of protection zones for riparian vegetation. For both species, detectability was strongly affected by time relative to sunset and may be taken into account whether delineating monitoring expeditions.

Resumo

As rãs são organismos geralmente associados a ambientes aquáticos, mas que também utilizam as porções terrestres das paisagens para diversas atividades durante todo seu ciclo de vida. A perda de hábitat e modificações da paisagem causadas por atividades humanas estão entre as principais causas do declínio global de anfíbios, influenciando a escolha de corpos d'água por rãs mesmo a 3.000m de distância. Desta forma, neste estudo nós investigamos a escala da interação entre duas espécies de rãs, *Leptodactylus bufonius* (Lb) e *L. chaquensis* (Lc), e a paisagem ao redor de corpos d'água (poças temporárias) com o objetivo de verificar se as modificações antropogênicas da paisagem estão afetando as probabilidades de ocupação, colonização e extinção local. Nós utilizamos modelos dinâmicos de ocupação de hábitat e Teoria da Informação para estimar as probabilidades de detecção, ocupação, colonização e extinção local em 50 poças temporárias no Chaco Brasileiro. Enquanto a ocupação de Lb foi dependente de características locais das poças e da paisagem, a ocupação de Lc foi apenas dependente de características da paisagem. As escalas de interação indicaram que a substituição e florestas nativas por pastos está influenciou a probabilidade de ocupação de habitat, a migração e dispersão de Lb. Colonizações e extinções locais não foram fortemente afetadas por covariáveis de hábitat entre duas estações reprodutivas consecutivas. Devido aos requerimentos específicos de hábitat para a reprodução, o impacto da modificação de hábitat é mais severo nas populações de Lb. Nós destacamos que assim como o observado para anfíbios de ambientes temperados, rãs de ambientes tropicais não estão necessariamente protegidas por leis delimitando o tamanho da área de proteção de matas ciliares. A detectabilidade das duas espécies foi fortemente influenciada pelo horário em relação ao pôr do sol e poderá ser ajudar no delineamento de futuras expedições de monitoramento.

Introduction

Landscape alterations, such as roads and farms, may decrease quality of the terrestrial habitats required by semiaquatic amphibians that reproduce in water bodies but use upland habitats while foraging, sheltering, migrating, and dispersing (e.g. Houlahan & Findlay 2003, Pellet et al. 2004, Becker et al. 2007, Eigenbrod et al. 2008, Cosentino et al. 2014). Accordingly, terrestrial variables distributed around rivers and ponds have been shown to be good predictors of frogs occupancy (e.g. Pellet et al. 2004, Eigenbrod et al. 2008), abundance (e.g. Houlahan & Findlay 2003, Lescano et al. 2015), and richness (e.g. Eigenbrod et al. 2008, Cosentino et al. 2014). Thus, it is not surprising that fragmentation and habitat loss caused by anthropogenic activities are among the main causes of amphibian declines worldwide (e.g. Young et al. 2001, Stuart et al. 2004, Young et al. 2004). Only in Brazil, many amphibian population declines have been reported and 56 species are nationally threatened (Eterovick et al. 2005, Haddad et al. 2008, Brasil 2014), most of them apparently related to habitat alterations (Silvano & Segalla 2005). Therefore, understanding the relationship between semiaquatic amphibians and the terrestrial environments surrounding water bodies is critical to inform management decisions for these threatened organisms.

The proportion of native forests is one of the most important abiotic factors determining the distribution of frogs in heterogeneous landscapes. By influencing the amount of leaf litter and sun light in the soil, pond scale canopy cover (i.e. trees and shrubs above and adjacent to the ponds) and forested areas in the landscape surrounding water bodies, strongly influence the quantity of nutrients input into water where tadpoles live and feed (Stoler & Relyea 2011, Provete et al. 2014), limit the distribution and abundance of understory plants such as cultivated grasses (Ludwig et al. 2001, Cole & Weltzin 2005), affect the distribution of invertebrates that

are food resources for adult frogs (e.g. McCauley et al. 2008), and sustain microclimates that reduce frogs desiccation and mortality particularly during migration and dispersal (Becker et al. 2007, Nowakowski et al. 2015a, Nowakowski et al. 2015b). Forested areas are also important for the structural and functional connectivity among ponds, affecting dynamic parameters such as colonization, extinction, migration, and gene flow (e.g. Adams et al. 2011, Nowakowski et al. 2015b). Additionally, because reproductive potential is one of the central parameters determining extinction thresholds and population persistence (Fahrig 2001, Peterman et al. 2013a), the availability and distribution of reproductive environments in the landscape (e.g. ponds or specific reproductive resources) are factors that may influence the distribution and connectivity of frogs (e.g. Rudolf & Rödel 2005, Heard et al. 2012).

Although it is clear that the complementation between the aquatic and terrestrial habitats is very important for the life cycle of amphibians (e.g. Cushman 2006, Becker et al. 2007), little is known about the scale of interaction between these organisms and their surrounding environment (Ficetola et al. 2009, Jackson & Fahrig 2014). A meta-analysis indicated that to fulfill all requirements of their life-history functions such as foraging and sheltering frogs need buffers between 200 m and 400 m radii of suitable habitats surrounding water bodies (Semlitsch & Bodie 2003). Other studies, however, highlighted that landscapes elements, such as native forests and roads, can influence frog's presence from as far as 3,000 m of the center of the water bodies, endorsing the importance of the landscape composition to longer migrations and dispersal (e.g. Houlahan & Findlay 2003, Ficetola et al. 2009). These findings are very important to guide future conservation plans and also allow the evaluation of currently conservation laws (Semlitsch & Bodie 2003). In Brazil, for example, only lotic water bodies of 200 m width or more must have at least 200 m of riparian vegetation (BRASIL 2012), resulting in reduced

protection for frog species associated to smaller water bodies whether we assume Semlitsch & Bodies' (2003) results.

However, most of the scientific knowledge on amphibian movements, habitat occupancy, and scale of interactions comes from data on temperate species (e.g. Semlitsch & Bodie 2003, Pellet et al. 2004, Denoël & Ficetola 2007, Eigenbrod et al. 2008, Zanini et al. 2008, Ficetola et al. 2009, Blomquist & Hunter Jr. 2010, Cosentino et al. 2014). Therefore, here we explored how landscape modifications are affecting population parameters of two Neotropical frog species with different habitat and reproductive requirements at the Brazilian Chaco. During the rainy season (from October to March), individuals of *Leptodactylus bufonius* and *L. chaquensis* aggregate in temporary ponds in order to reproduce employing distinct strategies (Crump 1995, Reading & Jofré 2003, Prado et al. 2005). *Leptodactylus bufonius* belongs to the *L. fuscus* group which includes species that reproduce in terrestrial mud chambers build by males at the bare soil in the periphery of the ponds wherein foam nests with eggs are deposited during amplexus (Heyer 1969, Crump 1995, Reading & Jofré 2003, Faggioni et al. 2011). Conversely, *L. chaquensis* belongs to the *L. latrans* group which includes species that form the foam nests direct on the surface of the water and thus do not require bare soil for mud chambers at ponds' shoreline (Heyer 1969, Prado et al. 2002). Although little information on habitat use is available for the studied species, *L. chaquensis* seems to use a wide range of habitats and ponds in open fields and shrublands (e.g. Areskoug 2001, Schaefer et al. 2006, Valdujo et al 2009), while *L. bufonius* is related to native shrubs and more forested areas (e.g. Areskoug 2001, Reading & Jofré 2003, Duré & Kehr 2004, Lescano et al. 2015).

Because replacement of native forests by cultivated pastures is among the main landscape alterations at the Brazilian Chaco (Tomas et al. 2015) our main goals were: (1) to identify key

habitat features driving species probabilities of occupancy, colonization, and local extinction of *L. bufonius* (Lb) and *L. chaquensis* (Lc) through site-occupancy dynamic models; (2) to determine the scale of interaction between the target species and their surrounding environment; (3) to investigate if the substitution of native forests by cultivated grasses is shaping population parameters of Lb and Lc; and (4) to investigate if specie-specific requirements related to habitat use and reproduction may result in stronger negative effects of the habitat alterations on Lb than on Lc populations. Our hypotheses were: (1) population parameters of Lb are influenced by the availability of bare soil and forested areas while Lc parameters will be dependent on shrublands; (2) the scale of interaction between species and the landscape is larger for Lb than for Lc, reflecting the higher permeability of the landscape for Lc migration and dispersal; (3) cultivated pastures are limiting the distribution of bare soil, shrubs, and forests in the landscape causing multiple scale impacts in Lb and Lc populations; and (4) habitat alterations are imposing stronger negative effects on Lb than on Lc populations due to its association to forested habitats in the landscape and bare soil for mud chambers (reproductive requirements) at the pond scale.

Methods

Study area

We conducted the fieldwork in the municipality of Porto Murtinho, Mato Grosso do Sul State, the only region in Brazil under the influence of the Chaco. The Gran Chaco encompasses almost 1,000,000 km^2, extending through four countries: Argentina, Paraguay, Bolivia and Brazil (Bucher & Huszar 1999, Pennington et al. 2000). Chacoan vegetation is composed by forests with shrubs, mainly of mimosoid species, and sparse herbaceous vegetation, mainly Bromeliaceae and Cactaceae, and some grass (Pennington et al. 2000). At the Brazilian Chaco,

the climate is “Aw” type according to Köppen (Alvares et al. 2013), with a hot rainy season from October to April and a dry season from May to September. During the rainy season, several temporary ponds are formed after sporadic rains where many frog species aggregate to reproduce (e.g. Faggioni et al. 2011, Schalk & Saenz 2016). Temporary ponds can hold water for a few days (ephemeral ponds) to many weeks, making them high variable in their persistence on the landscape (Schalk & Saenz 2016). Currently, replacement of native vegetation by cultivated pastures due to livestock practices is the main anthropogenic impact at the Chaco (Bucher & Huszar 1999, Souza et al. 2010). The Brazilian part of the Chaco is now reduced to about 13% of its original area, making it one of the most endangered ecoregions in Brazil (Tomas et al. 2015).

Sampling design

During the breeding seasons of 2012–2013 (S1) and 2013–2014 (S2), we conducted three visual surveys in 50 temporary breeding ponds (total of 300 surveys), located in private cattle farms in the municipality of Porto Murtinho (reference point:- 21.710079 ^{o} S, -57.721174^{o} W; Figure 1). Although at the study region summer rainfalls begin around September and last until March, we conducted our surveys between the months of October and February. Based on our experience with the target species and previous studies in the region (e.g. Prado et al. 2005, Souza et al. 2010, Faggioni et al. 2011), we believe that the occupancy status of breeding ponds was constant within this period. Ponds were systematically chosen in order to represent the full range of values from covariates of interest (e.g. from zero to 100% canopy opening). Distance between pairs of ponds ranged from 0.2 to 20 km. We conducted surveys between 1800 and 2400, during which two researchers actively searched for the target species for no more than 10 minutes by pond. At

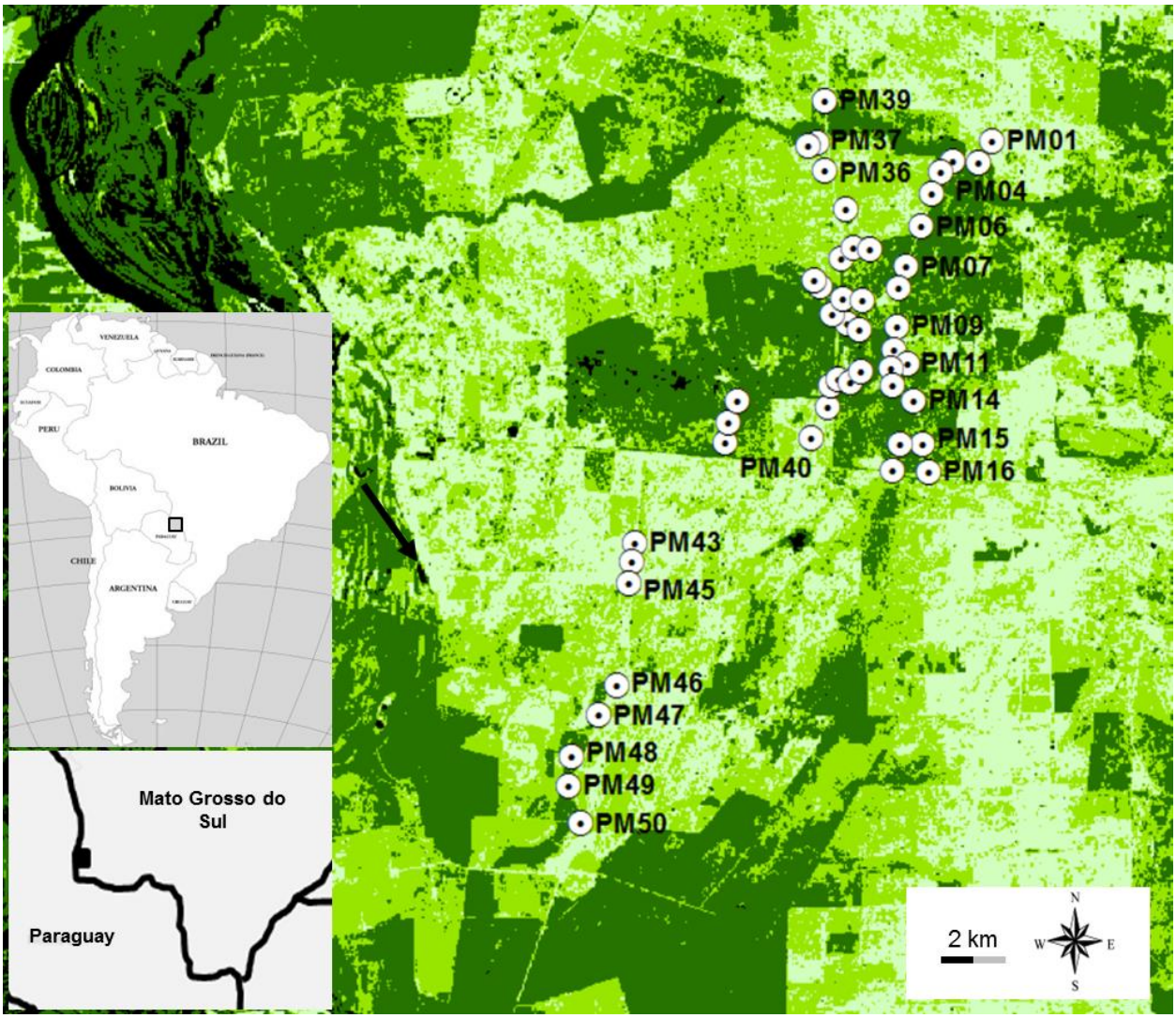

Figure 1 Location of the 50 temporary ponds (dots) sampled at the Brazilian Chaco in Porto Murtinho, Mato Grosso do Sul State, Brazil (some IDs are shown for geographic reference). Black: permanent water bodies (at left, Paraguay River and its flooded areas); dark green: forested areas; medium green: high density shrubs fields; light green: grass with sparse shrubs.

the end of a survey, both species were recorded as detected (1) or not detected (0) at the breeding pond, creating a detectability history for each species in every site. Estimates of population parameters of interest (Table 1) were calculated through site-occupancy dynamic models developed by MacKenzie et al. (2003).

Site-occupancy dynamic models

Given that many amphibians are cryptic, they may go undetected even when present (imperfect detection) in a site (e.g. Pellet & Schmidt 2005, Scherer et al. 2012). Site-occupancy dynamic models have the advantage of estimating population parameters while accounting for the probability of detecting the species in a site (MacKenzie et al. 2002, 2003). Estimating detectability is crucial not only to avoid biased calculation of population parameters (MacKenzie et al. 2006, Martin et al. 2009), but also to avoid incorrect conclusion regarding species' relation with covariates of interest (Gu & Swihart 2004, Mazerolle et al. 2005). To estimate detectability, site-occupancy models include sample covariates that can affect the chances of detecting the target species (MacKenzie et al. 2006). Sample covariates can vary significantly within in a season (e.g. sample effort, air temperature, or time of the day), and therefore are ideal to estimate detectability (MacKenzie et al. 2002, 2006). Conversely, site covariates should hold fixed values through the entire season, and are ideal to estimate the remaining parameters (MacKenzie et al. 2003, 2006). In order to estimate and correct for false-absence data, site-occupancy models require multiple sampling occasions in a period of time (season) short enough to ensure that local extinctions or colonization events do not occur (Gu & Swihart 2004, MacKenzie et al. 2006). So, during the whole season, sites are either occupied or unoccupied by the focal species (Mackenzie et al. 2006).

Table1 Parameters of interest, respective symbols, and applied definitions.

Parameter	Symbol	Definition
Detectability	p	Probability of detecting the species when it is present in a pond
Occupancy	ψ	Probability that a species is occupying a pond
Local colonization	γ	Probability that an unoccupied pond in S1 is occupied in S2
Local extinction	ε	Probability that an occupied pond in S1 is unoccupied in S2
Constant parameters	$\hat{p}$ (.), $\widehat{\psi}$ (.),$\hat{\gamma}$ (.), $\hat{\varepsilon}$ (.)	^ denotes parameters estimates without covariates (.)

Pond-scale: sample and site covariates

Covariates considered to potentially affect our chances to detect any of both species in a breeding pond were: (1) minutes relative to sunset (MRS); (2) air temperature (AT); (3) water temperature (WT); (4) water perimeter in the pond (WP); and (5) relative water depth (RD). We used a thermo-hygrometer to measure AT and WT, a GPS to measure water perimeter and minutes relative to sunset, and a metric tape to measure water depth in five points (center of the pond plus four points at one meter from the shoreline on north, south, east and west limits of the pond). For each survey, the value of RD for a pond was calculated as the ratio between observed depth at the sampling moment and maximum possible depth during the whole season and is a proxy for recent rainfalls. We expected that detectability of both species is less than 100% and related to sunset time and environmental conditions due to the nocturnal reproductive behavior of Lb and Lc. Relative water depth may also be an important predictor of species detectability (see predictions of sample covariates effects on detectability in Table 2). Site covariates at the pond scale considered to potentially influence parameters of interest were: (1) percentage of grass (Gr); (2) percentage of bare soil (BS); (3) percentage of leaf litter (LL); (4) percentage of shrubs (Shr); and (5) canopy opening (CO). Pond scale covariates Gr, BS, LL, and Shr were visually estimated always by the same person (GPF) along four transects of 10m (north, south, east and west), starting perpendicularly to the pond shoreline. The covariate Gr is represented by cultivated pastures. Canopy opening was measured with a convex densiometer at the center of the pond and at the end of each transect. All covariates measured in more than one point were averaged before entering the data set.

Table 2 Predictions of the covariate effects on detectability, occupancy, colonization, and extinction of *Leptodactylus bufonius* and *L. chaquensis* in 50 temporary ponds at the Brazilian Chaco. Pond scale covariates (BS, Gr, Shr, and CO) are predicted to affect only *L. bufonius* while landscape scale covariates (GrSc and Fd) are predicted to affect both species. MRS: minutes relative to sunset; AT: air temperature; WT: water temperature; WP: water perimeter; RD: relative depth; Gr: percentage of grass; BS: percentage of bare soil; LL: percentage of leaf litter; Shr: percentage of shrubs; CO: canopy opening; PWB: percentage of permanent water bodies; For: percentage of forested areas; Fd: percentage of high density shrubs fields; GrSc: percentage of grass with sparse shrubs. Gr and GrSc represent cultivated pastures. See text for details about covariates.

	Predictions	
Covariate	$\hat{p}$	$\hat{\psi}$, $\hat{\gamma}$, and $\hat{\varepsilon}$
MRS	Higher values of MRS increase detectability	-
AT	Higher temperatures increase detectability	-
WT	Lower temperatures decrease detectability	-
RD	Higher values of RD increase detectability	-
WP	Higher values of WP increase detectability	-
BS	-	Increasing percentage of BS around ponds increases occupancy and colonization, but decreases extinction
Gr	-	Increasing percentage of Gr around ponds decreases occupancy and colonization, but increases extinction
Shr	-	Increasing percentage of Shr around ponds increases occupancy and colonization, but decreases extinction
CO	-	Increasing percentage of CO around ponds decreases occupancy and colonization, but increases extinction
LL	-	Increasing percentage of LL around ponds increases occupancy and colonization, but decreases extinction
PWB*	-	Increasing percentage of PWB around ponds increases occupancy and colonization, but decreases extinction
GrSc*	-	Increasing percentage of GrSc around ponds decreases occupancy and colonization, but increases extinction
Fd*	-	Increasing percentage of Fd around ponds increases occupancy and colonization, but decreases extinction
For*	-	Increasing percentage of For around ponds increases occupancy and colonization, but decreases extinction

* Predictions are the same for all seven scales (30, 100, 400, 700, 1000, 1300, and 1600 m buffer radii; see text).

Multiscale covariates and spatial analyses

Landscape covariates were chosen based on the same criteria used for pond scale covariates, reflecting currently anthropogenic landscape modifications. In order to quantify landscape covariates we used a Landsat 8 satellite image of 30 x 30 m spatial resolution (EarthExplorer 2014) from August 2014. Using Geomatica (PCI 2012), we classified each pixel at the original image as one of the four chosen landscape covariates (see below). GPS control points together with scaled pictures took at the studied landscape were used as validation method. Next, to investigate the geographic scale of species' response (scale of effect *sensu* Jackson & Fahrig 2014) in occupancy, detectability, extinction, and colonization, we delimited seven concentric buffers of hierarchical radii length (30, 100, 400, 700, 1000, 1300, and 1600m) around each pond in ArcMap v10.3 (ESRI 2014). We delimited our larger buffer size according to results from previous studies (e.g. Semlitsch & Bodie 2003, Pellet et al. 2004, Hartel et al. 2010, Cayuela et al. 2012, Scherer et al. 2012, Jackson & Fahrig 2014). Regardless the overlap in concentric buffers, each circle covered its input area plus the area of any smaller buffer. Using ArcMap v10.3 (ESRI 2014), we calculated the percentage of area covered by four soil-cover classes inside each buffer around every pond: (1) percentage of permanent water bodies (PWB); (2) percentage of native forests (For); (3) percentage of high density shrub fields (Fd, mostly in set-aside pastures); and (4) percentage of grass with sparse shrubs (GrSc) representing areas of cultivated pastures (see predictions of landscape site covariates effects on occupancy, colonization, and extinction in Table 2).

