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The role of ecology in driving the diversification process along the Amazon-Cerrado gradient

A dissertation submitted in partial satisfaction

of the requirements for the degree

Doctor of Philosophy in Biology

by

Hilton Masaharu Oyamaguchi

2014

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ABSTRACT OF THE DISSERTATION

The role of ecology in driving the diversification process along the Amazon-Cerrado gradient

by

Hilton Masaharu Oyamaguchi

Doctor of Philosophy in Biology

University of California, Los Angeles, 2014

Professor Dr. Thomas B. Smith, Chair

How new species evolve is one of the most fundamental questions in biology. In particular, there has been considerable debate about the relative roles of genetic drift and natural selection in speciation. A salient example is the controversy surrounding the origins of Amazonian biodiversity. The Pleistocene has long considered one of the most important periods of diversification in the Amazon. According to the Pleistocene refugia hypothesis rainforest contracted into small refugia and genetic drift played the dominate role in driving speciation. However, the centers of diversity that would have provided evidence for these supposed refugia were never found. In addition, phylogenies and fossil records show that most lineages are older than the Pleistocene, refuting this simple allopatric model of speciation. Although multiple mechanisms such as geographical barriers, marine incursions, and ecological factors have been hypothesized to explain the Amazon's hyperdiversity there have been relatively few tests of these hypotheses.

Here I examined the importance of ecological factors in driving population divergence in the lesser tree frog (*D. minutus*) between the Amazon and Cerrado and along the gradient between these two biomes. Substantial temperature and precipitation differences between these

biomes raise the possibility that environmental factors drive population differentiation, potentially resulting in speciation.

In chapter one, I examined the importance of environmental variables in driving reproductive divergence in the lesser tree frog. Water availability and temperature emerged as important drivers of body size variation, with body size found to be negatively correlated with vocal traits. For frogs, vocalization is one of the most important traits for mating success. Thus, these results suggest that the contrasting selective pressures on body size between the Amazon and Cerrado influence vocalization, which may result in reproductive divergence and suggests how reproductive isolation may evolve as a result of divergent natural selection pressures in these distinct habitats.

In chapter two, I evaluated the relative roles of genetic drift and natural selection in driving divergence in the lesser tree frog along the Amazon-Cerrado gradient. I showed that environmental variables are more important than geographical distance for driving divergence in reproductive and morphological traits. This suggests that ecological differences along the gradient result in adaptive divergence. Such divergence in phenotypic traits indicates the dominant role of natural selection over neutral processes for speciation events in this region.

Finally, in the third chapter, I investigated the sources of phenotypic variation in response to environmental variation between the Amazon and Cerrado biomes. The goal was to understand the contributions of genetic and environment to *D. minutus* phenotypic variation in larval development between these two biomes. I conducted a common garden experiment to test whether phenotypic variation in *D. minutus* has a genetic component, whether tadpoles from the Cerrado develop faster due to a more seasonal habitat, and whether *D. minutus* shows plastic responses to different thermal conditions. This experimental work showed that both enviroment

and genetics play a role in phenotypic variation. Moreover, tadpoles from Cerrado populations developed faster than those from the Amazon populations. This is indicative of local adaptation to a more seasonal habitat by the Cerrado populations.

In summary, I showed that: 1) ecological factors are important drivers of phenotypic divergence; 2) natural selection appears to be more important than genetic drift in this divergence process between the Amazon and Cerrado; and 3) phenotypic differentiation is under genetic control, heritable, and can respond to selection. These findings indicate that environmental variation between habitats is likely an important factor in the speciation process. Furthermore, such environmental variation seems to be important in generating adaptive variation. Thus, preserving the habitat gradient between the Amazon and Cerrado is extremely important for the conservation of intraspecific adaptive variation, which could help species persist in the face of anthropogenic climate change.

The dissertation of Hilton Masaharu Oyamaguchi is approved.

Dr. Gregory Grether

Dr. Thomas Gillespie

Dr. Victoria Sork

Dr. Thomas B. Smith, Committee Chair

University of California, Los Angeles

2014

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CHAPTER 1

Environmental drivers of morphological and vocal divergence between the Amazon and Cerrado biomes in the lesser tree frog (*Dendropsophus minutus*)

Abstract

Environmental variation between habitats may impose divergent selection resulting in phenotypic divergence and can lead to reproductive divergence, especially if these same traits are favored by sexual selection. Anuran body size is an important trait in male mating success and affects spectral traits of call that frogs use in mate choice. Environmental differences in water availability and temperature influence anuran body size and shape and may result in reproductive incompatibility between populations and might cause speciation events. In this study, I investigated the importance of environmental differences between the Amazon and the Cerrado in driving reproductive divergence. I contrasted environmental effects on body size and shape variation in *Dendropsophus minutus* between the Amazon and the Cerrado biomes. In addition, I studied how body size is correlated with vocal frequencies. This results show that body size increases in more seasonal and colder climates. The most important variable in explaining body size variation is precipitation seasonality followed by annual precipitation, annual mean temperature, and temperature seasonality. Head shape variation is explained by annual mean precipitation, seasonality of temperature and annual mean temperature. Furthermore, body size is negatively correlated with vocal spectral traits. These results suggest that environmental differences between Amazon and Cerrado habitats may be an important driver of reproductive divergence through selection on body size.

Introduction

Environmental differences between habitats may result in divergent selection leading to phenotypic divergence and ultimately ecological speciation (Smith et al. 1997; Schneider et al. 1999; Slabbekoorn and Smith 2002; Thomassen et al. 2010; Schluter 2000; Coyne and Orr 2004; Rundle and Nosil 2005; Seehausen et al. 2008; Nosil 2012, Orr and Smith, 1998). Ecological speciation occurs when reproductive isolation between populations evolves as a result of adaptation to different environments or ecological niches (Schluter 2000; Rundle and Nosil 2005; Seehausen et al. 2008; Nosil 2012). Reproductive isolation may evolve as a by-product of divergent selection on traits important for non-random mating (Schluter 2000; Rundle and Nosil 2005; Servedio 2011; Nosil 2012). If a trait that causes non-random mating is also under divergent selection ('magic trait'), then recombination cannot break this association, making it effective in driving speciation events even with moderate levels of gene flow (Servedio 2011). Thus, understanding how environmental differences affect traits favored by sexual selection can provide important information about the processes that generate population divergence and potentially speciation events.

Anuran body size is an important trait in mate choice (Ryan 1992). A larger body indicates higher reproductive potential, because large males have larger breeding territories, greater survivorship, and larger offspring than small males (Berven 1982a). Females assess male body size by male vocalization (Gerhardt and Huber 2002), which is the most important form of communication in frogs (Duellman 1994). Frog vocalization attracts mates and therefore plays an important role in sexual selection (Wells 1977). In general, larger males produce lower pitches and females prefer those lower frequencies call (Ryan 1980; Ryan 1992). Consequently, environmental variables that affect anuran body size might be important drivers of reproductive divergence.

Environmental variables, such as precipitation (Nevo 1973; Olalla-Tarraga et al. 2009), hydroperiod (Denver et al. 1998; Mousseau 1997; Gomez-Mestre and Buchholz 2006), and temperature (Ashton 2002), influence body size in anurans. Amphibians are strongly dependent on water and most use aquatic and terrestrial habitats during their life cycle (Duellman 1994). Water availability affects body size and shape during larval stages (Gomez-Mestre and Buchholz 2006) and body size in the adult phase (Nevo 1973; Olalla-Tarraga et al. 2009). After metamorphosis, larger bodies reduce surface-to-volume ratio conferring higher desiccation tolerance in drier areas (Nevo 1973; Ashton 2002). During the larval stages, a more seasonal climate results in a shorter growth season in ectotherms (Mousseau 1997; Gomez-Mestre and Buchholz 2006) and also affects amphibian morphology (Denver et al. 1998). In particular, shorter developmental time results in smaller body and changes in body shape (Gomez-Mestre and Buchholz 2006; Székely et al. 2010).