Exploratory data analyses and final data set

Our first exploratory dataset included five samples and nine site covariates (four site covariates at multiple scales). Because our sample size was relatively small for the expected maximum number of estimated parameters (11 parameters in full models for 50 ponds, but see details below), we conducted exploratory analyzes prioritizing the reduction in the number of covariates. We excluded WP and WT from the sample covariates because of high number of sampled zeros. Spearman's correlations (r) among remaining sample covariates MRS × AT, MRS × RD, and AT × RD were low (r = -0.3, -0.2, and 0.2, respectively). We excluded LL from the site covariates because of high number of sampled zeros. Spearman's correlations among site covariates at the pond scale were relatively high ($r \geq \pm 0.6$) for Gr × BS (r = -0.8), Gr × CO (r = 0.6), and CO × Shr (r = -0.6). Correlation values were relatively low for CO × BS (r = -0.4), Shr × BS (r = 0.3), and Shr × Gr (r = -0.5).

We excluded PWB (at all scales) from the landscape covariates because of high number of sampled zeros. Because our modeling approach (see below) did not assume independence of the regressors from different scales (e.g. Mazerolle et al. 2005, Jackson & Fahrig 2014), we did not exclude covariates based on correlation coefficients of the same features (e.g. percentage of GrSc) among hierarchical buffers. While looking for the scale of an effect, assumptions about low correlation among covariates in hierarchical scales are unnecessary (Jackson & Fahrig 2014). This criterion can lead to an imprecise estimate of scale of effect and is one of the reasons for the relatively low number of buffer radii investigated in ecological studies (Jackson & Fahrig 2014). We excluded the variable Forest from the landscape covariates (at all scales) because of its high correlation value with GrSc through all the seven scales (range = -0.6 to -0.9) and with Fd at the three larger scales ($r \geq 0.6$ at 1000, 1300, and 1600m buffers). Correlation values among GrSc × Fd were low through all the seven scales (range = 0.1 to 0.5). After exploratory

analyses, our final data set was composed of three sample covariates (MRS, AT, and RD), four pond scale site covariates (BS, Gr, Shr, and CO) and two landscape scale site covariates (GrSc and Fd). All analyses were conducted in R 3.0.3 (R Development Core Team 2014), through the package "Hmisc" (Harrell Jr 2016).

Occupancy modeling and posterior analyses

Covariates were standardized by subtracting the mean and dividing by the standard deviation (e.g. Peterman et al. 2013a). Response parameters were estimated independently for each species. To investigate the relationships among occupancy, colonization, and local extinction and environmental and habitat covariates, we conducted information-theoretic (IT) approach followed by multi-model inference (MMI; Burnham and Anderson 2002, Mazerolle 2006, 2015a). Candidate models were fitted to the data and ranked according to Akaike's Information Criterion with a second-order bias adjustment (AICc = sample size/number of parameter ≤ 40; Burnham & Anderson 2002, MacKenzie et al. 2006). We assessed the goodness-of-fit of each of the best ranked models (including all general models) by comparing models' squared standard error (SSE) with a posterior random distribution of SSEs after 10,000 bootstraps (Burnham & Anderson 2002, MacKenzie et al. 2006). We were confident of a strong covariate effect on the response parameters if 95% of the unconditional confidence (95% Unc. CI) interval of the estimated regressor did not overlap zero (Mazerolle 2006, 2015a). We followed eight steps for modeling response parameters: (1) formulation of a set of candidate models and biological hypotheses related to response parameters and covariates (Table 3). Correlated covariates were never added to the same model; (2) modeling of all possible combination of covariates for detectability plus a constant model; (3) modeling of pond scale habitat occupancy upon the best ranked model of detectability plus a constant model; (4) modeling of pond scale colonization for

Table 3 Candidate set of habitat occupancy models for probability of detection, occupancy, colonization, and extinction of *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc) in 50 temporary ponds at the Brazilian Chaco. A summary of the biological hypotheses are presented. See text for details.

Parameter	Model	Biological hypotheses
$\hat{p}$	Constant	No effects of the sampled covariates
	MRS	Effect of survey time only (time of activity)
	AT	Effect of temperature on frogs activities only (Lc > Lb)
	RD	Effect of recent rains on frogs activity only (Lc > Lb)
	MRS+AT	Effect of survey time and temperature
	MRS+RD	Effect of survey time and recent rains
	AT+RD	Effect of air temperature and recent rains
	MRS+AT+RD	Effect of survey time, air temperature, and recent rains
Pond scale $\widehat{\psi}$, $\hat{\gamma}$, and $\hat{\varepsilon}$	Constant	No effects of the sampled covariates (expected for Lc)
	BS	Effect on Lb related to its reproductive mode
	Gr	Effect on Lb related to its reproductive mode
	Shr	Effect on both species related to local sheltering and foraging
	CO	Effect on Lb related to local sheltering and foraging
	BS+Shr	Effect on Lb related to reproduction and sheltering
	BS+CO	Effect on Lb related to reproduction and sheltering
	Gr+Shr	Effect on Lb related to reproduction and sheltering
Landscape scale $\widehat{\psi}$, $\hat{\gamma}$, and $\hat{\varepsilon}$*	Constant	No effects of the sampled covariates
	GrSc	Effect on Lb related to sheltering, foraging, and migration
	Fd	Effect on Lc related to sheltering, foraging, and migration
	GrSc+Fd	Effect on both species related to sheltering, foraging, and migration

* Models were repeated for all seven scales (30, 100, 400, 700, 1000, 1300, and 1600m buffer radii).

all covariates combination upon the best ranked model of occupancy while keeping local extinction constant, plus a constant model; (5) modeling of pond scale extinction for all covariates combination upon the best ranked model of occupancy while keeping local colonization constant, plus a constant model; (6) modeling of landscape general models (n = seven models). From step #6 we selected models that together summed 95% probability of containing the best scale (cumulative AICc weighted ($AIC_c w$) = 0.95). Following this approach, we were able to reduce the number of scales modeled in downstream analyses while keeping the most informative buffers; (7) we repeated steps #2 to #4 independently for each selected scale (n = four models for each scale); (8) MMI: calculation of the unconditional 95% confidence interval of the covariates regressors from models with $\Delta AICc \leq 2$ and/or above the constant models. For detectability and landscape scale estimates, but not for pond scale estimates, every set of candidate models presented the same number of models with *vs.* without each covariate, allowing us to calculate covariates importance (ranging from zero to unit) based on multi-modeling approach (Burnham & Anderson 2002, Mazerolle 2006, 2015a). All analyses were conducted in R 3.0.3 (R Development Core Team 2014), using the packages "unmarked" (Fiske et al. 2015) and "AICcmodavg" (Mazerolle 2015b).

After modeling and before any further interpretation of the results, we looked for possible spatial autocorrelation (SA) (e.g. Legendre 1993, Borcard & Legendre 2002). In order to verify whether SA was present in our system (i.e. closer ponds forming clusters of high or low probability of occupancy), we used the function "spline.correlog" from the package "ncf" (Bjornstad 2013) to calculate Moran's *I* and the respective standard error (SE) across a continuous class of Euclidian distances after 10,000 bootstraps (Legendre 1993, Zanini et al. 2008). We excluded chances of biased results due to SA because bootstrapped distribution along

Euclidian distance never excluded zero. All analyses were conducted in R 3.0.3 (R Development Core Team 2014).

Results

Detectability

We found no evidences of lack of fit for the models. *Leptodactylus bufonius* and *L. chaquensis* showed similar detection probabilities (Lb: $\hat{p} \pm SE = 52\% \pm 5$; Lc: $\hat{p} \pm SE = 59\% \pm 4$) and there was low uncertainty among detectability models for both species (one and two models with $\Delta AICc \leq 2$ for Lb and Lc, respectively; Table S1). The highest-ranked model indicated that minutes relative to sunset (MRS) was the main covariate affecting detectability of both species whereas there was also a weak influence of the ponds' relative depth (RD) in Lc probability of detection (Table S1). Our multi-model inference, however, indicated that only minutes relative to sunset was a good predictor of both species detectability (MRS in Lb: 95% Unc. CI = 0.39 – 1.15; MRS in Lc: 95% Unc. CI = 0.47 – 1.05; RD in Lc: 95% Unc. CI = -0.08 – 0.45; Tables 4 and 5; Figure 2).

Pond scale models: occupancy, colonization, and extinction

The proportion of sites occupied by Lb in S1 was 55% ($\widehat{\psi} \pm SE = 0.55 \pm 0.08$). From the set of candidate models for local occupancy estimates, three resulted in $\Delta AICc \leq 2$ (Table S1). Bare soil (BS) was present in all three models; shrubs (Shr) and canopy opening (CO) were present in one model each. The percentage of grass was in the first model with $\Delta AIC \geq 2$ and was also included in the multi-model inference (Table S1). Our results indicated that the percentage of bare soil had a positive effect on Lb probability of habitat occupancy while the percentage of

Table 4 Multi-model inference evaluated for covariates present at the highest-ranked models of detectability, occupancy, colonization, and extinction of *Leptodactylus bufonius* in 50 temporary ponds at the Brazilian Chaco. Covariates with strong effects on response parameters (i.e. unconditional 95% confidence interval excluded zero) in bold. Numbers sided to landscape scale covariates denote the length of buffer radii. Mod. Avg. = model-averaged regressor estimates; Unc. SE = unconditional squared errors (precision); Unc. 95% CI = unconditional 95% confidence interval.

Scale	Parameter	Covariate	Importance*	Mod. Avg.	Unc. SE	Unc. 95% CI
Unconditional to scale	$\hat{p}$	**MRS**	1.00	0.77	0.20	0.39 –1.15
Pond scale	$\widehat{\psi}$	**BS**	-	1.24	0.44	0.38 – 2.11
		Gr	-	-1.16	0.47	-2.08 – -0.25
		Shr	-	0.96	0.64	-0.3 – 2.21
		CO	-	-0.62	0.42	-1.43 – 0.2
	$\hat{\gamma}$	CO	-	-1.07	0.95	-2.94 – 0.8
	$\hat{\varepsilon}$	BS	-	-1.49	1.01	-3.47 – 0.49
		Shr	-	0.38	0.51	-0.62 – 1.39
Landscape scale	$\widehat{\psi}$	**GrSc400**	0.93	-1.01	0.42	-1.84 – -0.18
		GrSc700	0.98	-1.3	0.52	-2.33 – -0.28
		GrSc1000	0.99	-1.43	0.57	-2.55 – -0.30
		GrSc1600	0.97	-1.19	0.49	-2.15 – -0.23
	$\hat{\gamma}$	GrSc400	0.35	0.71	10.28	-58.92 – 20.86
		GrSc1000	0.59	-65.25	93.82	-249.13 – 118.62
		Fd400	0.33	-11.25	24.32	-58.92 – 36.42
		Fd1000	0.57	22.87	34.7	-45.14 – 90.88
	$\hat{\varepsilon}$	GrSc1600	0.47	-5.9	4.97	-15.64 – 3.84
		Fd1600	0.57	2.1	1.59	-1.02 – 1.58

*Importance can only be calculated when the number of models with *vs* without the covariate of interest are the same.

Table 5 Multi-model inference evaluated for covariates present at the highest-ranked models of detectability, occupancy, colonization, and extinction of *Leptodactylus chaquensis* in 50 temporary ponds at the Brazilian Chaco. Covariates with strong effects on response parameters (i.e. unconditional 95% confidence interval excluded zero) in bold. Numbers sided to landscape scale covariates denote the length of buffer radii. Mod. Avg. = model-averaged regressor estimates; Unc. SE = unconditional squared errors (precision); Unc. 95% CI = unconditional 95% confidence interval.

Scale	Parameter	Covariate	Importance*	Mod. Avg.	Unc. SE	Unc. 95% CI
Unc. to scale	$\hat{p}$	**MRS**	1.00	0.76	0.15	0.47 – 1.05
		RD	0.41	0.18	0.14	-0.08 – 0.45
Pond scale	$\widehat{\psi}$	Gr	-	34.99	47.24	-57.59 – 127.58
		Shr	-	-3.95	6.66	-17.01 – 9.11
	$\hat{\gamma}$	BS	-	27.62	36.25	-43.43 – 98.66
		Shr	-	-15.27	82.67	-177.3 – 146.76
		Gr	-	-1.41	37.1	-74.12 – 71.31
		CO	-	3.08	5.33	-7.37 – 13.52
	$\hat{\varepsilon}$	Gr	-	-24.49	34.39	-91.88 – 42.9
Landscape scale	$\widehat{\psi}$	**Fd400**	0.96	2.4	1.14	0.16 – 4.65
		Fd700	0.74	1.5	0.84	-0.14 – 3.14
		Fd1000	0.56	1.17	0.8	-0.41 – 2.74
		Fd1300	0.58	1.33	1.05	-0.73 – 3.39
		GrSc1000	0.32	-0.49	0.49	-1.46 – 0.48
		GrSc1300	0.39	-0.61	0.47	-1.53 – 0.31
	$\hat{\gamma}$	Fd400	0.75	-28.38	45.16	-166.86 – 60.13
		Fd700	0.86	-22.85	38.88	-99.06 – 53.36
		Fd1000	0.73	-20.99	38.90	-97.22 – 55.24
		Fd1300	0.55	-7.38	14.99	-36.76 – 22.01
		GrSc1000	0.36	4.35	13.00	-21.12 – 55.24
		GrSc1300	0.28	1.98	5.75	-9.29 – 13.25
	$\hat{\varepsilon}$	Fd400	0.78	3.19	3.03	-2.75 – 9.13
		Fd700	0.80	-	-	-
		Fd1000	0.81	18.07	30.74	-42.17 – 78.32
		Fd1300	0.70	16.24	27.64	-37.93 – 70.41
		GrSc700	0.63	61.83	73.48	-82.19 – 205.85
		GrSc1000	0.56	31.56	48.90	-64.29 – 127.41
		GrSc1300	0.39	11.31	20.64	-29.14 – 51.76

*Importance can only be calculated when the number of models with *vs* without the covariate of interest are the same. - NAs produced during calculations.

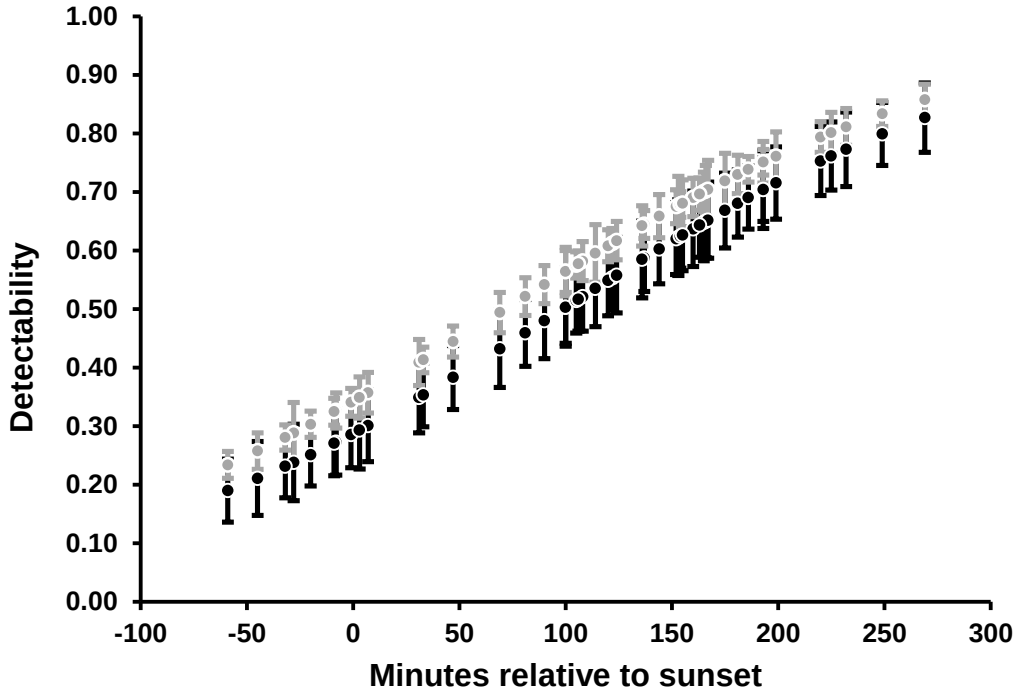

Figure 2 Detectability of *Leptodactylus bufonius* (black) and *L. chaquensis* (gray) as a function of sunset time in 50 temporary ponds at the Brazilian Chaco. Bars are squared errors. Models are the same for both species: detectability dependent on MRS ($\hat{p}$ (MRS)). Zero represent the sunset time.

grass imposed negative impacts (BS in Lb: 95% Unc. CI = 0.38 – 2.11; Gr in Lb: 95% Unc. CI = -2.08 – -0.25; Table 4, Figure 3). The 95% Unc. CI of the percentage of shrubs and canopy opening overlapped zero, indicating that these variables did not influenced Lb occupancy at the pond scale (Table 4). Conversely, the proportion of sites occupied by Lc in S1 was 89% ($\widehat{\psi} \pm SE = 0.89 \pm 0.06$). From the rank of Lc occupancy models, we selected Gr and Shr for multi-model inference (Table 5). The unconditional 95% CI of Gr and Shr overlapped zero (Table 5). Because the constant model for the probability of occupancy was among the best ranked (Table S1), we assumed that Lc occupancy was not affected by any of the pond scale variables.

Although one covariate for colonization (CO) and two covariates for extinction (BS and Shr) were present in models with $\Delta AIC \leq 2$ for Lb (Table S1), their unconditional 95% CI overlapped zero (Table 4) indicating no effects of these variables on Lb parameters. For Lc, the variables BS, Gr, Shr, and CO were all present in models ranked above the constant model for colonization, while only the model with Gr ranked above the constant model for extinction (Table S1). Our multi-model analysis, however, showed that the 95% unconditional CI of all variables overlapped zero, discarding any effect of the investigated variables on the colonization and extinction of Lc at the pond scale (Table 5).

Selection of the landscape scales

There was no evidence of lack of fit for the models. From the set of seven candidate general models for estimating landscape occupancy, colonization, and extinction, we selected four models for each species on the basis of cumulative probability of 95% of containing the best model as a subset of the general model. Selected scales were 400, 700, 1000, and 1600m for Lb and 400, 700, 1000, and 1300m for Lc (Table 6).

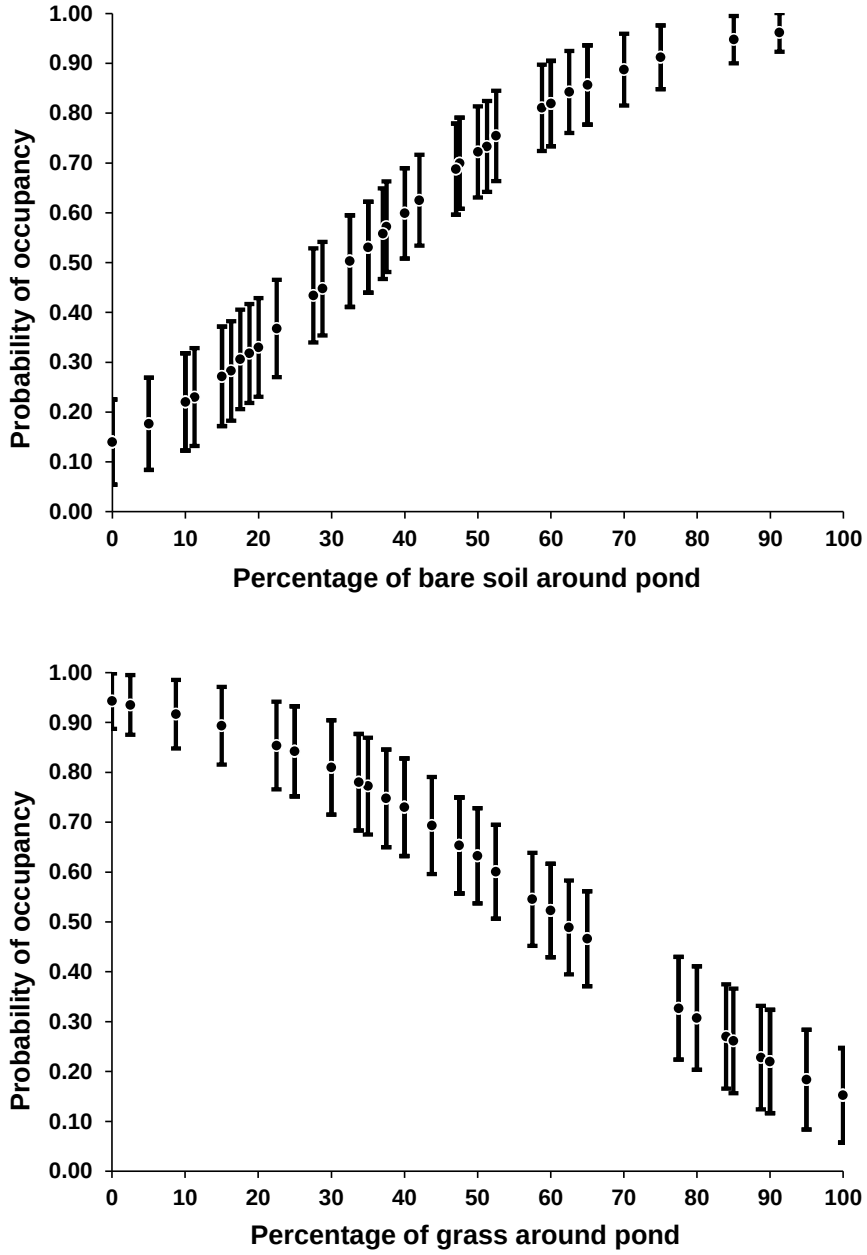

Figure 3 Pond scale probability of habitat occupancy by *Leptodactylus bufonius* in 50 temporary ponds at the Brazilian Chaco. Above: as a function of percentage of bare soil surrounding ponds. Below: as a function of percentage of grass surrounding ponds. Bars are squared errors.

Table 6 General landscape scale model selection table for probabilities of detection, occupancy, colonization, and extinction of *Leptodactylus bufonius* and *L. chaquensis* in 50 temporary ponds at the Brazilian Chaco. For every scale, general models were: $\hat{\psi}$ (GrSc+Fd) $\hat{\gamma}$ (GrSc+Fd) $\hat{\varepsilon}$ (GrSc+Fd) $\hat{p}$ (MRS). K = number of parameters; ΔAICc = Akaike's Information Criterion with the second bias adjustment; AICc*w* = AICc weighted; Cml. *w* = cumulative weight. Bold: Landscape scales selected for downstream analyses based on Cml. *w* of 0.95.

Species	Model	K	ΔAICc	AICc*w*	Cml. *w*
L. bufonius	**1000**	11	0.00	0.59	**0.59**
	1600	11	2.34	0.18	**0.77**
	400	11	3.57	0.10	**0.87**
	700	11	4.05	0.08	**0.95**
	1300	11	6.17	0.03	0.98
	30	11	7.14	0.02	0.99
	100	11	8.70	0.01	1.00
L. chaquensis	**700**	11	0.00	0.42	**0.42**
	1000	11	0.98	0.26	**0.68**
	400	11	2.00	0.16	**0.84**
	1300	11	2.46	0.12	**0.96**
	100	11	6.12	0.02	0.98
	1600	11	6.27	0.02	1.00
	30	11	14.66	0.00	1.00

Landscape scale models: occupancy, colonization, and extinction

The probability of habitat occupancy by Lb was negatively influenced by the proportion of grass (cultivated pastures) surrounding ponds in buffers of 400, 700, 1000, and 1600m (Table S2). Our multi-model inference showed that the importance of GrSc was high at all scales (Table 4). Accordingly, the unconditional 95% CI of GrSc excluded zero in all four scales and reached its stronger negative effect on occupancy at 1000m radii buffer (Table 4, Figure 4). For Lc, we selected the covariate Fd at all four scales and GrSc from 1000 and 1300m radii buffer (Table S3) for the multi-model inference (Table 5). Importance of Fd through the four scales was greater than 0.50, but reached its maximum value at 400m radii buffer (0.96, Table 5). Unconditional 95% CI of Fd excluded zero only at 400m scale, confirming the positive effect of shrublands on Lc occupancy at that scale (Table 5, Figure 5). For the remaining scales, unconditional 95% CI of Fd overlapped zero (Table 5, Figure 5).

For Lb colonization, we selected GrSc and Fd from 400 and 1000m radii buffer for the multi-model inference (Table S2). For extinction, we selected GrSc and Fd from 1600m radii buffer only. Unconditional 95% CI of all variables overlapped zero, discarding the influence the selected covariates on Lb probabilities of colonization and extinction (Table 4). For Lc colonization estimate (Table S3), we selected the covariate Fd at all four scales and GrSc from 1000 and 1300m radii buffer for multi-modeling (Table 5). Unconditional 95% CI of Fd and GrSc for colonization always overlapped zero (Table 5), indicating that Lc colonization was constant in relation to our set of covariates. For Lc extinction estimate, we selected the covariate Fd at all four scales and GrSc from 700, 1000, and 1300m radii buffer (Table 5). Because unconditional 95% CI of Fd and GrSc for extinction always overlapped zero (Table 5), we also assumed this parameter to be constant in relation to our set of covariates.

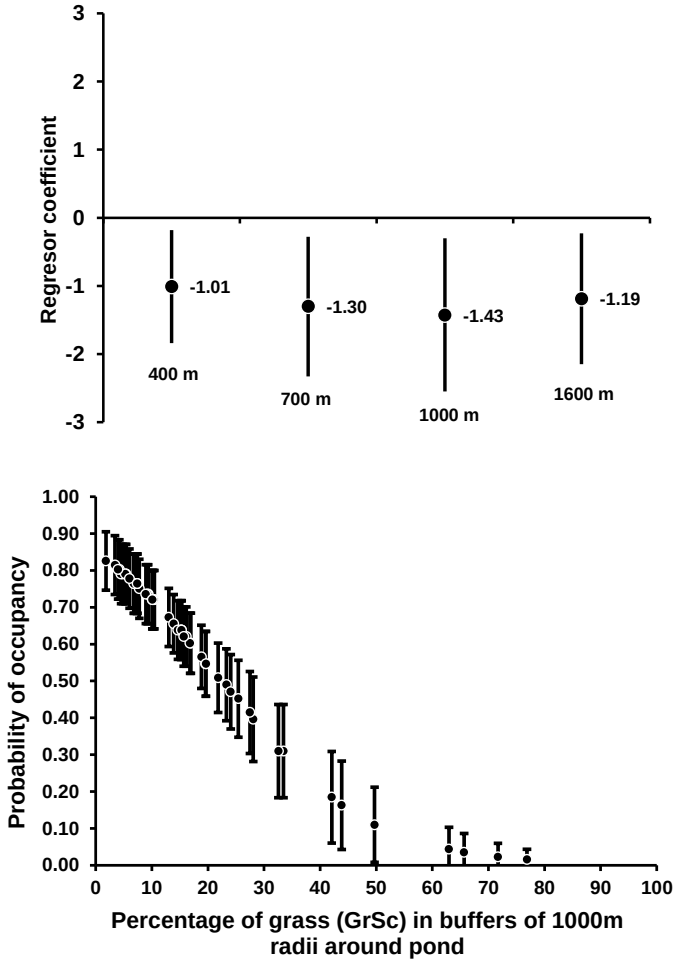

Figure 4 Landscape scale probability of habitat occupancy by *Leptodactylus bufonius* in 50 temporary ponds at the Brazilian Chaco. Above: model-averaged regressor coefficients (dots) and unconditional 95% confidence intervals (bars) for occupancy as a function of percentage of grass and sparse shrubs in buffers centered at the ponds (400, 700, 1000, and 1600m). Models are the same for every scale: detectability dependent on MRS and occupancy dependent on the percentage of grass around ponds ($\hat{p}$ (MRS) $\widehat{\psi}$ (GrSc)). Below: Landscape scale probability of habitat occupancy as a function of percentage of grass and sparse shrubs in buffers of 1000m radii length. Model: $\hat{p}$ (MRS) $\widehat{\psi}$ (GrSc1000) $\hat{\gamma}$ (.) $\hat{\varepsilon}$ (.). Bars are squared errors.

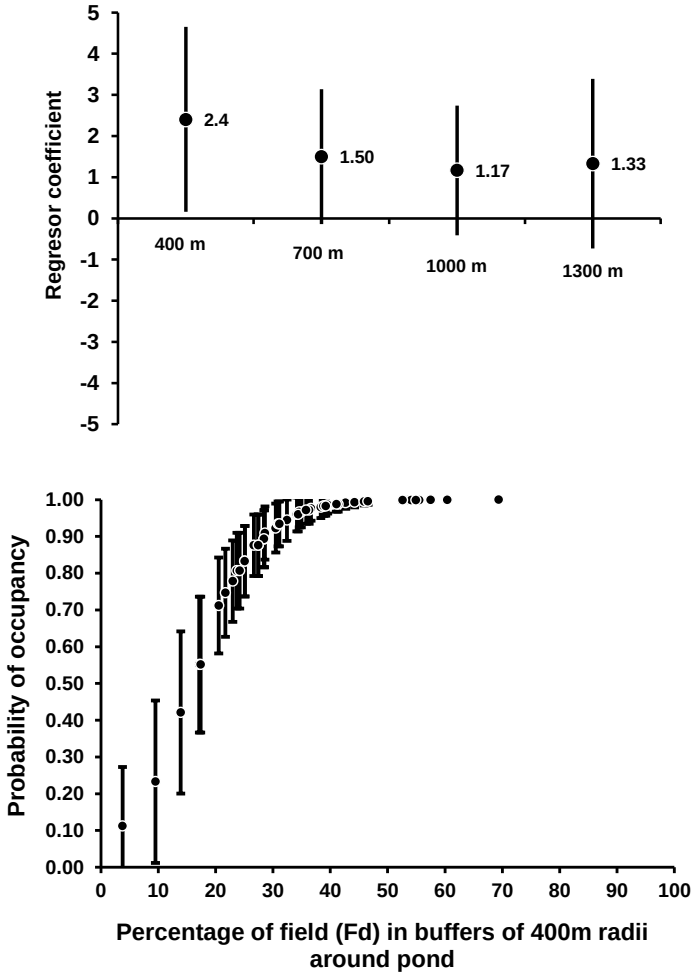

Figure 5 Landscape scale probability of habitat occupancy by *Leptodactylus chaquensis* in 50 temporary ponds at the Brazilian Chaco. Above: model-averaged regressor coefficients (dots) and unconditional 95% confidence intervals (bars) for occupancy as a function of percentage of fields (dense shrub lands) in buffers centered at the ponds (400, 700, 1000, and 1300m). Models are the same for every scale: detectability dependent on MRS and occupancy dependent on high density shrub lands ($\hat{p}$ (MRS) $\hat{\psi}$ (Fd)). Below: Landscape scale probability of habitat occupancy as a function of percentage of fields in buffers of 400m radii length. Bars are squared errors

Discussion

Detectability

At the Brazilian Chaco, *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc) showed slightly different probabilities of visual detection in temporary breeding ponds, but always below 100% (Lb: 52%; Lc: 59%). Since the seminal paper by MacKenzie et al. (2002) on habitat occupancy models, hundreds of studies have confirmed that detectability is a common outcome from field studies; however, ignoring chances of imperfect detection (false-negatives) can lead to biased estimates of parameters with important consequences for applied conservation (e.g. Bailey et al. 2004, Mazerolle et al. 2007, Bailey et al. 2009, 2014, Guillera-Arroita et al. 2010, Durso et al. 2011, Weir et al. 2014, Fellers et al. 2015). As expected, because Lc is dependent on water surface to deposit its foam nest (Prado et al. 2002, Prado & Haddad 2005), the relative depth of ponds (RD), used as proxy to recent rains, had a marginally positive effect on Lc detectability, but not on Lb. These results endorse the higher dependence of water for reproduction of Lc in relation to Lb as observed by previous studies (Heyer 1969, Crump 1995, Perotti 1997, Prado et al. 2000, 2002). Our multi-model inference, however, revealed that only time of survey (measured as minutes relative to sunset, MRS) had a strong effect on both species detectability. For monitoring proposes, financial costs with field expeditions could be reduced and detectability increased by sampling after 9 p.m. (≈ 180 MRS). For example, one visit at 11 p.m. or three visits during sunset time will result in the same detection probability of 90%.

Probability of habitat occupancy: pond and landscape scales

At the Brazilian Chaco, habitat features associated with anthropogenic modification of the landscape were important predictors of ponds occupancy by Lb and Lc at multiple scales. At the

pond scale, the replacement of native forests by cultivated grasses has limited the availability of bare soil required by males of Lb to shape the mud chambers for reproduction at the periphery of the ponds. Conversely, because Lc deposits foam nests direct on the water (Prado et al. 2002, Haddad & Prado 2005), none of the sampled covariates at the pond scale affected its probability of occupancy. At the landscape scales, our results endorsed a shifting paradigm in herpetology which recognizes that the habitat of semiaquatic species goes beyond the water limit and thus may expose these organisms to the stronger impacts of habitat alterations than previous supposed (Gibbons 2003, Semlitsch & Bodie 2003, Ficetola et al. 2009, Scherer et al. 2012, Lescano et al. 2015).

Estimating species-specific habitat's sizes and requirements of amphibians allows insights on how different species interact with the surrounding landscape and may reflect spatial relationships between habitat features and population processes (Gibbons 2003, Semlitsch & Bodie 2003, Eigenbrod et al. 2008, Ficetola et al. 2009). Based on distances moved by frogs, Semlitsch & Bodie (2003) estimated that buffer radii ranging between 200 and 400 m are required to protect both the aquatic and terrestrial part of the landscape used by these semiaquatic organisms during their complex life cycle. Consequently, while buffers of radii lengths ranging between 200 – 400m are expected to reflect the size of terrestrial habitat, larger buffers are potentially related to longer migration and dispersal events (Semlitsch & Bodie 2003, Ficetola et al. 2009, Scherer et al. 2012). For Lc, the probability of habitat occupancy was only influenced by the proportion of shrubs at the 400m scale. Thus, this scale of effect indicates that despite been considered a habitat generalist species (Valdujo et al. 2009), Lc may select sites based on habitat features in accordance to recent results including other generalist *Leptodactylus* species (e.g. Silva et al. 2011, 2012). For Lb, however, the scales of interaction indicated that the

substitution of native vegetation by cultivated grasses is potentially reducing habitat quality (pond scale and 400m scale) and individual movements (≥400m scales). Moreover, because the percentage of native forests was negatively correlated with the percentage of cultivated grass with sparse shrubs (GrSc), we can directly link the anthropogenic alterations to the multiple negative effects observed in Lb populations at the studied area. However, we believe that Lc populations are more exposed to periodically changes in habitat distributions because, unlike areas of native forests, areas of dense shrub vegetation are not protected by legal reserves at the studied region.

Species-specific habitat requirements play a major role in the relationship between amphibians´ response parameters and abiotic variables (Cushman 2006, Ficetola et al. 2009). However, the influence of abiotic factors and the scales of interaction between amphibians and their surrounding environment can be strongly dependent of particularities of the studied landscapes, limiting models transferability. For example, in a study conducted with six species in five landscapes with contrasting proportions of the same habitat features, highest-ranked models always included an interaction term between covariates and geographic regions (Zanini et al. 2009). At the Brazilian Chaco, our results identified the buffer size of 1000m as the scale with the stronger negative effect of cultivated pastures (negatively related to native forests) on Lb populations. At the same time, in the Arid Chaco sub-region of Argentina, a recent study found that the amount of forest in buffers of 1000m radii (the only landscape scale investigated) marginally affected abundancy of Lb in temporary ponds (Lescano et al. 2015). Therefore, while Lb and Lc presented contrasting responses in relation to the same variables at the Brazilian Chaco, the proportion of native forests seems to be consistently influencing Lb across its geographic distribution despite landscapes particularities. Thus, our results reinforce the

importance of forests and shrublands as key features influencing adult frog site selection (e.g. Provete et al. 2014, Lescano et al. 2015).

Much information on landscape ecology of frogs come from temperate species (e.g. Ficetola et al. 2009, Scherer et al. 2012) providing the opportunity to look for similarities and differences among anthropogenic impacts, scales of interaction, and effectiveness of conservation laws for species from contrasting climates. For Lb, the positive effects of native vegetation and negative effects of habitat modification were confirmed, while our results for Lc did not allowed us to directly link habitat alterations to its response parameters. Similarly to Lb, some frog species from temperate environments have shown consistent patterns in relation to this general expectation. For example, *L. pipiens* (mainly at 2000m; Eigenbrod et al. 2008), *Pelophylax lessonae* and *R. dalmatina* (at 200 and 300m respectively; Ficetola et al. 2009), and *L. catesbeianus* (Cosentino et al. 2014) were all negatively affected by roads or traffic intensity, while in the same studies, *R. dalmatina* (mainly at 50m), *L. sylvaticus* (at 100m), and *L. clamitans* were all positively related to forest cover. However, these general relationships are not always as clear. For example, in a study conducted in Canada, *L. clamitans* occupancy was positively affected by the percentage of forest cover in buffers of 1000m radii, while the same covariate had a negative effect at 250m (Mazerolle et al. 2005). Also, Houlahan & Findlay (2003) observed that although *Lithobates clamitans*, *L. sylvaticus*, and *L. septentrionalis* were positively related to forests, *L. catesbeianus* was positively associated to intensive land use and was negatively affected by increasing forest cover at 100m scale. Thus, the scales of interactions have been shown that, despite a few exceptions, frog species from both temperate and tropical regions are not fully protected by laws stating the size of protection zones for riparian vegetation;

even species inhabiting small water bodies may need riparian buffers as large as 400m to fulfill all life requirements.

Probabilities of colonization and extinction: pond and landscape scales

In the present study, despite constant models have indicated negative effects on the probability of colonization and extinction between S1 and S2 for Lb, but not for Lc, estimated parameters were not strongly affected by any sampled covariate. More specifically, only the percentage of bare soil had a marginally negative effect on Lb extinction. In other words, for Lb, ponds surrounded by greater amounts of bare soil tended to have lower chances of local extinctions. This is not surprising given the strong effect of bare soil on the probability of occupancy (see above). Because amphibians are usually strongly related to climatic characteristics (Duellman & Trueb 1994, Prado et al. 2005, Wells 2007), variables such as temperature and rainfall are of great importance not only for detectability (e.g. Pellet & Schmidt 2005, Cayuela et al. 2012, Fellers et al. 2015), but also for population rates such as colonization and extinction (e.g. Cayuela et al. 2012, Randall et al. 2015). In southern France, for example, rainfall events between seasons increased colonization and decreased local extinction of tadpoles of some frog species (Cayuela et al. 2012).

The lack of strong covariate effects on Lb vital parameters may be explained by the fact that we did not include environmental covariates (e.g. rainfall amount) or habitat covariates (e.g. vegetation around ponds in areas converted to pastures) that could vary between S1 and S2. Moreover, it is worth to note that short-term trends may not be representative of long-term dynamics (Blaustein et al. 1994, Randall et al. 2015). Fluctuations in occupancy due to measured habitat characteristics of ponds and adjacent landscape may occur at a rate too slow to be

detected between two consecutive reproductive seasons. Finally, occurrence data are less sensitive to vital rates change than abundance (Randall et al. 2015). For example, Lb and Lc abundance could be negatively affected by covariates, but if one single individual last it could be detected, masking the negative effect on occurrence data sets (Randall et al. 2015). Future long-term studies are required to describe the possible relationships among Lb and Lc population vital rates and environmental or habitat covariates (e.g. Cayuela et al. 2012, Weir et al. 2014).

Main conclusions and future directions for conservation

For Lb, our results indicated that when the mud zone, located at the ponds' shorelines, is replaced by grass, males may not be able to shape the mud nest, attract females, and reproduce. Moreover, habitat modification seems to be influencing site selection, migration, and dispersal of Lb. Therefore, habitat alteration imposed stronger negative effects on Lb than on Lc populations due to the specific habitat requirements for reproduction and mobility of Lb. Importantly, although considered to be a habitat generalist, Lc probability of habitat occupancy was strongly affected by the proportion of shrublands indicating a possible habitat requirement for this species. The scales of interactions reinforced the need for conservation laws taking into account the requirements of semiaquatic organisms.

Altogether, our results indicated that to guarantee 50% probability of pond occupancy by Lb, its reproduction, and mobility through the landscape: (1) each pond should be immediately surrounded by 35% of bare soil which could be achieved by preserving the riparian vegetation and (2) pastures should represent no more than 30% of the area in buffers measuring between 400m and 1000m radii surrounding ponds. For Lc, if areas of dense shrubs surrounding wetlands (400m buffer) are reduced to $\leq 20\%$, the average probability of occupancy can decrease to up to

50%, potentially reaching zero when dense shrubs areas are reduced to 10%. Because both species are considered to be tolerant to habitat modification (Heyer et al. 2004a, 2004b) our results may be related to particularities of the Brazilian Chaco such as habitat configuration in relation to the whole geographic distribution of the species.

Studies focusing on larger geographic scales will help to test generalizations of the observed effects we observed here, including the occurrence of contrasting thresholds values of habitat loss for specialist *vs* generalist species such as Lb and Lc (Banks-Leite et al. 2014). We believe that given the restricted distribution of *L. bufonius* in Brazil (mainly at Porto Murtinho and borders of Paraguay River; Pansonato et al. 2011, Frost 2016), future monitoring programs and conservation efforts should include this species. Finally, because detectability of Lb and Lc was below 100% at any survey time, we suggest caution while estimating population parameters of Lb and Lc in future studies. For monitoring proposes, the number of field expeditions could be greatly reduced while chances of detection increased by starting surveys after 9 p.m.

2. Isolation and characterization of microsatellite markers for two South American frogs (*Leptodactylus bufonius* and *L. chaquensis)* using next generation sequencing

Abstract

Leptodactylus bufonius (Vizcacheras' White-lipped Frog) and *L. chaquensis* (Cei's White-lipped Frog) are pond-breeding frogs that inhabit the Chaco and surrounding savanna-like formations in South America. Throughout the Chacoan plain, the combined impacts of livestock and forestry practices have led to a highly fragmented landscape and an impoverished ecological system, threatening local species. We cloned and characterized new microsatellite markers for both species. These markers will be useful for behavioral studies of reproductive strategies and conservation genetic studies of populations throughout this threatened habitat.

Resumo

As espécies de rã *Leptodactylus bufonius* e *L. chaquensis* ocorrem no Chaco e em outras formações de áreas abertas da América do Sul. Por todo o Chaco, os impactos combinados da criação de animais e exploração de madeira resultaram em uma paisagem fragmentada e instável, ameaçando as populações locais. Desta forma, nós clonamos e caracterizamos novos marcadores de regiões microssatélites para as duas espécies. Estes marcadores serão de grande utilidade para estudos de comportamento ligado a estratégias reprodutivas e estudos de genética da conservação de populações nesta região tão ameaçada.

Introduction

The 75 frog species in the New World genus *Leptodactylus* Fitzinger, 1826, are distributed across highly variable habitats throughout South America, and at least 12 of them inhabit the Chacoan plain (Frost 2016). *Leptodactylus bufonius* (Vizcacheras' White-lipped Frog) and *L. chaquensis* (Cei's White-lipped Frog) are sympatric in the Chaco of Bolivia, Argentina, Paraguay, and the state of Mato Grosso do Sul in western Brazil (Frost 2016). The genus *Leptodactylus* exhibits broad morphological and behavioral diversity (e.g. Heyer 2005, Ponssa 2008) and has been divided into four species groups (*L. latrans, L. pentadactylus, L. fuscus* and *L. melanonotus* groups) based on a continuum of reproductive modes ranging from fully aquatic to fully terrestrial development of tadpoles (Heyer 1969, Prado et al. 2002). Species in the *L. latrans* group, including *L. chaquensis*, represent the most extreme aquatic reproductive mode, with eggs laid in a foam nest on the surface of water and aquatic tadpole development. Species of the *L. fuscus* group, including *L. bufonius*, represent an intermediate stage, with terrestrial foam nest inside mud chambers built by males and aquatic tadpoles (Heyer 1969, Prado et al. 2002). For both species, previous observation of larger relative testes size compared to other leptodactylids (Prado & Haddad 2003, Faggioni et al. unpubl. data) and multimale spawning strongly suggest the occurrence of polyandry for both species (Prado & Haddad 2003, Faggioni et al. 2011). Because species-specific habitat requirements and skewed reproductive success may be important mechanisms underlying population genetic structure (Myers & Zamudio 2004, Holman & Kokko 2013, Nowakowski et al. 2015a), our main go was to characterize

polymorphic microsatellite markers that could be applied for the study of the population genetics of *L. bufonius* and *L. chaquensis*.