Temperature also influences body size and shape in the aquatic and terrestrial phases (Berven 1982b). In the terrestrial phase, body size is important for thermoregulation (Olalla-Tarraga and Rodriguez 2007). Larger size is associated with higher latitudes and lower temperatures to minimize heat loss (Bergmann 1847). During the larval stages, higher temperatures increase the rate of development and result in smaller body size (Berven 1982a; van der Have 1996).

Environmental differences in precipitation and temperature between the Amazon and Cerrado biomes created strong selection on phenotypic divergence in frogs. The Amazon rainforest and the Cerrado are the largest biomes in South America (Da Silva and Bates 2002; Da

Silva et al. 2005) and form an extensive ecotone (extending about 2800 km). The Amazon, at more than 6 million km^2, is the largest rainforest and river system in the world (Da Silva and Bates 2002; Wesselingh et al. 2009) and has a heterogeous landscape (Tuomisto et al. 1995; Tuomisto 1997; Tuomisto 2007). The wet conditions in the Amazon biome are due to the hydrological cycle in which the moist air from the tropical Atlantic is trapped by the Andes (Salati and Vose 1984). After the Amazon rainforest, the Cerrado is the second largest biome in South America at 1.86 million km^2 (Da Silva and Bates 2002) and harbors high number of endemic species (Myers et al. 2000). The Cerrado occupies the central region of South America and has a tropical wet and dry climate (Klink 2005). This biome has vegetation that varies from dense dry forests (Cerrado *sensu stricto*) to open grassland (known as Campo limpo) (Eiten 1972; Oliveira-Filho and Ratter 2002).

From the northwest of the Amazon to the southeast in the Cerrado biome, the climate varies from continuously rainy to wet/dry with a long dry season (Davidson et al. 2012). This region presents similar conditions to the African savanna-rainforest ecotone where divergent natural selection has shown to be important in divergence and speciation (Smith et al. 1997; Smith et al. 2005a; Smith et al. 2005b). In a similar fashion, the vast contact zone between Amazon rainforest and Cerrado in South America may also play an important role in generating and maintaining rainforest diversity. However, the evolutionary importance of this area is poorly understood (Smith et al. 2005a).

The broad goal of this study was to explore the importance of environmental differences between the Amazon and the Cerrado in driving reproductive divergence. I investigated how traits important for non-random mating in *Dendropsophus minutus* (e.g. body size and vocalization) can be affected by differences in water availability and temperature between the

Amazon and the Cerrado biomes. The specific objectives of this study were to test: 1) whether Amazon and Cerrado populations of *D. minutus* are morphologically different, 2) two potential mechanisms to explain this morphological variation, including: a) water availability (Nevo 1973; Ashton 2002; Olalla-Tarraga et al. 2009), and b) heat balance: a negative relationship between body size and temperature is expected in ectotherms due to efficiency in thermoregulation (Olalla-Tarraga and Rodriguez 2007), and 3) whether variation in morphology affects vocalization traits.

Material and Methods

Target species

Dendropsophus minutus is widely distributed in South America occurring in the lowlands east of the Andes, Brazil, Paraguay, and Uruguay (Frost 2013). This species is found in open and forested areas (Silvano 2010), at the edges of forests, and in clearings (Lima et al. 2006), and occurs in the Amazon and as well as the Cerrado. Reproduction occurs from November to May during the rainy season, with males calling from shrubs near ponds (Lima et al. 2006), and females depositing eggs in still water (Haddad and Prado 2005). This species shows intraspecific variation among populations in vocal traits in different habitats (Cardoso and Haddad 1984) and in morphology (Kaplan 1994; Hawkins 2007), suggesting that ecological differences between habitats might shape these differences (Cardoso and Haddad 1984).