Methods

Study area and sampling

The Chaco plain, where our focal populations occur, is a region of important conservation concern (Tálamo & Caziani 2003). In southwestern Mato Grosso do Sul, Brazil, the combined impacts of livestock and forestry practices have led to a highly fragmented landscape and an impoverished ecological system (Bucher & Huszar 1999, Souza et al. 2010), thus threatening the few remaining areas of Brazilian Chaco (Pott & Pott 2003).

We collected 24 individuals of each species at three neighboring ponds (mean distance between ponds = 5 km), in the municipality of Porto Murtinho, State of Mato Grosso do Sul, Brazil (SISBIO license number 36741-1). Individuals were euthanized with 10% Lidocaine, fixed in 10% formalin, preserved in 70% ethanol and deposited in the Coleção Zoológica of the Universidade Federal de Mato Grosso do Sul (ZUFMS), Brazil. Muscle tissue samples were preserved in 100% ethanol.

Isolation of the new markers

In the laboratory, we extracted genomic DNA from muscle tissue samples from one individual of each species with a Qiagen DNeasy Tissue Kit. We digested the genomic DNA with the restriction enzyme *HincII* to obtain small fragments of DNA (150–900 bp). After digestion we ligated a modified double-strand SNX linker to the ends of obtained

fragments in the presence of the restriction enzyme *PmeI* to avoid linker-linker ligations (modified from Hamilton et al. 1999). To enrich DNA libraries for microsatellites, the fragments were hybridized to biotinylated repeat probes, captured by streptavidin-coated magnetic beads and amplified with Platinum *Taq* polymerase and a SNX-forward primer. We ligated 500 nanograms of PCR-amplified product to one microliter of a Titanium Rapid Library MID adapter (10 μM) and removed small fragments with Ampure beads. We sequenced this library using 454 shotgun pyrosequencing on a Titanium GS-FLX platform (454 Life Sciences, Branford, CT, USA). To assemble sequence reads we imported the raw read data to SeqMan NGen (version 4.1.0.147). We used the assembled .fasta file from SeqMan on msatcommander 1.0.3 to design primers for the potential dimeric, trimeric and tetrameric microsatellites. Specifically, we chose a product size range of approximately 150–450 bp, primers 22–23 bp in length, and melting temperatures (Tm) of 60–62ºC.

Characterization of the new markers

To test for microsatellite polymorphism, we extracted whole genomic DNA from the remaining 23 individuals in 150 μL 5% Chelex solution with 20 ng Proteinase K, incubated at 55°C for 120 minutes, and 99°C for 10 minutes. The supernatant from the Chelex extraction was used directly as template in polymerase chain reactions (PCR) for microsatellite amplification and genotyping. We used a three-primer method for genotyping by including a 20 bp tag on the 5' end of the forward primer and co-amplifying with a fluorescently tagged third 'universal' oligonucleotide that hybridizes to the 20 bp tail (Schuelke 2000). This procedure allowed us to pool PCR products from different loci on

the same plate for multiloaded genotyping. We performed all PCRs in 10µL reaction volumes, with 1µL of template DNA (1–10ng), 1X buffer, 1.5µM $MgCl_2$, 0.4mM dNTPs, 0.1µM of the forward primer, 0.1µM of the reverse primer, 0.3µM of the universal fluorescent primer (6-FAM, NED, PET or VIC), and 0.25 U *Taq* polymerase. Some primers required different quantities of $MgCl_2$ and/or Bovine Serum Albumin (BSA 10x) (Tables 1 and 2). PCRs consisted of an initial denaturation step for five minutes at 94°C, 35 cycles of 1 min at 94°C, 1 min at primer specific annealing temperature, one minute at 72°C, and a final extension for 5 min at 75°C.

We determined individual genotypes using 1.0µL pooled PCR product, 0.15µL GeneScan LIZ500, and 18.85µL Hi-Di Formamide solution on a 3730 Genetic Analyzer (Applied Biosystems). We used the software GeneMarker version 2.4.1 (SoftGenetics, State College, PA, USA) to check electropherograms and score alleles to automatically generated size bins after setting for Local Southern as sizing method and allele sizes of 100–500 bp. To assess the quality of genotypes at optimized loci we searched for the presence of null alleles and allelic dropout using Micro-Checker 2.2.3 (Oosterhout et al. 2004) and used Genepop 4.2 (Rousset & Raymond 1995) to test for deviations from HWE (Tables 1 and 2). We conducted preliminary Bayesian assignment tests implemented in Structure v1.2 (Pritchard et al. 2000, Falush et al. 2003) from K=1 to K=6 to test for possible population structure in our sample.

Results

Our library sequencing resulted in 130,461 sequence reads for *L. bufonius* and 75,440 for *L. chaquensis*. Following our specification for product size, primers length, and melting temperature, the analysis in msatcommander returned 6,894 and 5,280 microsatellite loci for *L. bufonius* and *L. chaquensis*, respectively. From those, we optimized 17 polymorphic loci for *L. bufonius* and 16 for *L. chaquensis* (Tables 1 and 2).

We found no evidence of genetic structure among ponds for *L. chaquensis*. For *L. bufonius*, we found evidence of three genetic demes; however, individual assignments (coefficient of membership) to respective demes was very low (average 62%; data not shown). Thus, for both species, we grouped individuals from all three ponds for preliminary analysis. All loci were polymorphic for both species and allele sizes ranged from 162–496 bp for *L. bufonius* (Table 1) and from 166–482 bp for *L. chaquensis* (Table 2). The *L. bufonius* loci Lbufo120 and Lbufo261, as well as *L. chaquensis* locus Lchaq99, had one or more alleles over 500 bp that should be confirmed with Gene Scan LIZ600. The number of alleles per locus ranged from five to 25 for *L. bufonius* (Table 1) and from three to 26 for *L. chaquensis* (Table 2). We found evidence for potential null alleles in 13 *L. bufonius* loci and in eight *L. chaquensis* loci (Tables 1 and 2). We did not find evidence of allelic dropout.

Discussion

Different habitat requirements for reproduction can lead to differences in evolutionary population viability in co-occurring species inhabiting landscapes threatened by land-cover changes (Gagné & Fahrig 2007, Richardson 2012). Both genetic population viability and reproductive strategies can be assessed through analyses of microsatellite

Table 1 Forward (F) and reverse (R) primer sequences, annealing temperature (T_a), Bovine Serum Albumin (BSA), number of alleles/number of individuals (n_A/n), heterozygosity (H_o: observed; H_e: expected) and deviation from HWE in 17 loci of *Leptodactylus bufonius* collected at three ponds (57.7053ºS, 21.6384ºW, 57.7211ºS, 21.7100ºW, and 57.7340ºS, 21.6609ºW) in the vicinity of Porto Murtinho, Mato Grosso do Sul, Brazil. Values in bold in n_A are loci with putative null alleles according to Microchecker.

Locus (GenBank #)	Primer sequence (5' - 3')	Repeat Motif (5' - 3')	Size Range (bp)	T_a (ºC)	BSA 10x (µL)	n_A/n	H_o/H_e	HWE p-value
Lbufo17 (KJ125273)	F:[1]CAGAATTTGTTGGTATCATTGCGG R: ACCAACTTCAACTACTCCTCCAG	$(AGT)_6$ … $(AGT)_8$	284–310	55	3	**7**/21	0.476/0.723	n.s.
Lbufo27 (KJ125274)	F:[1]TATGGCGCGGTTACTGAAATATG R: AAACTGACCCAACCCTTACTCTG	$ATCT_{10}$	388–483	50	2	**18**/21	0.762/0.944	n.s.
Lbufo33 (KJ125275)	F:[1]AAAGCCAACTGTTTACAACTCTG R: ATGGCCCATACATAGAAGGCTAG	$(ATCT)_{11}$ … $(ATCT)_{10}$ … $(ATCT)_{12}$	179–259	53	2	9/22	0.609/0.579	n.s.
Lbufo36 (KJ125276)	F:[1]GTAGGGTCAGTTGGTAGGATCTC R: AGGGATAAAGGGACACTCAGATC	$ATCT_6$	423–491	60	2	**17**/19	0.526/0.934	*
Lbufo55 (KJ125277)	F:[1]AAACTGACCCAACCCTTACTCTG R: TATGGCGCGGTTACTGAAATATG	$AGAT_9$	379–483	60	2	**19**/22	0.727/0.940	*****
Lbufo57 (KJ125278)	F:[1]AAACAAAGAAACGCGGCAATTC R: ACACATGAGATGCTGCTGAAATAG	$(ATCT)_7$ … $(GT)_{23}$	407–496	58	3	**21**/22	0.727/0.967	*
Lbufo61 (KJ125279)	F:[1]ATGATTGGTGCCTAATATGTGGG R: CTGACTATGGTTTCGTCTAGTGC	$ATCT_{10}$	162–182	50	2	5/21	0.429/0.374	n.s.
Lbufo75 (KJ125280)	F:[1]AAAGCCAACTGTTTACAACTCTG R: ATGGCCCATACATAGAAGGCTAG	$(ATCT)_{12}$ … $(ATCT)_{11}$ … $(ATCT)_{10}$	317–381	61	2	**17**/22	0.500/0.946	*****
Lbufo91 (KJ125281)	F:[1]ATTTAGGGCAAAGATGAACTCGG R: GTTAAACTGTGCTGTTCTGAAGC	$ATCT_{14}$	287–323	60	2	10/23	0.956/0.876	n.s.
Lbufo113 (KJ125282)	F:[1]CAATTGCTCTCTAGTGGACCATG R: GCGTATGAGTAGACTGCAGTAAG	AC_{12}	278–309	60	2	**14**/22	0.636/0.891	*
Lbufo120 (KJ125283)	F:[1]ATCCCTACAGTCATGAAACCACC R: ACATTTACTTGACGGCAGATTCC	$(AT)_6$ … $(ATCT)_{12}$ … $(ATCT)_{11}$	289–533	60	2	**25**/23	0.435/0.971	*****

Lbufo261 (KJ125284)	F:[1]TTGGCAGATACAGCAAAGAAGAC R: AGGCCCTGAAGACTTACCTATTC	ATT_5	314–508	60	2	8/23	0.696/0.776	n.s.
Lbufo286 (KJ125285)	F:[1]GAAAGATAGACAACATGGCCCAG R: ACTGGTTTCCGTCTAAAGATGTG	AC_{12}	391–410	58	2	**11**/20	0.550/0.901	*
Lbufo287 (KJ125286)	F:[1]CAGAGACATTGTATTGGGAAGGG R: TCCACTTGTCTGCAGATGAAATG	$(ATT)_5$ … $(ACT)_{10}$	297–350	64.5	2	**15**/22	0.227/0.931	*
Lbufo324 (KJ125287)	F:[1]CCTCCATACTCGCATACATTATTG R: ACGTTGAAACAGGTGAACTATCG	$(GT)_6$ … $(ATCT)_9$	315–352	58	2	**15**/23	0.695/0.889	n.s.
Lbufo456 (KJ125288)	F:[1]CAGTGCTAGCAGTTAACAGGAAG R: ACCTGGCCACTTATTTCCATAAC	AGG_5	235–416	50	2	**10**/21	0.571/0.797	n.s.
Lbufo756 (KJ125289)	F:[1]AGACTGTTAGACCAAGGCTAGTG R: ATTTGGATTGCAACATCACAGTG	$(AGT)_5$ … $(AGT)_5$	265–411	60	2	**12**/21	0.714/0.886	n.s.

PCR reactions for loci Lbufo75 and Lbufo287 included a final concentration of 2mM of $MgCl_2$.

[1] 20 bps tag added at the 5' end of the forward primer: CGAGTTTTCCCAGTCACGAC.

*$p < 0.001$ (adjusted p-value ≤ 0.0029 after Bonferroni correction).

Table 2 Forward (F) and reverse (R) primer sequences, annealing temperature (T_a), Bovine Serum Albumin (BSA), number of alleles/number of individuals (n_A/n), heterozygosity (H_o: observed; H_e: expected) and deviation from HWE in 16 loci of *Leptodactylus chaquensis* collected at three ponds (57.6954ºS, 21.6338ºW, 57.7211ºS, 21.7100ºW, and 57.7265ºS, 21.6589ºW) in the vicinity of Porto Murtinho, Mato Grosso do Sul, Brazil. Values in bold in n_A are loci with putative null alleles according to Microchecker.

Locus (GenBank #)	Primer sequence (5' - 3')	Repeat Motif (5' - 3')	Size Range (bp)	T_a (ºC)	BSA 10x (µL)	n_A/n	H_o/H_e	HWE p-value
Lchaq13	F:[1]GAATTGTGCTAGGACCAGGATTG	$AGAT_{12}$	395–432	58.5		13/22	0.818/0.904	n.s.
(KJ125290)	R: GCACTAAAGAGAAAGGGATCCAG							
Lchaq15	F:[1]CCATAGGAATAGGAAGGTTATGGC	$ATCT_{11}$	440–463	57		**9**/22	0.454/0.833	*
(KJ125291)	R: ACCAACCCGTGATCAGTATAGAG							
Lchaq18	F:[1]TCCATAATACAGTGCCAAGGTTG	$AAAT_7$	307–322	57		5/23	0.783/0.629	n.s.
(KJ125292)	R: ATGTCAGTGGGAGGAAACAAATG							
Lchaq25	F:[1]ACAGCTTCCATGATTCATTGGAC	$ATCT_7$	377–410	55		**17**/22	0.590/0.932	*
(KJ125293)	R: CTGTGGGAATTATACACCATCGG							
Lchaq36	F:[1]CTACCTAACATAACGTGGTTGCC	$AGAT_8$	383–434	60		**12**/17	0.750/0.919	n.s.
(KJ125294)	R: AAATACTGTCACATCACCTGCTG							
Lchaq57	F:[1]GTTTGCTTTACTTAGATCCACCTG	$ATCT_{12}$	352–390	58.5		11/19	0.737/0.888	n.s.
(KJ125295)	R: TGCAAAGCCTTTATCTGACTGAC							
Lchaq63	F:[1]AAATATATTCCAGGCCTCCCACC	$AGAT_{13}$	336–482	58.5		**19**/19	0.210/0.961	*
(KJ125296)	R: AAGCCTACGTGCAATTGACTTAG							
Lchaq99	F:[1]CATGGCTTTGGTATTGGTAAGGG	$ATCT_5$	424–550	65	2	13/22	0.350/0.917	n.s.
(KJ125297)	R: CATTACTAGTCTGTCGCCTTCAG							
Lchaq103	F:[1]CTACCTAACATAACGTGGTTGCC	$AGAT_9$	352–399	60		14/22	0.818/0.926	n.s.
(KJ125298)	R: CCAGGACTAAATACTGTTGCACC							
Lchaq115	F:[1]AGCTGCTACCTTTGATGCTAAAC	$AGAT_{11}$	167–207	65	2	**10**/22	0.435/0.844	*
(KJ125299)	R: AAAGGGCACAGCTAAATGTTCG							
Lchaq283	F:[1]GCAAGTAACATTGCCCATAAAGC	$(AG)_7$ … $(ATCT)_9$	215–287	57		**26**/23	0.826/0.968	n.s.
(KJ125300)	R: AATTATATCTGCAGTTCCAATGGC							
Lchaq404	F:[1]CCTGGTGTTAGCGGTATACATTG	$(AG)_8$ … $(AG)_6$	444–470	65		**8**/22	0.476/0.778	n.s.

(KJ125301)	R: TACTGTCCACCATCTTCTCTAGC						
Lchaq618	F:[1]TAGCATGTGATTGTAACCAGCAC	AGG_5	209–226	64.5	3/18	0.167/0.160	n.s.
(KJ125302)	R: TGACACCAGATCATGTGTTCAAC						
Lchaq642	F:[1]TGCAGAATGACAATGAATGGAGG	AC_8	263–291	55	11/23	0.739/0.854	n.s.
(KJ125303)	R: GGTATCTGATATGGAGGTTTGCC						
Lchaq708	F:[1]TTGCTACACTAGTAAATTGGATGC	CT_8	166–179	55	**6**/23	0.391/0.707	*
(KJ125304)	R: TGAAACACTGGCAATGTAAAGGG						
Lchaq3572	F:[1]AACCAAATCAACCTCAAGATCCC	AC_6	280–304	55	12/22	0.909/0.898	n.s.
(KJ125305)	R: CGACGTTCCAACACTACATCAAG						

PCR reactions for loci Lchaq57, Lchaq63 and Lchaq618 included a final concentration of 2mM of $MgCl_2$.and for locus Lchaq13, a final concentration of 2.5 mM MgCl2.

[1]20 bp tag added at the 5' end of the forward primer: CGAGTTTTCCCAGTCACGAC.

*$p < 0.001$ (adjusted p-value ≤ 0.0031 after Bonferroni correction).

loci, highlighting the importance of these markers for conservation purposes (e.g. Myers & Zamudio 2004, Angelone & Holderegger 2009). Despite the broadly diversity of reproductive modes and the great distribution of our focal Genus through the Neotropics (e.g. Prado et al. 2002, Lucas et al. 2009), specie-specific microsatellite markers are available for only two species of the genus *Leptodactylus*. The markers described here are the first microsatellites characterized for *L. bufonius*, whereas our markers for *L. chaquensis* add to the 12 polymorphic microsatellites published by Arruda et al. (2010).

The new microsatellites characterized here were high polymorphic. For example, previous isolated microsatellite markers from 20 Lc individuals collected in the Cerrado were less polymorphic (2–7 alleles by loci; Arruda et al. 2010) than our markers applied here in 23 Lc individuals (3–26 alleles by loci). The deficiency of heterozygotes observed in some loci could result from unidentified structure among sampled ponds, reduction of the genetic variability, or small sample sizes.

The new markers will be useful for comparative population studies and conservation strategies of *L. bufonius* and *L. chaquensis* in the threatened Brazilian Chaco. Isolation and characterization of new specie-specific markers together with tests of cross amplifications (e.g. Duryea et al. 2009) will increase our capacity to look for the effects of land-cover changes and guide conservation efforts of this diverse genus.

3. Effects of the habitat modification on the genetic diversity and functional connectivity of two Neotropical pond-breeding frogs

Abstract

Amphibians are particularly susceptible to habitat modifications due to their limited dispersal capacity, contrasting life stages, and physiological constrains. However, much of our understanding of the interfaces among demographic processes, genetic diversity, landscape alterations, and functional connectivity of amphibians come from temperate species. The lack of studies including tropical species represents a significant gap in the current knowledge on amphibian conservation genetics in a scenario of alarming extinction rates in tropical ecosystems. Here we investigate if past and/or current landscape alterations have been shaping the distribution of genetic diversity of two tropical pond-breeding frogs, *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc), in human-altered landscape. We used microsatellite markers to look for signatures of past bottlenecks and current inbreeding and applied resistance surfaces representing species-specific hypothesis to investigate possible differences in the relationship between landscape structure and functional connectivity of Lb and Lc. Populations (ponds) of both species showed high levels of inbreeding despite the great allelic richness. Bottlenecks possibly linked to clearing of native vegetation about 50 years ago may explain current levels of inbreeding. Landscape structure among breeding ponds led to high gene flow among populations of Lc, but to moderate functional connectivity of Lb. While the studied landscape seems very permeable for Lc dispersal among breeding ponds, path-ways for Lb individuals are potentially becoming narrowed by human activities.

Resumo

Anfíbios são particulamente suscetíveis a alterações de hábitat devido a sua limitada capacidade de dispersão, estágios de vida contrastantes e limitações fisiológicas. No entanto, muito do que sabemos sobre as interações entre processos demográficos, diversidade genética, impactos das alterações da paisagem e conectividade funcional de anfíbios é baseado em espécies de climas temperados. A falta de estudos incluindo espécies de ambientes tropicais representa uma importante lacuna no conhecimento sobre a genética e conservação de anfíbios frente ao alarmante ritmo de extinções nesses ecossistemas. Neste trabalho, nós investigamos se modificações passadas e/ou características atuais da paisagem de estudo moldaram a distribuição da diversidade genética de duas rãs tropicais que se reproduzem em poças temporárias, *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc), em uma paisagem modificada por atividades humanas. Nós utilizamos marcadores microssatélites para procurar por evidências de gargalos genéticos passados e endogamia atual e também aplicamos superfícies de resistência representando hipóteses espécie-específicas para investigar possíveis diferenças nas relações entre a estrutura da paisagem e a conectividade funcional entre as espécies Lb e Lc. Embora as populações (poças) das duas espécies tenham apresentado grande riqueza alélica, ambas as espécies apresentaram endogamia na área de estudo. Gargalos genéticos passados com atual recuperação da riqueza alélica parece um cenário plausível para explicar os níveis de endogamia observados. A estrutura da paisagem entre as poças possibilitou grande fluxo gênico entre as populações de Lc, mas moderada conexão funcional entre as populações de Lb. Enquanto a paisagem estudada parece ser permeável a dispersão de Lc entre poças, as conexões estruturais da paisagem estão se estreitando para Lb devido a perda hábitat e modificação da paisagem.

Introduction

Habitat loss and modification can significantly decrease effective population sizes (i.e. bottleneck), increase isolation in subdivided populations, and accelerate the negative effects of genetic drift and inbreeding; altogether increasing the risk of extinction (Frankham 1995, 2005, Vos et al. 2001, Halverson et al. 2006). Patchily distributed amphibians, such as many pond-breeding frogs and salamanders, are particularly susceptible to these negative impacts of the habitat modification due to their limited dispersal capacity, specific habitat requirements, contrasting life stages, and physiological constrains (Smith & Green 2005, Becker et al. 2007, Holderegger & Wagner 2008, Allentoft & O'Brien 2010). Consequently, human development over natural areas is among the main drivers of the current amphibian population declines (Stuart et al. 2004, Cushman 2006, Whitfield et al. 2016). However, natural landscape topography (e.g. Funk et al. 2005) and species intrinsic factors such as water dependency (e.g. Mims et al. 2015), may influence the distribution of amphibians' genetic diversity and should also be considered in studies on altered landscapes (e.g. Crosby et al. 2008, Peterman et al. 2014, Nowakowski et al. 2015). Advancing our knowledge on the complex relationships between amphibian species and their surrounding landscape is therefore urgent to forecast the consequences of anthropogenic disturbances and critical to guide conservation actions for these threatened organisms.

Similarly to amphibian conservation genetics, one of the main goals of landscape genetics is to elucidate how modern landscape changes (e.g. anthropogenic disturbance) have affected patterns of genetic structure at relatively small geographic scales (Holderegger & Wagner 2008, Storfer et al. 2010, Manel & Holderegger 2013). Challenges for landscape genetic analyses remain on how to assign species-specific resistance values to different elements such as forests and roads (i.e. parameterization of a resistance surface), as the actual effects of such

elements in ecological traits are usually unknown (Spear et al. 2010, Koen et al. 2012). Assigning resistance costs based on field experiments (e.g. Nowakowski et al. 2015a), species-specific ecological tolerances (e.g. Peterman et al. 2014, Mims et al. 2015), and demographic models (e.g. Peterman et al. 2014, Nowakowski et al. 2015a) have proven to be biologically meaningful approaches. By doing so, researchers can create ecologically explicit surfaces to test the relative support of multiple landscape genetic hypotheses in species presenting contrasting life-history characteristics (Peterman et al. 2014, Mims et al. 2015).

In this regard, recent landscape genetic studies have highlighted the rule of physiological limitations and ecological strategies in the population genetics of amphibians. In tropical and temperate environments, the replacement of native forests and shrublands by human settlements and farms seems to be an important process that limits gene flow among populations due to physiological limitations (e.g. Peterman et al. 2014, Zancolli et al. 2014, Nowakowski et al. 2015a). The presence of forests and shrublands in the landscape increases the amount of leaf litter and shade in the soil (Ludwig et al. 2001, Cole & Weltzin 2005), buffering microclimates that reduce amphibians' mortality due to desiccation, especially during migration and dispersal, which may promote functional connectivity (Becker et al. 2007, Nowakowski et al. 2015a, Nowakowski et al. 2015b). In arid environments, however, water and aquatic habitats represent landscape elements that strongly influence the genetic structure of frog populations (Chan & Zamudio 2009, Mims et al. 2015). In such environments, frog species that require daily access to water and show longer larval period are usually associated to perennial water bodies and tend to show site fidelity (philopatry), leading to reduced gene flow and strong genetic structure among populations (Mims et al. 2015). Conversely, populations of frog species adapted to ephemeral aquatic resources are usually well connected due to the higher mobility of individuals through

the unfavorable dry matrix (Chan & Zamudio 2009, Mims et al. 2015). Tadpoles of these species may quickly develop into adults due to the ephemeral nature of small ponds (Mims et al. 2015).

Despite the worrying extinction rates in tropical ecosystems we still lack fundamental information about the impacts of human activities on the genetic diversity and connectivity of tropical amphibians (e.g. Dixo et al. 2009, Storfer et al. 2010, Zancolli et al. 2014, Nowakowski et al. 2015a). Therefore, here we focused on two frogs species from the highly diverse Neotropical genus *Leptodactylus.* Frogs of this genus exhibit reproductive modes ranging from fully aquatic, such as in species of the *L. latrans* group, to partially terrestrial found in the *L. fuscus* group (Heyer, 1969; Prado et al., 2002). *Leptodactylus bufonius* (Lb) belongs to this last group and thus deposits its foam nests inside terrestrial chambers built by males at the periphery of the breeding ponds (Crump 1995, Reading & Jofré 2003, Faggioni et al. 2011). After hatching, Lb tadpoles can survive inside the terrestrial chambers for over 40 days without water before stochastic rainfalls carry them out to the closest pond where they will develop into adults in 20–30 days (Philibosian et al. 1974, Crump 1995, Prado et al. 2000). Conversely, *L. chaquensis* belongs to the *L. latrans* group and deposits its foam nests direct on the surface of the water after rains; after hatching tadpoles will develop inside the aquatic environment for about 60 days (Prado et al. 2002, Fabrezi 2011, Martinuzzi et al. 2016). Both species are sympatric at the studied landscape which has been intensely impacted by the replacement of native dry forests and shrublands by cultivated pastures for at least 50 years. However, while forests and shrublands represent niche requirements for Lb reproduction and mobility through the landscape (e.g. Areskoug 2001, Reading & Jofré 2003, Duré & Kehr 2004, Lescano et al. 2015, Chapter 1), Lc seems to use a wide range of habitats and ponds in open fields and shrublands (e.g. Areskoug 2001, Schaefer et al. 2006, Valdujo et al 2009, Chapter 1).

Our main goals were to look for demographic impacts of the habitat alteration on genetic parameters of Lb and Lc and evaluated whether and how these impacts are shaping the distribution of the genetic diversity in two ecologically distinct Neotropical species. We expected that gene flow of Lb and Lc will be promoted through favorable landscape characteristics, accordingly to connectivity patterns of amphibians from altered landscapes in tropical and temperate environments. Contrary to species from arid environments, gene flow of Lb and Lc will be hampered by unfavorable elements. Specifically, we predicted that if the effective population sizes of Lb and Lc had decreased between 50 and 30 years ago there will be a deficiency of heterozygotes within and overall ponds. We also expect to find a heterozygosity excess or deficiency relative to the allelic richness, as expected after bottlenecks. Although both species may present the common pattern of isolation-by-distance, we expect that Lb gene flow will be facilitated through terrestrial forested habitats, but limited through cultivated pastures. Conversely, we expected that Lc gene flow will be facilitated through pathways for overflowing water due to increased chances of tadpoles transportation (longer larval period) and stronger association between adults of Lc and aquatic environments.

Methods

Study area and sampling populations

We conducted our study in a landscape of the Brazilian Chaco located east of the Paraguay River and the remaining Gran Chaco (Souza et al. 2010). The Gran Chaco extends for almost 1,000,000 km^2, covering regions of Argentina, Paraguay, Bolivia and Brazil (Bucher & Huszar 1999, Pennington et al. 2000). The local vegetation is composed by forests with shrubs, mainly of mimosoid species, and sparse herbaceous vegetation, mainly Bromeliaceae and Cactaceae,

and some grass (Pennington et al. 2000). The climate is “Aw” type according to Köppen (Alvares et al. 2013), with a hot rainy season from October to April and a dry season from May to September. During the last 50 years, replacement of native vegetation by cultivated pastures due to livestock practices has been the main anthropogenic impact in the Brazilian Chaco (Bucher & Huszar 1999, Souza et al. 2010, Tomas et al. 2015). Forest cover at the Brazilian part is now reduced to about 13% of its original area, making the Chaco one of the most endangered ecoregions in Brazil (Tomas et al. 2015). Although some endemic frog species from the Chaco also occur at the Brazilian part (e.g. Souza et al. 2010, Sugai et al. 2013), most of the frog species are typical from open habitats and also occur in other neighbouring formations, such as the Cerrado (Souza et al. 2010). During the rainy season, some of these species, including *L. bufonius* and *L. chaquensis*, aggregate in temporary ponds to reproduce (e.g. Faggioni et al. 2011, Schalk & Saenz 2015). Such temporary ponds can hold water from a few days (ephemeral ponds) to many weeks, making them high variable in their persistence on the landscape (Schalk & Saenz 2015). Occasionally during the rainy season, ponds may overflow after strong rains, connecting most of the breeding sites by water flow (GPF pers. obs.).

During the breeding seasons of 2012–2013 and 2013–2014, we collected adults of Lb and Lc (license: Sisbio #36741) in temporary breeding ponds, distributed in private cattle farms at the municipality of Porto Murtinho, Mato Grosso do Sul State (reference point:- 21.710079^{o} S, -57.721174^{o} W; Figure 1A), southwestern Brazil. The study area is a heterogeneous mosaic of native forests and cultivated pastures surrounded by the Alumiador Mountains at the east, the Paraguay River at west, the APA River at south, and the Amonguijá River at north. Therefore, the majority of the studied system is found inside an anthropogenic modified landscape, making it an interesting system to look for the effects of habitat alteration in demographic and genetic

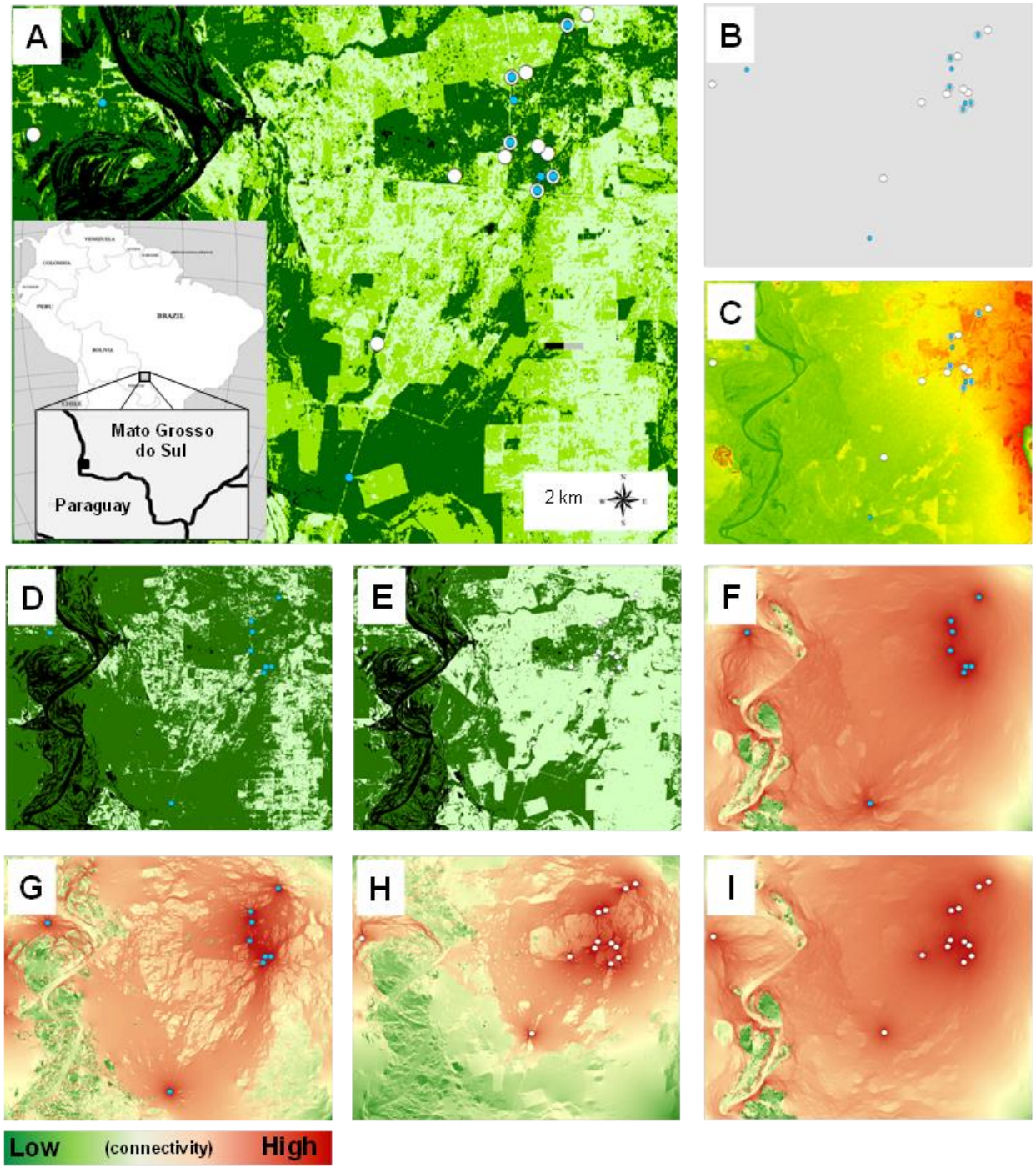

Figure1 Map of the study area at the borders of Brazil and Paraguay showing: A) sampled breeding ponds of *Leptodactylus bufonius* (Lb; blue circles), *L. chaquensis* (Lc; white circles), or both (blue circles inside white circles). Black: permanent water bodies

(mainly Paraguay River); dark green: forested areas; medium green: high density shrubs fields; light green: grass with sparse shrubs. B), C), D), and E) Space, WatRes, EcoRes for Lb, and EcoRes for Lc surfaces respectively. In C, dark green represent the higher cost. F), G), H), and I) Pairwise resistance rasters based on WatRes for Lb, EcoRes for Lb, EcoRes for Lc, and WatRes for Lc, respectively. Green and red represent the range from low to high gene flow (horizontal bar). See Appendix A for details.

parameters of species with contrasting life-histories. Besides the temporary ponds located at the Brazilian Chaco, we included in our genetic analyses five individuals of each species collected in two temporary ponds located in Paraguay (PMParaguay for Lb and PMParaguay2 for Lc).

Laboratory protocols and genotyping

In the laboratory, we euthanized individuals with 10% Lidocaine, preserved muscles samples in 100% ethanol, fixed individuals in 10% formalin, and preserved specimens in 70% ethanol. Specimens were deposited in the Coleção Zoológica de Referência of the Universidade Federal de Mato Grosso do Sul (ZUFMS), Brazil. We extracted whole genomic DNA from muscle samples with 150 µL 5% Chelex solution and 20 ng Proteinase K, incubated at 55°C for 120 minutes, and 99°C for 10 minutes. In order to accesses the genetic diversity and estimate the parameters of interest, we used 16 microsatellite loci for *L. bufonius* and 15 loci for *L. chaquensis*, all of them previously isolated and characterized (Faggioni et al. 2014). We scored individual genotypes using Genemarker v2.4.1 (SoftGenetics, State College, PA, USA). To avoid any tendency to score similar alleles sizes within or among neighboring ponds, we randomly scored individuals. For both species, individuals with missing data in three or more loci were genotyped a second time (for all loci) to estimate the average genotyping error (27 and 90 duplicates for *L. bufonius* and *L. chaquensis*,
respectively). We estimated the error rate by comparing the scores at the loci that worked at both first and second genotyping. The average error rate for Lb and Lc were 1.54% (range 0% to 9.09%) and 5.38% (range 0% to 12.50%), respectively. Finally, we merged the results from the first and second runs to define our final data set. We used Genepop v.4.2 (Rousset & Raymond 1995) to check for linkage disequilibrium between all pair of loci. We used a Markov-Chain

method (Guo & Thompson 1992) with 10 000 dememorization steps and 100 batches of 5,000 iterations to determine the significance. We used Micro-Checker v2.2.3 (Van Oosterhout et al. 2004) to look for evidences of null alleles, large allele dropouts and scoring error by stuttering within ponds.

Population genetic analyses

Within-ponds and global patterns of genetic diversity. We used Genalex v.6.5 (Peakall & Smouse 2012) to calculate the expected (H_e) and observed (H_o) heterozygosity, the mean number of different alleles (N_a), and the effective number of alleles (N_e) by pond. We performed the *U*-score test for heterozygote deficiency in Genepop v.4.2 (Rousset & Raymond 1995) and accessed the significance of the analyses after 10,000 dememorization steps and 100 batches of 5,000 iterations. We used the software HP-Rare (Kalinowski 2005) to estimate the allelic (AR_r) and private allelic richness (PAR_r) while accounting for variation in sample sizes (Kalinowski 2005). We estimated fixation indexes (inbreeding coefficients) F_{is} and F_{it} (Weir & Cockerham 1984) in Fstat v.2.9.3.2 (Goudet 2002). We accessed the 95% CI around the estimated mean after 1,000 bootstraps. We also used Genalex to calculate N_a, N_e, H_e, H_o, and the inbreeding index G_{is} by loci considering all ponds (global values). The calculation of G_{is} followed the formula of Wright's F_{is} with the correction of Nei & Cheeser (1983) for small population size and inbreeding, applied in the calculation of H_e (Meirmans & Hedrick 2011). To investigate whether the presence of null alleles or sample sizes could lead to erroneous conclusions in further genetic and demographic analyses, we re-estimated the fixation indexes and performed Hardy-Weinberg Equilibrium (HWE) tests after excluding the loci that showed recurrent evidence of null alleles and/or had few genotyped individuals within ponds. This step reduced our data set from 16 to 11

loci for *L. bufonius* and from 15 to eight loci for *L. chaquensis* (Table S1). Because both data sets led us to the same conclusions (Table S2), we considered the evidences of null alleles an artefact of the analyses within some ponds and used the larger data sets for the remaining analyses. Next, we used Bottleneck v1.2.02 (Piry et al. 1999) to look for evidences of current or past (few generations) demographic bottlenecks using the Sign and the Wilcoxon rank tests (Cornuet & Luikart 1996, Luikart et al. 1998). We determined the significance of the Wilcoxon test with a two-tail test. Because different loci may follow different mutation models, both the two-phase (TPM) and the step-wise mutation (SMM) models were used in the Sign and Wilcoxon tests (Cornuet & Luikart 1996, Dudaniec et al. 2012, Scherer et al. 2012). For the TPM model, we set the variance to 12.00, the probability of single-step mutations to 95%, and 10,000 iterations (Piry et al. 1999, Krug & Pröhl 2013, Peterman et al. 2013b). Finally, to investigate the relationship between patterns of inbreeding and philopatry we calculated the pairwise relatedness index RQG (Queller & Goodnight 1989) between individuals within subpopulations also in Genalex. Significance values and the 95% CI were estimated after 9,999 permutation and 10,000 bootstraps respectively.

Among-ponds patterns of genetic diversity. To look for evidences of genetic structure among ponds, we estimated the global genetic fixation index G''_{st} (Meirmans & Hedrick 2011). G''_{st} is an extension of the F_{st} of Wright (1951), with adjustments for multiallelic markers (Nei 1973), variable mutation rate among markers (Hedrick 2005), and sample size (Meirmans & Hedrick 2011). The 95% CI for G-statistics were estimated after 999 bootstraps.

We used the genetic differentiation index D_{est} (Jost 2008) to calculate the pairwise genetic divergences among ponds. D_{est} is based on the effective number of alleles and mirror differentiation due to processes such as migration and mutation; it is sensitive to the number of

subpopulations but not by subpopulation sizes (Jost 2008, 2009, Meirmans & Hedrick 2011, Peterman et al. 2014, Wang 2015). Pairwise values and the 95% CI were estimated after 1,000 bootstraps through the package "DEMEtics" (Gerlach et al. 2010) in the R statistical environment v.3.0.3 (R Development Core Team 2014). To investigate whether locus-specific effects (variation in mutation rates) could lead to bias in the pairwise calculations, we performed a correlation analysis between H_e and D_{est} by loci (Wang 2015, Table S3, Figure S1). For both species, the correlation was neither strong nor significant (Lb: r=- 0.10; p=0.71; Lc: r=0.11; p=0.69), allowing us to correlate genetic differentiation with demographic processes (Wang 2015). We were confident of a covariate effect on response parameters when 95% CI of the estimated value did not overlap zero (Jost 2009, Meirmans & Hedrick 2011). However, because of the small number of individuals collected in some ponds, we also considered the statistical significance (p-values) for the pairwise comparisons to avoid misinterpretations (Gerlach et al. 2010).

Finally, we conducted individual-based Bayesian assignment tests implemented in Structure v1.2 (Pritchard et al. 2000, Falush et al. 2003) to test for population subdivision (genetic demes) among breeding ponds. Because of the small geographic scale of our study, where the Paraguay River represents the single presumed major geographic barrier to individuals of Lb and Lc due to its width (between 250 and 500m), we did not consider each breeding pond as a putative genetic deme. Instead, we performed Structure analysis from K=1 to K=8 for each species to look for hidden genetic structures due to geographic isolation. The maximum number of eight allowed us to track the second-order rate of change in the log-likelihood beyond the geographic expected K=2. For each K, 25 iterations were run for 2,000,000 cycles with a burn-in of 500,000 cycles. We considered a model with admixture and correlated allele frequencies. The

most likely K was determined using the ΔK method (Evanno et al. 2005), in which the most likely number of genetic clusters is assessed by the second-order rate of change in the log-likelihood.

Landscape parameterization and resistance surfaces

We used a Landsat 8 satellite image of 30 x 30 m spatial resolution from August 2014 and a SRTM satellite topography map at the same resolution (EarthExplorer 2014) to construct three landscape resistance surfaces, which were expected to mirror geographic distances (Space), water flow direction (WatRes), and species-specific ecological characteristics (EcoRes) in ArcMap v10.3 (ESRI 2014). Parameterization of the Space-only surface was set by assigning the resistance value of 1 for every pixel in the raster grid, representing an analogue to isolation-by-distance pattern (Figure 1B). The WatRes surface was built up on the topographic map and the surface parameterization followed the original metrics with some adjustments (Figure 1B; Appendix A). To define the EcoRes surface, we first used the software Geomatica (PCI 2012) to classify each pixel at the original image as one of the four main landscape elements of the studied area: permanent water bodies (PWB); forested areas (For); high density shrubs fields (Fd); and grass with sparse shrubs (GrSc) (Figure 1A, D, and E). GPS control points together and scaled pictures took at the studied landscape were used as validation method. Next, we scored each landscape element based on both, habitat occupancy models (Faggioni et al. Chapter 1.) and natural history characteristics of the studied species (e.g. Areskoug 2001, Prado & Haddad 2003, Reading & Jofré 2003, Duré & Kehr 2004, Schaefer et al. 2006, Valdujo et al 2009, Lescano et al. 2015). Parameterization details are provided in Appendix A. Briefly, for Lb, lower resistance values were assigned to pixels of forest and high density shrubs fields, while higher values,

representing higher resistance to movements, were set to pixels of grass and water bodies (Figure 1D). In contrast, for Lc, lower resistance values were assigned to pixels of shrubs and grass, while higher values were set to forest and water pixels (Figure 1E).

For each surface, we used Circuitscape v.4.0 (McRae et al. 2013) to calculate pairwise resistance values among sampled ponds. Circuitscape combines electrical circuit and graph theories to simulate migration (i.e. electric current) among sites considering all possible pathways (McRae 2006, McRae et al. 2008, Figure 1F, G, H, and I). Recently, theoretical and empirical studies have demonstrated that the amount of current connecting two nodes in an electric circuit can be related to movement ecology via random-walk theory (e.g. McRae 2006, McRae & Beier 2007, McRae et al. 2008, Mims et al. 2015, Nowakowski et al. 2015a). Moreover, because gene flow among real populations is rarely restricted to a single best pathway, allowing for multiple connections between two sites represents more ecologically realistic patterns of habitat connectivity (McRae & Beier 2007, McRae et al. 2008, Mims et al. 2015, Nowakowski et al. 2015a).

Landscape genetic models

We evaluated the magnitude of the relationships among genetic differentiations (pairwise D_{est}), spatial distribution of breeding ponds, and landscape layout through Information-Theory (Burnham & Anderson 2002, 2004, Mazerolle 2006). Based on the results from the correlation analysis between H_e and D_{est} (Table S3, Figure S1), we were confident to correlate the pairwise genetic differentiation values with the pairwise resistance values calculated by Circuitscape. A lack of correlation between H_e and D_{est} indicates that, despite the mutation model under consideration, loci under investigation will reflect pure demographic parameters (Wang 2015).

Because of the nonindependence in pairwise values and the relative small number of subpopulations (nine and 13 breeding ponds for Lb and Lc, respectively), we conducted a conservative approach to access the relative support of each resistance surface. Our modeling approach followed: (1) formulation of a few candidate models and the respective biological hypotheses (Table 1); (2) for both species, we combined the competing models in two separated structures. The first one included the effects of Space in genetic differentiation (Genetic) and EcoRes, and also the direct effect (unbiased by Space, i.e. autocorrelation) of EcoRes in Genetic, and was named Terrestrial structure (Figure 2). The second one included the effects of Space in Genetic and WatRes, and also the direct effect of WatRes in Genetics, and was named Aquatic structure. To keep a small number of parameters we did not estimate the indirect effects of Space in Genetics; (3) estimation of the regressor coefficients through structural matrix analysis (path analysis). At this point, in order to control for the pseudoreplication, we conducted 1,000 bootstraps steps of nine and 13 pairwise values from Lb and Lc matrices, respectively. Therefore, we avoided pseudoreplication without overestimating the degrees of freedom. Regressor coefficients and AIC_c values were computed after each bootstrap step; (4) ranking of the candidate Structural Models according to Akaike's Information Criterion with a second-order bias adjustment (AIC_c = sample size/number of parameter ≤ 40; Burnham & Anderson 2002); and (5) calculation of the 95% confidence intervals of the regressors for the best structure. All analyses were conducted in the R statistical environment v.3.0.3 (R Development Core Team 2014).

Table 1 Candidate set of models evaluated to explain the pairwise genetic differentiation among breeding ponds of *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc) in the Brazilian Chaco. A summary of the biological hypotheses are presented. Space: resistance surface analogue to isolation-by-distance surface; EcoRes: resistance surface based on specie-specific habitat requirements and natural history; WatRes: resistance surface based on water flow. See text for details.

Models	Lb	Lc	Biological hypotheses
Space in EcoRes	yes	no	The spatial distribution of the ponds is affecting the resistance values through terrestrial habitats;
Space in WatRes	no	yes	The spatial distribution of the ponds is affecting the resistance values through water flow;
Space in Genetic	no	yes	Lc migration rate among ponds is not affected by landscape alterations; Lb migration among ponds is narrowed by the landscape alteration and spatial distribution has only indirect effects;
EcoRes in Genetic	yes	no	Lb migration rate among ponds is narrowed by landscape alteration to specific terrestrial habitats; Lc migration rate among ponds is not affected by landscape alterations;
WatRes in Genetic	no	yes	Lc connectivity by means of tadpoles migration in overflowing water; Lb migration rate is not facilitate by water flow;

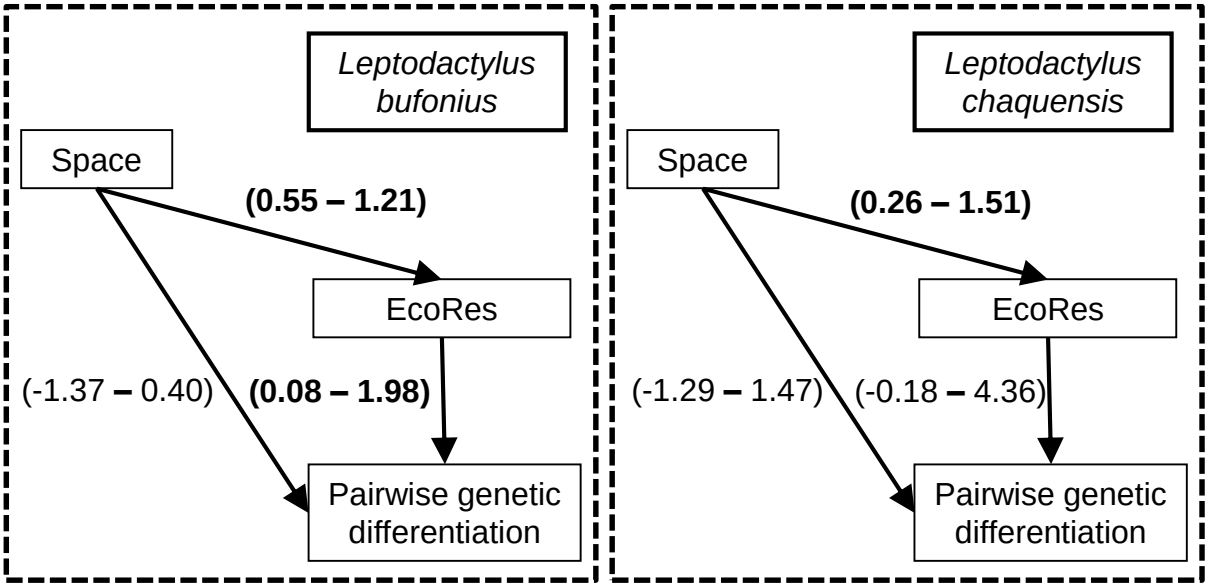

Figure 2 Terrestrial structures used for modeling pairwise genetic differentiation of *Leptodactylus bufonius* and *L. chaquensis* through path analysis. For each species the structure contains a set of three models linked by arrows. Number are 95% CI. In bold are 95% CI that excluded zero. See text for details on path analysis.

Results

Within-ponds and global patterns of genetic diversity

We collected a total of 126 individuals of *L. bufonius* and 161 individuals of *L. chaquensis*, distributed in nine and 13 temporary breeding ponds, respectively (Figure 1A). Geographic distance among ponds ranged from 0.61 km to 24.79 km for Lb and from 0.65 km to 33.53 km for Lc. For Lb, only a pair of loci (Lbufo27 and Lbufo55) showed evidence of linkage disequilibrium, but it was in a single site (PM17), suggesting that linkage may not be the main explanation for this result. No pairs of loci showed evidence of linkage disequilibrium for Lc. For both species, our microsatellite markers were highly polymorphic. The average number of alleles by loci was 28.4 for Lb (range: 6–82) and 22.9 for Lc (range: 4–54); within ponds the number of alleles ranged from 5.75 (PMParaguay) to 15.25 (PM22) and from 5.07 (PMParaguay2) to 11.87 (PM01) for Lb and Lc, respectively (Tables 2 and 3). For Lb, within-ponds genetic diversity was high as indicated by expected heterozygosity (H_e range: 82%–86%), effective number of alleles (N_e range: 5.59–7.194), and rarified allelic richness (AR_r range: 3.15–3.33; Table 2). However, individuals from PMParaguay showed higher levels of private allelic richness and slightly higher effective number of alleles (Table 2), suggesting larger population size and lower population connectivity. Although slightly lower than Lb, within-ponds genetic diversity of Lc was also high (H_e, N_e, and AR_r ranges: 78%–85%, 4.63–6.62, and 3.03–3.27, respectively; Table 3). It is worth noting that for Lc, individuals from Paraguay did not present a different pattern of genetic richness compared to individuals from Brazil (Table 3). For both species, all ponds showed significant deviations from HWE due to heterozygotes deficient, leading to high levels of inbreeding (Tables 2 and 3). Both species also presented high and similar global levels of genetic diversity as indicated by the analysis across loci including all

Table 2 Population genetic parameters, Hardy-Weinberg Equilibrium test (HWE), and fixation indexes (F_{is} and F_{it}) across 16 microsatellite loci of *Leptodactylus bufonius* collected in temporary breeding ponds in the Brazilian Chaco. n: number of individuals. Parameters are: expected (H_e) and observed (H_o) heterozygosity; mean number of different alleles (N_a) and effective number alleles (N_e); rarified allelic (AR_R) and private allelic (PAR_R) richness. Rarified values were standardizing for a population of five individuals. 95% LCI and 95% UCI represent the lower and upper limits of the 95% confidence intervals.

Breeding pond	n	H_e	H_o	N_a	N_e	AR_R	PAR_R	HWE	F_{is}	F_{it}
PM03	15	0.841	0.653	10.875	6.289	3.260	0.750	*	0.230	-
PM15	14	0.834	0.630	10.875	6.024	3.230	0.790	*	0.252	-
PM18	14	0.821	0.647	10.750	5.586	3.200	0.730	*	0.219	-
PM17	23	0.856	0.683	14.813	6.944	3.330	0.830	*	0.206	-
PM22	24	0.858	0.664	15.250	7.042	3.330	0.880	*	0.230	-
PM32	15	0.840	0.722	12.438	6.250	3.270	0.810	*	0.145	-
PMcigano	7	0.840	0.589	6.688	6.250	3.210	0.760	*	0.319	-
PMbnovo	9	0.821	0.660	7.563	5.586	3.150	0.670	*	0.208	-
PMparaguay	5	0.861	0.619	5.750	7.194	3.240	1.240	*	0.318	-
Overall	126	0.841	0.652	10.556	6.352	3.250	0.829	*	0.222	0.225
95% LCI	-	-	-	-	-	-	-	-	0.158	0.160
95% UCI	-	-	-	-	-	-	-	-	0.293	0.295

*p<0.0005.

Table 3 Population genetic parameters, Hardy-Weinberg Equilibrium test (HWE), and fixation indexes (F_{is} and F_{it}) across 15 microsatellite loci of *Leptodactylus chaquensis* collected in temporary breeding ponds in the Brazilian Chaco. n: number of individuals. Parameters are: expected (H_e) and observed (H_o) heterozygosity; mean number of different alleles (N_a) and effective number of alleles (N_e); rarified allelic (AR_R) and private allelic (PAR_R) richness. Rarified values were standardizing for a population of five individuals. 95% LCI and 95% UCI represent the lower and upper limits of the 95% confidence intervals.

Breeding pond	n	H_e	H_o	N_a	N_e	AR_R	PAR_R	HWE	F_{is}	F_{it}
PM03	12	0.804	0.586	8.067	5.102	3.090	0.400	*	0.282	-
PM14	10	0.810	0.587	6.733	5.263	3.070	0.340	*	0.290	-
PM15	19	0.843	0.618	10.800	6.369	3.240	0.380	*	0.274	-
PM17	13	0.810	0.654	9.133	5.263	3.130	0.430	*	0.199	-
PM22	7	0.849	0.675	6.933	6.623	3.250	0.540	*	0.221	-
PM32	8	0.784	0.515	6.533	4.630	3.030	0.460	*	0.361	-
PM34	15	0.830	0.632	10.400	5.882	3.200	0.480	*	0.246	-
PM01	21	0.832	0.638	11.867	5.952	3.210	0.410	*	0.238	-
PM13	17	0.832	0.634	10.467	5.952	3.200	0.450	*	0.245	-
PM48	8	0.847	0.608	7.933	6.536	3.270	0.510	*	0.300	-
PM20	14	0.818	0.600	9.800	5.495	3.150	0.470	*	0.275	-
PM40	12	0.810	0.701	8.200	5.263	3.140	0.580	*	0.140	-
PMParaguay2	5	0.813	0.653	5.067	5.348	3.040	0.530	*	0.235	-
Overall	161	0.822	0.623	8.610	5.668	3.160	0.460	*	0.251	0.251
95% LCI	-	-	-	-	-	-	-	-	0.147	0.148
95% UCI	-	-	-	-	-	-	-	-	0.355	0.355

*p<0.0005.

ponds (Tables 4 and 5). For Lb, the overall expected heterozygosity was 85% and the effective number of alleles was 6.762, while for Lc, the overall expected heterozygosity was 84% and the effective number of alleles was 6.289. The global test of HWE indicated a significant level of heterozygotes deficient and inbreeding for Lb (G_{is} mean: 0.232; 95% CI: 0.168–0.296; Table 4) and Lc (G_{is} mean: 0.254; 95% CI: 0.144–0.358; Table 5). For both species, the Bottleneck analyses strongly suggested a pattern of heterozygosity deficient (high allelic richness) despite the mutation model under consideration. For Lb, both Wilcoxon two-tailed tests for bottlenecks were significant (p=0.0023 and 0.0085, under the SMM and TPM models, respectively). The same pattern was observed for Lc (p=0.0001 and 0.0053, under the SMM and TPM models, respectively). For both species and despite the mutation model, not all loci showed significant heterozygosity deficient. For example, under the SMM model, seven loci out of 10 negatives and five loci out of 13 negatives were significantly different from zero for Lb and Lc, respectively (Table 6). Taken together, the Bottleneck results suggested that both species are out of mutation-drift equilibrium, showing a slightly but significant increase in the allelic richness. Finally, for both species, the index of relatedness, RQG, indicated no pattern of high relatedness among individual within-ponds (Figure 3), suggesting that pond philopatry is not the causal process of the observed levels of inbreeding.

Among-ponds patterns of genetic diversity

The standardized index of genetic structure, G''_{st}, indicated that for Lc, the total genetic diversity is not subdivided among ponds (G''_{st} mean: 0.012; 95% CI: -0.018–0.048; Table 5), suggesting high connectivity among sampled sites. Conversely, estimates of G''_{st} for Lb slightly overlapped zero, suggesting lower connectivity among Lb breeding ponds (mean: 0.071; 95% CI: -0.007–

Table 4 Genetic parameters and Hardy-Weinberg Equilibrium test (HWE) in 16 microsatellites loci of *Leptodactylus bufonius* collected in temporary breeding ponds in the Brazilian Chaco. n: number of genotyped individuals. Parameters are: expected (H_e) and observed (H_o) heterozygosity; mean number of different alleles (N_a) and effective number of alleles (N_e); inbreeding coefficient (G_{is}) and fixation index (G''_{st}). 95% LCI and 95% UCI represent the lower and upper limits of the 95% confidence intervals.

Loci	n	H_e	H_o	N_a	N_e	HWE	G_{is}	G''_{st}
Lbufo17	115	0.726	0.562	5.444	3.124	***	0.226	-0.111
Lbufo27	117	0.934	0.746	13.222	8.169	***	0.201	0.118
Lbufo33	119	0.638	0.562	6.111	2.528	**	0.119	0.012
Lbufo36	121	0.974	0.846	18.000	12.257	***	0.131	0.061
Lbufo55	118	0.876	0.699	9.333	5.756	***	0.202	0.136
Lbufo57	117	0.966	0.875	16.444	11.719	***	0.094	0.181
Lbufo61	121	0.372	0.266	3.333	1.520	*	0.285	0.363
Lbufo75	119	0.954	0.536	14.111	8.940	***	0.438	0.277
Lbufo91	122	0.848	0.811	7.222	5.148	ns	0.044	0.002
Lbufo113	117	0.889	0.692	9.556	6.039	***	0.221	0.044
Lbufo120	116	0.987	0.650	16.667	12.403	***	0.342	-0.176
Lbufo261	112	0.840	0.690	8.111	4.732	***	0.179	0.082
Lbufo286	112	0.883	0.702	8.444	5.815	***	0.204	-0.102
Lbufo287	109	0.923	0.406	10.444	6.523	***	0.560	0.125
Lbufo324	111	0.911	0.612	10.667	6.593	***	0.328	-0.009
Lbufo756	117	0.906	0.776	11.778	6.922	***	0.143	0.013
Overall	-	0.852	0.652	10.556	6.762	***	0.232	0.071
95% LCI	-	0.763	0.512	8.451	5.161	-	0.168	-0.007
95% UCI	-	0.908	0.721	12.500	8.403	-	0.296	0.172

*p<0.05; **p<0.005; ***p<0.0005; ns: not significant.

Table 5 Genetic parameters and Hardy-Weinberg Equilibrium test (HWE) in 15 microsatellites loci of *Leptodactylus chaquensis* collected in temporary breeding ponds in the Brazilian Chaco. n: number of genotyped individuals. Parameters are: expected (H_e) and observed (H_o) heterozygosity; mean number of different alleles (N_a) and effective number of alleles (N_e); inbreeding coefficient (G_{is}) and fixation index (G''_{st}). 95% LCI and 95% UCI represent the lower and upper limits of the 95% confidence intervals.

Loci	n	H_e	H_o	N_a	N_e	HWE	G_{is}	G''_{st}
Lchaq57	154	0.927	0.920	11.462	8.411	*	0.008	-0.044
Lchaq618	142	0.464	0.306	3.385	1.755	***	0.342	-0.026
Lchaq63	120	0.967	0.455	11.077	7.809	***	0.530	0.191
Lchaq103	150	0.913	0.870	10.077	7.322	*	0.047	-0.014
Lchaq115	159	0.791	0.494	6.615	3.824	***	0.376	0.029
Lchaq25	138	0.912	0.499	9.077	5.706	***	0.453	-0.209
Lchaq3572	156	0.900	0.850	9.538	6.812	ns	0.055	0.021
Lchaq36	122	0.894	0.823	8.385	5.597	**	0.079	0.155
Lchaq708	154	0.830	0.676	7.692	4.562	***	0.186	0.113
Lchaq99	138	0.970	0.791	14.308	10.521	***	0.185	-0.014
Lchaq15	153	0.867	0.648	8.538	5.367	***	0.252	-0.029
Lchaq18	154	0.545	0.666	3.462	2.100	ns	-0.224	0.022
Lchaq283	149	0.961	0.473	12.231	8.915	***	0.508	0.077
Lchaq404	141	0.756	0.277	5.615	3.085	***	0.634	0.014
Lchaq642	159	0.826	0.600	7.692	4.455	***	0.273	0.005
Overall	-	0.835	0.623	8.610	6.289	***	0.254	0.012
95% LCI	-	0.763	0.529	7.195	4.976	-	0.144	-0.018
95% UCI	-	0.900	0.718	10.005	7.657	-	0.358	0.048

*p<0.05; **p<0.005; ***p<0.0005; ns: not significant.

Table 6 Results from the Sign test to detect an excess or deficit in the allelic richness relative to the expected heterozygosity due to a recent demographic bottleneck. Only individuals from Brazil were grouped for analyses in both species. SMM: step-wise mutation model; TPM: two-phase mutation model; Sig: direction of the signal test ((-): heterozygosity deficient; (+): heterozygosity excess); Prob: *p*-value. See text for details.

	Leptodactylus bufonius					*Leptodactylus chaquensis*				
	SMM		TPM			SMM		TPM		
Loci*	Sign	Prob	Sign	Prob	Loci*	Sign	Prob	Sign	Prob
Lbufo17	(-)	0.003	(-)	0.010	Lchaq57	(-)	0.059	(-)	0.113
Lbufo27	(-)	0.074	(-)	0.151	Lchaq618	(-)	0.009	(-)	0.018
Lbufo33	(-)	0.000	(-)	0.000	Lchaq103	(-)	0.123	(-)	0.257
Lbufo55	(-)	0.022	(-)	0.047	Lchaq115	(-)	0.095	(-)	0.212
Lbufo61	(-)	0.000	(-)	0.003	Lchaq25	(-)	0.311	(+)	0.528
Lbufo75	(-)	0.075	(-)	0.108	Lchaq3572	(-)	0.393	(+)	0.449
Lbufo91	(+)	0.338	(+)	0.208	Lchaq36	(-)	0.136	(-)	0.265
Lbufo113	(-)	0.178	(-)	0.314	Lchaq708	(-)	0.003	(-)	0.000
Lbufo261	(-)	0.016	(-)	0.043	Lchaq15	(-)	0.004	(-)	0.008
Lbufo286	(+)	0.475	(+)	0.385	Lchaq18	(-)	0.368	(+)	0.438
Lbufo287	(+)	0.388	(+)	0.236	Lchaq283	(-)	0.343	(+)	0.470
Lbufo324	(-)	0.014	(-)	0.024	Lchaq404	(-)	0.000	(-)	0.003
Lbufo756	(-)	0.026	(-)	0.053	Lchaq642	(-)	0.007	(-)	0.027
Total and # of significant (-)	10	7/10	10	6/10		13	5/13	9	5/13

* Loci Lbufo36, Lbufo57, Lbufo120, Lchaq63, and Lchaq99 were excluded from the BOTTLENECK analyses because of calculation limitations;

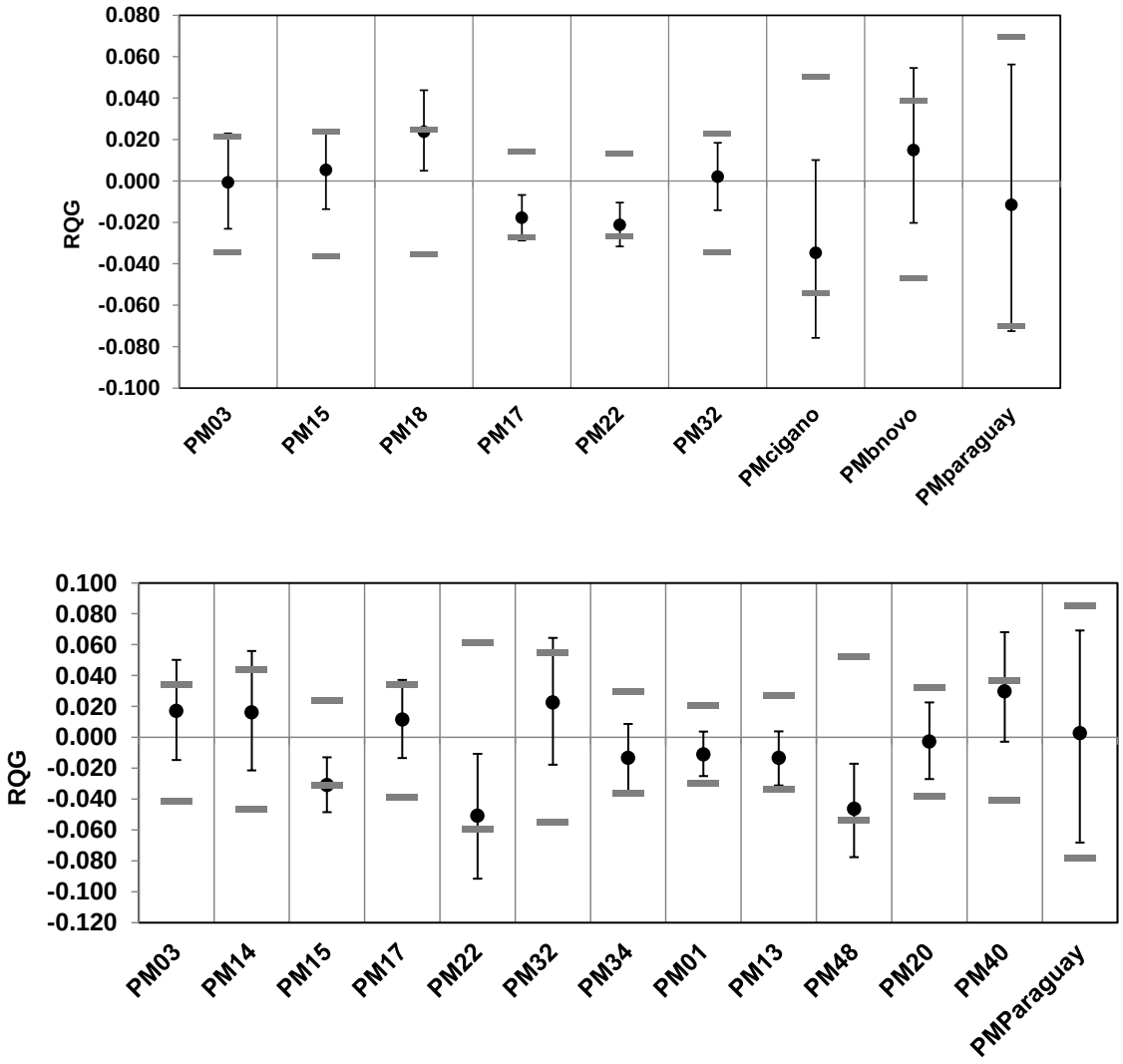

Figure 3 Mean within-population relatedness, RQG, of (A) *Leptodactylus bufonius* and (B) *L. chaquensis* collected in temporary breeding ponds in the Brazilian Chaco. Gray bars are 95% upper and lower confidence values generated from 999 permutations of data from all populations and enclose the values expected in breeding aggregations that are panmictic, show relatively even reproductive success, and are not philopatric.

0.172; Table 4). For both species, genetic differentiation, D_{est}, among pairs of populations were all non-significant after the sequential Bonferroni correction (Tables 7 and 8), supporting the global G''_{st} results. The individual-based Bayesian assignment test of population structure indicated lack of genetic structure for Lc and moderate levels for Lb. According to the ΔK method, there was evidence of three genetic demes for Lb (Table S4) supported by a moderate level of individual assignment (coefficient of membership) to respective demes (average 75%). However, because of the high genetic admixture found within-ponds (all three genetic demes were well represented in mostly ponds), we considered all 126 individual of Lb as part of one single genetic deme (Figure 4). The ΔK method indicated that K=4 represented the best model for Lc. However, the mean individual assignment to respective demes was very low (average 40%). Therefore, we assumed that all 161 individuals of Lc came from a single genetic deme, because we considered that the suggested number of genetic clusters was an artefact of the analysis likely due to the small geographic scale and the lack of biological barriers.

Landscape genetic models

According to the AIC ranks, the Terrestrial structure was the most likely model for both species (Table 9), suggesting a possible effect of the current habitat configuration on functional connectivity. The Aquatic structure presented high values of ΔAIC_c (≥4) and very low probabilities (Lb: w=0.02; Lc: w=0.01; Table 9); thus we did not proceed with this model for further estimates. Isolating models grouped within the Terrestrial structure allowed us to directly compare the magnitude of the relationships among alternative models and the response parameter. For both species, the genetic differentiation among breeding ponds (Genetic) was independent from the direct effects of geographic distance (Space surface), showing no pattern of

Table 7 Pairwise genetic differentiation based on the effective number of allele (D_{est}) of *Leptodactylus bufonius* collected in temporary breeding ponds in the Brazilian Chaco are shown above the diagonal. Pairwise Euclidian distances (km) are shown below the diagonal. P-values were all non-significant after Bonferroni correction (adjusted p-value=0.0031) and are not shown.

	PM03	PM15	PM18	PM17	PM22	PM32	PMcigano	PMbnovo	PMparaguay
PM03	-	0.202	0.183	0.043	0.150	0.114	0.159	0.204	0.275
PM15	7.34	-	0.107	0.056	0.046	0.189	0.098	0.140	0.296
PM18	7.43	0.61	-	0.093	0.050	0.084	0.091	0.133	0.402
PM17	8.13	1.05	0.70	-	0.029	0.061	0.114	0.176	0.376
PM22	6.40	2.78	2.32	2.72	-	0.071	0.082	0.097	0.390
PM32	3.88	5.26	5.04	5.69	3.13	-	0.023	0.118	0.277
PMcigano	24.64	18.02	17.67	17.00	18.24	21.08	-	0.060	0.378
PMbnovo	4.58	4.21	3.96	4.52	2.04	1.11	20.14	-	0.392
PMparaguay	24.79	23.99	23.39	23.30	21.58	21.59	22.25	21.69	-

Table 8 Pairwise genetic differentiation based on the effective number of allele (D_{est}) of *Leptodactylus chaquensis* collected in temporary breeding ponds in the Brazilian Chaco are shown above the diagonal. Pairwise Euclidian distances (km) are shown below the diagonal. P-values were all non-significant after Bonferroni correction (adjusted p-value=0.0031) and are not shown.

	PM03	PM14	PM15	PM17	PM22	PM32	PM34	PM01	PM13	PM48	PM20	PM40	PMParaguay2
PM03	-	0.166	0.114	0.113	0.159	0.148	0.098	-0.001	0.113	-0.012	0.185	0.179	0.234
PM14	6.27	-	0.045	0.128	0.181	0.254	0.132	0.100	0.120	0.080	0.132	0.208	0.307
PM15	7.34	1.15	-	0.042	0.083	0.161	0.046	0.064	0.062	-0.019	0.034	0.080	0.115
PM17	8.13	1.87	1.05	-	0.091	0.131	0.100	0.054	0.066	0.061	0.106	0.033	0.100
PM22	6.40	2.05	2.78	2.72	-	0.265	0.161	0.099	0.106	0.155	0.182	0.209	0.148
PM32	3.88	4.14	5.26	5.62	3.13	-	0.076	0.100	0.186	0.057	0.176	0.250	0.312
PM34	3.15	4.06	5.21	5.71	3.46	0.81	-	0.041	0.098	-0.050	0.048	0.200	0.226
PM01	1.14	7.02	8.03	8.88	7.37	5.00	4.25	-	0.086	0.003	0.072	0.183	0.184
PM13	6.01	0.65	1.70	2.17	1.45	3.57	3.57	6.83	-	0.039	0.051	0.075	0.264
PM48	18.34	12.85	12.27	11.22	11.98	14.66	15.25	19.35	12.77	-	-0.036	0.086	0.063
PM20	7.14	2.26	2.70	2.36	0.74	3.83	4.20	8.10	1.81	11.26	-	0.123	0.210
PM40	9.39	5.05	5.20	4.44	3.37	5.61	6.25	10.45	4.64	9.07	2.83	-	0.194
PMParaguay2	32.43	30.93	31.23	30.47	28.95	29.16	29.96	33.53	30.39	24.03	28.67	26.04	-

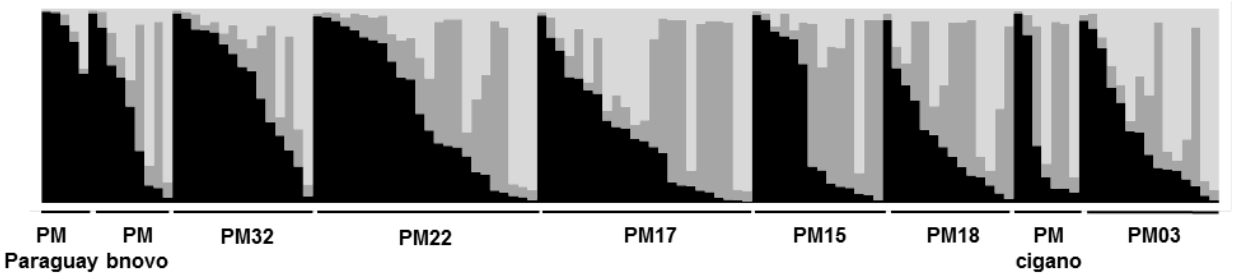

Figure 4 Bayesian individual-based analysis of population structure for 126 individual of *Leptodactylus bufonius* collected in the Brazilian Chaco. Bars represent individuals. Shades of gray represent the three distinct genetic demes suggested by STRUCTURE. The mean individual assignment to one single deme was moderate (75%), indicating high admixture within and among ponds.

Table 9 Model selection table for landscape genetics of *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc) collected in temporary breeding ponds in the Brazilian Chaco. Alternative models are grouped in two competing structures: Terrestrial and Aquatic.

Species	Response parameter	Structure	K	AIC_c	ΔAIC_c	*w*
L. bufonius						
	Pairwise genetic differentiation					
		Terrestrial	5	36.56	0.00	0.97
		Aquatic	5	43.52	6.96	0.02
L. chaquensis						
	Pairwise genetic differentiation					
		Terrestrial	5	57.42	0.00	0.99
		Aquatic	5	66.93	9.51	0.01

isolation-by-distance. However, because of its influence on EcoRes, the geographic distance played an important role on the Terrestrial structure of both species (Table 10, Figure 2). For Lc, there was no direct effect of EcoRes in Genetics (despite the strong asymmetry on the 95% CI; Table 10). Interestingly, for Lb, the EcoRes surface was an important predictor of the pairwise genetic differentiation independently of the spatial distribution of the breeding ponds. In fact, the mean regressor coefficient was higher for EcoRes in Genetics than for Space in EcoRes (1.06 and 0.88, respectively; Table 10), strongly suggesting that Lb individuals disperse among ponds through forested areas, avoiding crossing open fields dominated by grass.

Discussion

Within-ponds and global patterns of genetic diversity

Our results showed that *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc) have high genetic diversity within and overall ponds supported by the expected high heterozygosity (H_e), the high effective number of alleles, and rarefied allelic richness within-ponds and overall loci. However, both species also showed a significant deviation from HWE caused by a deficiency of heterozygotes and an increased level of inbreeding within and overall ponds, despite the low relatedness among individuals within ponds. Results from the pairwise individual relatedness within-ponds and inference from the bottleneck tests suggest that the observed values of inbreeding were not due to site philopatry and may be a result from a recent demographic bottleneck, leading to a current recovery of the genetic diversity.

Based on the high levels of H_e in Lb and Lc, it may be appealing to exclude chances of past demographic impacts because bottlenecks should reduce genetic diversity. For example, in many European regions characterized by intense anthropogenic habitat fragmentation, the tree

Table 10 Modeling results for the best structure of the landscape genetics of *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc) collected in temporary breeding ponds in the Brazilian Chaco. Genetic: pairwise genetic differentiation D_{est}. Space: resistance surface analogue to isolation-by-distance; EcoRes: resistance surface based on specie-specific habitat requirements and natural history. Mean and 95% lower and upper confidence values (95% LCI and 95% UCI respectively) of the regressors are presented.

	Model	Mean	95% LCI	95% UCI
L. bufonius				
	Space in Genetic	-0.4212	-1.3735	0.3991
	EcoRes in Genetic	1.0600	0.0830	1.9769
	Space in EcoRes	0.8760	0.5505	1.2069
L. chaquensis				
	Space in Genetic	-0.4272	-1.2881	1.4744
	EcoRes in Genetic	0.7795	-0.1842	4.3572
	Space in EcoRes	0.8332	0.2576	1.5071

frog *Hyla arborea* showed reduced levels of genetic diversity (e.g. Angelone & Holderegger 2009, Krug & Pröhl 2013). In Brazil, the toad *Rhinella ornata* showed lower levels of genetic diversity in smaller forest fragments of the highly modified Atlantic Forest (Dixo et al. 2009). However, the expected heterozygosity (H_e or H_s) is dependent on both, population size (*N*) and mutational rate (μ) (Wang 2015). Therefore, the high levels of H_e observed for Lb and Lc could have resulted from a high mutational rate of our markers even if the population sizes were not too large. In fact, with a few exceptions, our microsatellite markers were highly polymorphic, which could have masked genetic signatures of small population sizes.

Analyses of the genetic variation can be more informative. Our results indicated a significant deficiency of heterozygotes (HWE), significant levels of inbreeding (F_{is} and G_{is}), and excess of allelic richness (bottleneck analyses) for both species. It is well established that a significant reduction in the effective population size (demographic bottlenecks) will generally decrease the genetic variation and expose the remaining reduced population to the stronger effects of genetic drift and inbreeding (Frankham 1995, Frankham et al. 2005, Broquet et al. 2010, Luquet et al. 2011). Ultimately, these processes can lead to a lack of adaptability, reduced fitness, and increased risk of extinction (Frankham 2005, Halverson et al. 2006, Allentoft & O'Brien 2010). Reduced fitness due to inbreeding depression has been empirically proven to decrease the average survivorship of larvae of amphibians mainly in wild populations (Andersen et al. 2004, Halverson et al. 2006), which could be related to the harsh nature of the environmental unpredictability (Halverson et al. 2006, Zamudio & Wieczorek 2007). Moreover, the explosive reproduction of many amphibians may decrease the effective population sizes due to the high differential reproductive success among individuals, i.e., not all reproductive mature individual will equally contribute for the genetic profile of the next generation (Tennessen &

Zamudio 2003, Zamudio & Wieczorek 2007). Because Lb and Lc are very dependent on unpredictable rains to reproduce in temporary ponds (Prado & Haddad 2003, Crump 1995, Reading & Jofré 2003), the observed deficiency of heterozygotes could be related to a skewed reproductive success and high mortality rate of tadpoles (e.g. Andersen et al. 2004). Future studies on the heterozygosity-fitness relationship in regions of stochastic climates are needed to improve our understanding on the impacts of the habitat modification in the genetic patterns of these species.

Bottleneck tests suggested that a past demographic bottleneck leading to ongoing increase of the genetic diversity is a plausible explanation for the observed excess of rare alleles relative to the expected heterozygosity. Demographic bottlenecks due to the lack of suitable habitats were already detected in amphibian populations located in high fragmented landscapes (e.g. Andersen et al. 2004, Crosby et al. 2008, Krug & Pröhl 2013). However, inference based on bottleneck tests can lead to two potential issues. First, it was recently recognized that in cases of complete population isolation (i.e. no immigration), a genetic pattern of demographic bottlenecks will be detected even when population size is constant, confounding the effects of a reduction in population size and genetic drift in isolated populations (genetic bottleneck “sensu” Broquet et al. 2010). However, because the modifications of the landscape did not isolate the studied area from the remaining geographic distribution of the studied species, we had no reasons to expect a recent genetic bottleneck caused by population isolation. Second, Cornuet & Luikart (1996) warned for the fact that it may be hard to distinguish between a post-bottleneck recovering of the allelic richness from an increase in the allelic richness due to population expansion (Figures 1B and C in Cornuet & Luikart (1996)). However, the excess of allelic richness resulted from a population in expansion is expected to reach much larger values than the one observed in a post-

bottleneck recovering process, mainly if high levels of immigration are allowed (Cornuet & Luikart 1996). Because most loci were congruent in signing an ongoing increase of the allelic richness, but only a sub-sample of those were significant, we believe that the studied population of Lb and Lc are recovering from a past demographic bottleneck caused by the replacement of native vegetation by cultivated pastures about 50 years ago. Consequently, because anthropogenic modification of the habitat neither isolates the studied region from the remaining geographic distribution nor causes a reduction in the effective population sizes and intra-deme connectivity, both species may reach the expectations of HWE in the future.

Among-ponds patterns of genetic diversity

In our study, breeding ponds as far as 25 km were not genetically distinct, indicating high functional connectivity among breeding ponds of both species. The geographic scale of a genetic structure is dependent on species-specific intrinsic aspects, such as life history characteristics, and also on extrinsic factors such as landscape composition and topography (e.g. Prugnolle & Meeus 2002, Andersen et al. 2004, Funk et al. 2005, Richardson 2012, Mims et al. 2015). Therefore, it is hard to predict at which geographic distance two breeding sites will represent genetic differentiated populations (genetic demes) or how many genetic clusters are grouped in a sampled region (e.g. Austin et al. 2004, Jehle et al. 2005, Zamudio & Wieczorek 2007, Crosby et al. 2008, Chan & Zamudio 2009, Mims et al. 2015). For instance, the genetic differentiation was greater among breeding sites of the salamander *Ambystoma maculatum* than for the frog *Rana sylvatica* in the same landscape (Richardson 2012). Also, studying two frogs from arid environments, Chan & Zamudio (2009) found that arid-adapted species have higher functional

connectivity in fine to moderate scales than frogs from mesic environments, which was partially attributed to the stochasticity of arid-environments.

Our results showed that populations of Lc and Lb sampled in Paraguay, at the other side of the Paraguay River, did not represent a different genetic deme, suggesting that this river may not represent a barrier to gene flow for these species. Indeed, for Lc, all conducted tests were consistent and strongly suggested that the 161 studied individuals were part of a single genetic deme. For Lb, while the pairwise genetic differentiation analyses did not indicate any significant difference among ponds, G''_{st} and individual-based Bayesian assignment tests tended to indicate low to moderate levels of genetic structure. However, the evidences for genetic structure were weak likely due to the high levels of gene flow among ponds and lack geographic correspondence. Therefore, for Lb, we also considered that all analyzed individual were part of a single genetic deme. Combined, our results indicated higher gene flow rates among ponds for Lc compared to Lb, likely due to ecological differences between both species: while Lc individuals occupy and move through a wide range of habitats and ponds (Areskoug 2001, Schaefer et al. 2006, Valdujo et al 2009, Chapter 1), Lb individuals are related to more forested areas and avoid crossing open fields (Areskoug 2001, Reading & Jofré 2003, Duré & Kehr 2004, Lescano et al. 2015, Chapter 1). The high gene flow among breeding sites of Lb and Lc supports previous findings of high connectivity among breeding sites of amphibians that form aggregations and depend on environmental stochasticity for reproduction in arid environments (Chan & Zamudio 2009, Mims et al. 2015).

Landscape genetic models

Recently, the use of resistance surfaces became very popular in landscape genetics of amphibians (e.g. Peterman et al. 2013b, Titus et al. 2014, Zancolli et al. 2014, Mims et al. 2015); leas-cost

path (e.g. Igawa et al. 2013, Coster et al. 2015) or circuit-theory (e.g. Peterman et al. 2014, Nowakowski et al. 2015a) are then usually employed to calculate pairwise resistances. Regardless of the approach, the key challenge for landscape genetic studies remains on how to assign biological meaningful costs to different landscape features, as the actual effects of such features are generally unknown (Spear et al. 2010, Koen et al. 2012, Nowakowski et al. 2015a). Our results highlight that costs surfaces based on demographic models and life history strategies can generate biologically meaningful parameterizations (Mims et al. 2015, Nowakowski et al. 2015a). By following this approach, we were able to explicit use "a priori" knowledge about the studied species to identify key habitat features contributing to shape their genetic distribution. This procedure was especially successful for Lb, while the contrasting results for Lc endorse the importance of multi-species approach in landscape genetics studies looking for the impacts of anthropogenic activities.

Our path analysis showed that, for both species, the genetic distribution among ponds did not follow the pattern of isolation-by-distance. A weak or even lack of isolation-by-distance can occur when the geographic scale is too small and gene flow among sites is high; alternatively, it may indicate that processes others than isolation by distance are more important in shaping the distribution of the genetic diversity, especially in heterogeneous landscapes (e.g. Dixo et al. 2009, Peterman et al. 2014, Titus et al. 2014, Mims et al. 2015, Nowakowski et al. 2015a). However, the pairwise genetic differentiation of Lc was neither affected by the aquatic nor the terrestrial hypotheses. Therefore, our results showed that aquatic pathways are not promoting functional connectivity of Lc among breeding ponds despite its stronger dependence on aquatic environments. Explanations for this lack of association may reside in the frequency of

overflowing, tadpole mortality, scale of investigation, and high adult migration through the plain terrestrial topography, but more detailed studies are required.

Conversely, life history characteristics of Lb were good predictors of its functional connectivity. Similar results were found for the litter frog *Craugastor bransfordii* and the poison frog *Oophaga pumilio* in a human-modified landscape from Costa Rica (Nowakowski et al. 2015a). Both the litter frog *C. bransfordii* and Lb are similar in their preferences for forested areas and avoidance of pastures, whereas the poison frog *O. pumilio* and Lc are similar in their capacity of crossing pastures (Areskoug 2001, Nowakowski et al. 2015a, Faggioni et al. Chapter 1). While the genetic diversity of Lb and the litter frog were shaped by the contemporary landscape, the genetic diversity of Lc and the poison frog were not (Nowakowski et al. 2015a, this study). However, it is important to note that composite resistance surfaces (e.g. based on demography data) can represent movement and/or physiology limitations; thus disentangle their relative contribution "a posteriori" may be a difficult task (Spear et al. 2010, Nowakowski et al. 2015a). Costs derived from field-experiments represent an important advance in solving this problem before parameterization. For example, a resistance surface derived from demography models had strong support and showed that functional connectivity of *C. bransfordii* occurs through forest patches (Nowakowski et al. 2015a). However, further investigation of surfaces based on field experiments, found that gene flow was facilitated through forested areas due to a decreased risk of mortality linked to microclimate variation across land uses (Nowakowski et al. 2015a). A similar approach was used to study the salamander *Plethodon albagula* in a well preserved area in the USA (Peterman et al. 2014). The authors found great support for the resistance surface representing the rate of water loss during the summer and lack of isolation-by-distance pattern (Peterman et al. 2014). In our study, land use costs in the ecological surface

were derived from habitat occupancy models that mirrored the reproductive requirements of Lb (bare soil in forested areas: Faggioni et al. Chapter 1.). Thus, further investigations are needed to evaluate the relative importance of physiological, physical, and reproductive requirements on the functional connectivity of Lb.

Finally, it is currently recognized that pseudo-replicated data and collinearity are common caveats when dealing with landscape genetic data; recent studies have been proposing interesting solutions for analytical methods and model inference (e.g. Richardson 2012, Titus et al. 2014, Mims et al. 2015). For instance, bootstrap procedures, mixed-effect models, multiple regression with distance matrices, and random forest algorithm can all be found in studies of landscape genetic of amphibians (e.g. Titus et al. 2014, Mims et al. 2015, Coster et al. 2015). Our study showed that when a few hypotheses are tested, the combination of bootstrap procedures and path analysis offers a robust and simple way to deal with pseudo-replication and collinearity, generating estimates that can be directly compared.

Main conclusions and future directions

Our results indicated that clearing for pastures in the last decades may have caused significant reduction of genetic diversity of Lb and Lc. However, the anthropogenic fragmentation did not isolate the studied breeding ponds from the remaining geographic distribution of the species. Fortunately, due to the high gene flow observed for both species, it is possible that the studied populations are recovering the genetic diversity. Despite current allelic richness, our analyses showed that both species presented high within-ponds and global levels of inbreeding, probably not caused by philopatry, reinforcing the chances of past bottlenecks.

Path-ways for Lb individuals may be becoming narrowed by human activities whereas the studied landscape seems to be very permeable for Lc individuals. Such a result is very important for to guide conservation efforts and supports the conclusions based on habitat occupancy models developed for the same species in the studied area (Faggioni et al. Chapter 1.). Structural connectivity through forested corridors must be maintained to avoid genetic and demographic impacts, protecting the habitat required by Lb for reproduction at the same time. Future studies with multiple species, especially those from tropical regions, will help to test the generalization of our findings.

General conclusion

At the Brazilian Chaco, our results suggest that human habitat modification is causing negative impacts on demographic and genetic parameters of *Leptodactylus bufonius* (Lb), whereas *L. chaquensis* (Lc) seems to be adapted to the current landscape configuration. Importantly, the effects of the conversion of the native vegetation to pastures can be predicted by their ecological strategies. The more generalist use of the habitat by Lc, reflected in the high probability of occupancy and high functional connectivity among breeding ponds, facilitate its permanence in the altered landscape. On the other hand, the anthropogenic modification of the landscape is clearly reducing the amount of suitable habitats to fulfill the life requirements of Lb. When forested habitats are converted to pasture, the mud zone, located at the ponds' shorelines, is replaced by grass. Without mud, males of Lb are not able to shape the mud nest, attract females, and reproduce. At the same time, the spread of pastures narrow the structural and functional connectivity of Lb among breeding ponds. Therefore, structural connectivity through forested areas must be maintained to avoid genetic and demographic impacts, also protecting the habitats required by Lb for reproduction. Fortunately, due to the high gene flow of both species, our analyses indicated that the studied populations are probably recovering their genetic diversity. Because many *Leptodactylus* species present similar reproductive modes and habitat requirements, future studies can test the generalizations of the relationships between ecological strategies and anthropogenic impacts described in the present dissertation for *L. chaquensis* and *L. bufonius*.

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Supporting information: Chapter 1

Table S1 Pond scale model selection table for probabilities of detection, occupancy, colonization, and extinction of *Leptodactylus bufonius* and *L. chaquensis* in 50 temporary ponds at the Brazilian Chaco. K = number of parameters; ΔAICc = Akaike's Information Criterion with the second bias adjustment; AICc*w* = AICc weighted; Cml. *w* = cumulative weight. Models selected for multi-model inference (see Tables 8 and 9) are in bold.

Species	*Leptodactylus bufonius*					*Leptodactylus chaquensis*				
Parameter	Model	K	ΔAICc	AICc*w*	Cml. *w*	Model	K	ΔAICc	AICc*w*	Cml. *w*
$\hat{p}$	**MRS**	5	0.00	0.61	0.61	**MRS**	5	0.00	0.45	0.45
	MRS+RD	6	2.44	0.18	0.78	**MRS+RD**	6	0.70	0.32	0.76
	MRS+AT	6	2.56	0.17	0.95	MRS+AT	6	2.26	0.14	0.91
	MRS+AT+RD	7	5.13	0.05	1.00	MRS+AT+RD	7	3.19	0.09	1.00
	Constant	4	16.64	0.00	1.00	AT	5	28.26	0.00	1.00
	AT	5	17.08	0.00	1.00	Constant	4	28.64	0.00	1.00
	RD	5	19.12	0.00	1.00	AT+RD	6	30.14	0.00	1.00
	AT+RD	6	19.66	0.00	1.00	RD	5	30.48	0.00	1.00
$\hat{\psi}$	**BS+Shr**	7	0.00	0.37	0.37	**Gr**	6	0.00	0.59	0.59
	BS	6	0.80	0.25	0.61	Gr+Shr	7	2.80	0.15	0.74
	CO+BS	7	1.00	0.22	0.84	Constant	5	3.94	0.08	0.82
	Gr	6	2.71	0.10	0.93	CO+BS	7	4.83	0.05	0.87
	Gr+Shr	7	3.96	0.05	0.98	BS+Shr	7	5.41	0.04	0.91
	Shr	6	7.25	0.01	0.99	BS	6	5.77	0.03	0.95
	CO	6	8.32	0.01	1.00	CO	6	5.87	0.03	0.98
	Constant	5	12.26	0.00	1.00	Shr	6	6.47	0.02	1.00
$\hat{\gamma}$	**Constant**	6	0.00	0.37	0.37	**BS**	6	0.00	0.38	0.38
	CO	7	1.25	0.20	0.56	BS+Shr	7	2.31	0.12	0.49
	Shr	7	2.37	0.11	0.68	Gr+Shr	7	2.32	0.12	0.61
	Gr	7	2.62	0.10	0.78	CO+BS	7	2.42	0.11	0.72
	BS	7	2.63	0.10	0.88	Shr	6	2.59	0.10	0.83
	CO+BS	8	3.94	0.05	0.93	Constant	5	3.08	0.08	0.91
	Gr+Shr	8	4.34	0.04	0.97	Gr	6	3.40	0.07	0.97
	BS+Shr	8	4.93	0.03	1.00	CO	6	5.36	0.03	1.00
$\hat{\varepsilon}$	**BS**	7	0.00	0.34	0.34	**Gr**	6	0.00	0.47	0.47
	Constant	6	0.74	0.23	0.57	**Constant**	5	1.19	0.26	0.73
	BS+Shr	8	1.87	0.13	0.71	Gr+Shr	7	2.73	0.12	0.86
	CO+BS	8	2.69	0.09	0.79	Shr	6	3.75	0.07	0.93
	Gr	7	3.31	0.06	0.86	CO	7	5.49	0.03	0.96
	Shr	7	3.32	0.06	0.92	BS	7	5.68	0.03	0.99
	CO	7	3.45	0.06	0.98	BS+Shr	8	8.27	0.01	0.99
	Gr+Shr	8	6.02	0.02	1.00	CO+BS	8	8.44	0.01	1.00

Table S2 Landscape scales model selection table (only models with ΔAICc ≤ 2 are shown) for probabilities of detection, occupancy, colonization, and extinction of *Leptodactylus bufonius* in 50 temporary ponds at the Brazilian Chaco. Scale = length of the buffer radii centered at the pond; K = number of parameters; ΔAICc = Akaike's Information Criterion with the second bias adjustment; AICc*w* = AICc weighted; Cml. *w* = cumulative weight.

Parameter	Scale	Model	K	ΔAICc	AICc*w*	Cml. *w*
$\hat{\psi}$	400 m	GrSc400	6	0.00	0.68	0.68
	700 m	GrSc700	6	0.00	0.74	0.74
	1000 m	GrSc1000	6	0.00	0.77	0.77
	1600 m	GrSc1600	6	0.00	0.77	0.77
$\hat{\gamma}$	400 m	Constant	6	0.00	0.51	0.51
		GrSc400+Fd400	8	1.98	0.19	0.70
	700 m	Constant	6	0.00	0.53	0.53
	1000 m	GrSc1000+Fd1000	8	0.00	0.49	0.49
		Constant	6	0.82	0.32	0.81
	1600 m	Constant	6	0.00	0.61	0.61
$\hat{\varepsilon}$	400 m	Constant	6	0.00	0.58	0.58
	700 m	Constant	6	0.00	0.60	0.60
	1000 m	Constant	6	0.00	0.60	0.60
	1600 m	GrSc1600+Fd1600	8	0.00	0.36	0.36
		Constant	6	0.26	0.32	0.68
		Fd1600	7	1.04	0.21	0.89

Table S3 Landscape scales model selection table (only models with ΔAICc ≤ 2 are shown) for probabilities of detection, occupancy, colonization, and extinction of *Leptodactylus chaquensis* in 50 temporary ponds at the Brazilian Chaco. Scale = length of the buffer radii centered at the pond; K = number of parameters; ΔAICc = Akaike's Information Criterion with the second bias adjustment; AICc*w* = AICc weighted; Cml. *w* = cumulative weight.

Parameter	Scale	Model	K	ΔAICc	AICc*w*	Cml. *w*
$\hat{\psi}$	400 m	Fd400	6	0.00	0.76	0.76
	700 m	Fd700	6	0.00	0.56	0.56
	1000 m	Fd1000	6	0.00	0.40	0.40
		Constant	5	0.70	0.28	0.68
		GrSc1000+Fd1000	7	1.84	0.16	0.84
		GrSc1000	6	1.86	0.16	1.00
	1300 m	Fd1300	6	0.00	0.37	0.37
		Constant	5	0.90	0.24	0.61
		GrSc1300+Fd1300	7	1.16	0.21	0.82
		GrSc1300	6	1.40	0.18	1.00
$\hat{\gamma}$	400 m	Fd400	7	0.00	0.60	0.60
	700 m	Fd700	7	0.00	0.69	0.69
	1000 m	Fd1000	7	0.00	0.43	0.43
		GrSc1000+Fd1000	8	0.71	0.30	0.73
		Constant	6	1.35	0.22	0.94
	1300 m	Fd1300	7	0.00	0.36	0.36
		Constant	6	0.07	0.35	0.72
		GrSc1300+Fd1300	8	1.30	0.19	0.91
$\hat{\varepsilon}$	400 m	Fd400	7	0.00	0.57	0.57
	700 m	GrSc700+Fd700	8	0.00	0.64	0.64
	1000 m	GrSc1000+Fd1000	8	0.00	0.43	0.43
		Fd1000	7	0.29	0.38	0.81
	1300 m	Fd1300	7	0.00	0.50	0.50
		GrSc1300+Fd1300	8	1.80	0.20	0.70
		GrSc1300	7	1.91	0.19	0.89

Supporting information: Chapter 3

Table S1 Full and reduced data sets for *Leptodactylus bufonius* (Lb) and *L. chaquensis* (Lc). Loci in bold were exclude from the full data set based on evidences of null alleles (Null alleles: ponds with evidence of null alleles/total number of ponds sampled) and/or few genotyped individuals across nine and 13 breeding ponds of Lb and Lc respectively. Nvalid must be higher than Nmin.

L. bufonius			*L. chaquensis*		
Loci	Null alleles	Nvalid/Nmin	Loci	Null alleles	Nvalid/Nmin
Lbufo17	1/9	5/3	Lchaq57	1/13	5/2
Lbufo27	2/9	5/2	Lchaq618	2/13	5/5
Lbufo33	0/9	5/2	**Lchaq63**	10/13	4/6
Lbufo36	0/9	4/2	Lchaq103	0/13	5/2
Lbufo55	0/9	5/3	**Lchaq115**	8/13	5/1
Lbufo57	1/9	5/3	**Lchaq25**	9/13	2/4
Lbufo61	0/9	2/3	Lchaq3572	1/13	5/2
Lbufo75	7/9	5/2	**Lchaq36**	0/13	2/7
Lbufo91	0/9	5/1	Lchaq708	2/13	5/2
Lbufo113	1/9	5/2	**Lchaq99**	4/13	5/7
Lbufo120	7/9	4/4	Lchaq15	5/13	5/1
Lbufo261	1/9	4/4	Lchaq18	0/13	5/1
Lbufo286	2/9	4/5	**Lchaq283**	12/13	5/3
Lbufo287	8/9	5/4	**Lchaq404**	10/13	2/3
Lbufo324	3/9	5/3	Lchaq642	6/13	5/2
Lbufo756	1/9	5/2			

Table S2 Global values estimated from the full and the reduced data sets for *Leptodactylus bufonius* and *L. chaquensis*. Values are mean (lower 95% CI–upper 95% CI). Excluded loci are indicated in Table S1.

		By pond overall loci		By loci overall ponds		Overall
	# Loci	F_{is}	F_{it}	G_{is}	G''_{st}	HWE
L. bufonius						
Full data set	16	0.222 (0.158–0.293)	0.225 (0.160–0.295)	0.234 (0.168–0.296)	0.071 (-0.007–0.172)	***
Reduced data set	11	0.156 (0.117–0.195)	0.157 (0.118–0.195)	0.172 (0.124–0.219)	0.021 (-0.025–0.072)	***
L. chaquensis						
Full data set	15	0.251 (0.147–0.355)	0.251 (0.148–0.355)	0.254 (0.144–0.358)	0.012 (-0.018–0.048)	***
Reduced data set	8	0.123 (0.020–0.229)	0.124 (0.020–0.228)	0.117 (0.030–0.251)	0.008 (-0.019–0.041)	***

***p<0.0005.

Table S3 D_{est} and H_e by loci used in the correlation analysis suggested by Wang 2015 (Figure S1).

Leptodactylus bufonius			*Leptodactylus chaquensis*		
Loci	H_e	D_{est}	Loci	H_e	D_{est}
Lbufo17	0.726	-0.078	Lchaq57	0.927	-0.041
Lbufo27	0.934	0.111	Lchaq618	0.464	-0.012
Lbufo33	0.638	0.008	Lchaq63	0.967	0.186
Lbufo36	0.974	0.059	Lchaq103	0.913	-0.012
Lbufo55	0.876	0.121	Lchaq115	0.791	0.024
Lbufo57	0.966	0.176	Lchaq25	0.912	-0.189
Lbufo61	0.372	0.175	Lchaq3572	0.900	0.019
Lbufo75	0.954	0.267	Lchaq36	0.894	0.141
Lbufo91	0.848	0.002	Lchaq708	0.830	0.097
Lbufo113	0.889	0.040	Lchaq99	0.970	-0.014
Lbufo120	0.987	-0.174	Lchaq15	0.867	-0.025
Lbufo261	0.840	0.069	Lchaq18	0.545	0.012
Lbufo286	0.883	-0.089	Lchaq283	0.961	0.075
Lbufo287	0.923	0.116	Lchaq404	0.756	0.011
Lbufo324	0.911	-0.008	Lchaq642	0.826	0.004
Lbufo756	0.906	0.012			

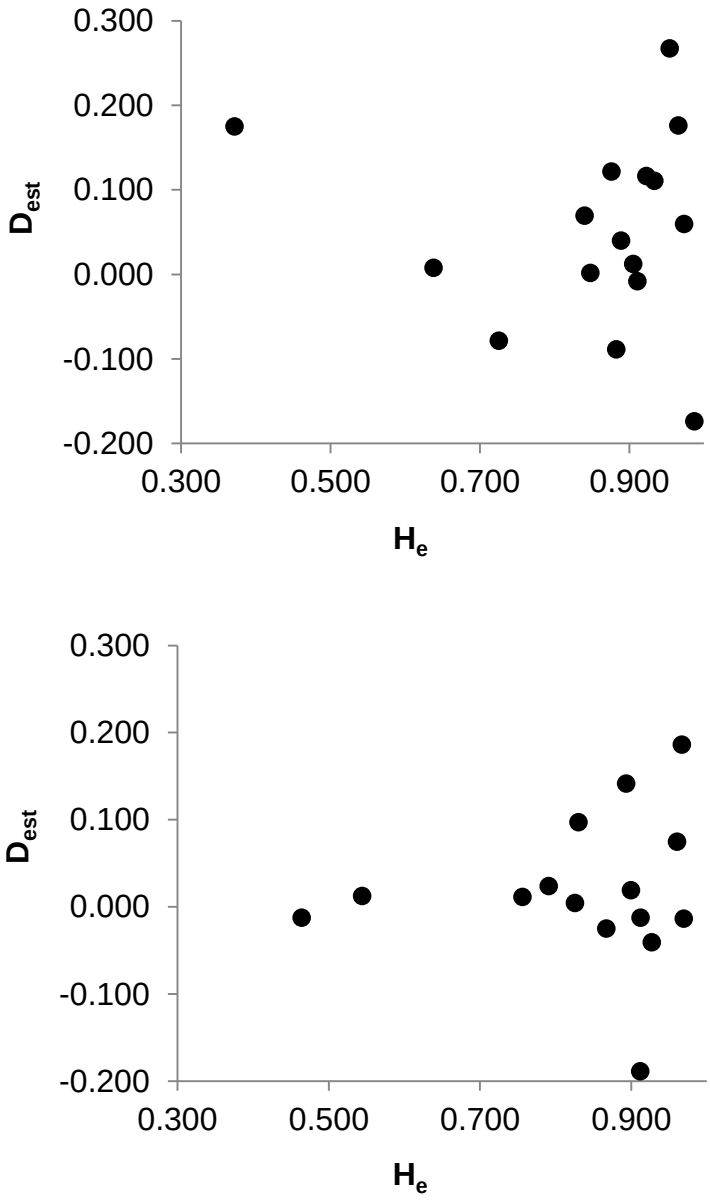

Figure S1 Correlations between D_{est} and H_e across microsatellites loci for *Leptodactylus bufonius* (above) and *L. chaquensis* (below).

Table S4 STRUCTURE results and ΔK calculations for *Leptodactylus bufonius* and *L. chaquensis*. The best ΔK is presented in bold for each species.

Species	K	Mean LnP(K)	Std.dev LnP(K)	Ln'(K)	[Ln''(K)]	ΔK
Leptodactylus bufonius						
	1	-9891.74	0.9526	NA	NA	NA
	2	-9876.692	3.5015	15.048	55.668	15.898158
	3	**-9805.976**	**7.9435**	**70.716**	**205.392**	**25.85657**
	4	-9940.652	107.1894	-134.676	273.244	2.549171
	5	-10348.572	173.5028	-407.92	311.688	1.796444
	6	-10444.804	221.4615	-96.232	163.692	0.739144
	7	-10377.344	238.8536	67.46	50.108	0.209785
	8	-10259.776	217.9566	117.568	87.016	0.399236
Leptodactylus chaquensis						
	1	-12178.696	0.7536	NA	NA	NA
	2	-12215.296	26.5216	-36.6	70.144	2.644791
	3	-12181.752	145.3754	33.544	111.22	0.765054
	4	**-12036.988**	**16.9634**	**144.764**	**60.972**	**3.594332**
	5	-11953.196	26.5605	83.792	63.344	2.384893
	6	-11932.748	17.7703	20.448	19.056	1.072351
	7	-11931.356	20.9011	1.392	4.456	0.213194
	8	-11925.508	23.2774	5.848	5.052	0.217035

Appendix A Parameterization of the resistance surfaces.

Euclidian distance surface (Space): the parameterization of the Space surface was built up in a map of 30 meters ground resolution from the studied area downloaded from USGS EarthExplorer (accessed on July 2014). In order to construct a resistance surface analogue to the geographic distance between breeding ponds, we set each pixel the value of 1. Thus, pairwise resistance values for Space mirror the pure accumulative effect in a homogenous landscape, representing the isolation-by-distance hypothesis.

Ecological strategies surface (EcoRes): the parameterization of the EcoRes surface was built up in a map of 30 meters ground resolution from the studied area (EarthExplorer 2014). We used the software Geomatica (PCI 2012) to classify each pixel at the original image as one of the four main landscape elements of the studied area: permanent water bodies (PWB); forested areas (For); high density shrubs fields (Fd); and grass with sparse shrubs (GrSc). Habitat occupancy models evaluated for 50 breeding ponds at the same studied area (Faggioni et al. Chapter 1.) and previous knowledge about the natural history and habitat use of each species (e.g., Areskoug 2001; Prado & Haddad 2003; Valdujo et al 2009; Lescano et al. 2015), were considered for the parameterization. Occupancy models for Lb resulted in a range of probabilities of occupancy (15% – 94%) inversely linked to the range of grass percentage surrounding ponds (0% – 100%). Because the percentage of grass and forest were highly negatively correlated (range = -0.6 – -0.9 through increasing buffer radii; Faggioni et al. Chapter 1.), we used the ration 94/15=6 to determine the contrast between forest and grass pixels. Because high-density shrub areas did not affect migration of Lb (Faggioni et al. Chapter 1.), we set them the same resistance value of forested areas. Finally, because of the terrestrial use of habitat by Lb, water pixels were set to the value of 100 (Mims et al. 2015, Appendix B). The final ratio among cover types was 100:1:1:6

for water, forest, high density shrubs, and grass respectively. On the other hand, occupancy models for Lc resulted in a range of probabilities of occupancy (23% – 93%) positively linked to the range of the percentage of high density shrubs surrounding ponds (4% – 72%). Because the percentage of high density shrubs was not correlated with percentage of forest and grass surrounding ponds (Faggioni et al. Chapter 1.), and also because landscape alteration does not seem to limit Lc movements (Areskoug 2001; Prado & Haddad 2003; Valdujo et al 2009; Faggioni et al. Chapter 1.), we used the ration 93/23=4 to determine the contrast between high density shrubs and forest and grass pixels. Finally, because Lc is an aquatic breeder, water pixels were arbitrarily set to the value of 50 to contrast with the terrestrial breeder Lb. The final ratio among cover types were 50:4:1:1 for water, forest, high density shrubs, and grass respectively.

Water flow surface (WatRes): the parameterization of the water flow surface was based on a topographic map of 30 meter resolution from the studied area downloaded from USGS EarthExplorer (accessed on July 2014). Because it represents the hypothesis of tadpole connectivity through overflowing water, we used the same surface for both species. In the topographic map, the lowest values (1–6 representing the lowest resistance) were found inside the Paraguay River, which should represent the greatest barrier for tadpoles in our studied system. The remaining values (7–180) represented the variation in topography outside the river. Therefore, pixels inside the river were transformed previous to analyses. The transformation aimed to keep the same ration of 100:6 and 50:4 for the water and non-habitat pixels in the EcoRes of Lb and Lc respectively. Therefore, in order to find a mean value (related to both species EcoRes) for water pixels we (1) calculated the ratio between water and non-habitat pixels, W:N-H, for both species (Lb: 100/6=17; Lc: 50/4=13); (2) calculated arithmetic mean of the product between W:N-H and the highest altitudinal value in the studied area (180 meters; Lb:

17*180=3060; Lc: 13*180=2340; (3060+2340)/2=2700). Thus, the final resistance values ranged from 1–180 outside the Paraguay River and a fixed value of 2700 inside the river.

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