Data collection

I collected morphological data on *D. minutus* from museum collections (Smithsonian Natural History Museum, American Natural History Museum, Museu de Zoologia da

Universidade de São Paulo, Universidade Estadual Paulista, and the Universidade Federal do Mato Grosso). I examined *D. minutus* morphology (n = 164) from the Amazon and the Cerrado biomes from 23 localities (Table S1, Fig. 1-1). Only adult males were analyzed to avoid confounding effects of sexual dimorphism. Using dial calipers, calibrated to the nearest 0.01 mm, I measured snout-vent length (SVL), head length (HL), head width (HW), thigh length (THL), tibia length (TBL), tarsus (Ta), foot length (FL), hand length (HaL), and forearm length (FA). All measurements followed Heyer *et al.* (1994).

I recorded vocalizations of 20 males *D. minutus* from six localities in the Cerrado (Table 1-2S) with a digital recorder (Marantz PDM660) coupled to a shotgun microphone (Sennheiser ME66) at about 1 m from the calling frog at a sampling frequency of 44.1 kHz and 16 bits resolution. *Dendropsophus minutus* vocalization presents three types of notes, A, B, and C, which are combined forming advertisement and territorial calls depending on the social context (Haddad 1987). The emission of notes A and B are associated with an advertisement call and C with a territorial call (Haddad 1987). I measured dominant frequency (DM), fundamental frequency (FF), minimum frequency (MiF), and maximum frequency (MaF) of each type of note using spectrogram and waveform in the Sound Ruler software (Gridi-Papp 2007). After each recording, I captured the calling frog and measured snout-vent length (SVL), head length (HL), and head width (HW). In addition, I also measured the air temperature with a digital thermometer to the nearest 0.5° C to test the effect of temperature on spectral traits. To evaluate if morphology affects vocalization traits, I tested correlation between morphological (SVL, HL and HW) and spectral traits (DM, FF, MiF, and MaF) using linear regression.

Environmental predictors

Out of 23 sampling sites from museum collections, 19 had geographical coordinates. I excluded the four sites without coordinates (which only had municipality locations) from the museum data to avoid spatial error influence (Graham et al. 2008). I extracted four bioclimatic predictors (annual mean precipitation, precipitation seasonality, annual mean temperature, and temperature seasonality) from the WorldClim database for each of these 19 sites (Hijmans et al. 2005). The four bioclimatic variables were obtained from raster layers using Quantum GIS (Development Team 2010). I used the environmental data to test two hypotheses. First, the water availability hypothesis contends that annual mean precipitation and precipitation seasonality are the main drivers of body size. Second, according to the heat balance hypothesis, body size should depend on mean annual temperature and seasonality in temperature.

I classified each of the 19 sampling sites as belonging to either the Amazon or Cerrado biomes using the biome classification of Dinerstein et al. (1995) (Fig. 1-1). I used classification tree regression (Liaw 2002; R-Development-Core-Team 2008) to test whether the four bioclimatic variables predict climatic differences between Cerrado and Amazon sites. I built a classification tree (n = 5000) based on four bioclimatic predictors (annual mean temperature, temperature seasonality, annual precipitation, and precipitation seasonality) extracted from each sampling site and used biome type as a response variable. Using this model, I accurately classified 89.5% (17/19) sites as Cerrado or Amazon.

Data analysis

Divergence between biomes

I conducted a principal component analysis (PCA) to reduce redundancy in the morphological traits and then used relevant principal components to summarize the results and to visually explore variation in body size and shape. To test for significant morphological differences between specimens from the Amazon and the Cerrado, I used a discriminant function analysis in the CanDisc and Mass R packages (Friendly 2013; Ripley 2013), which maximizes the total variance between groups. The degree of morphological divergence between these two biomes was estimated from the standardized coefficient value of each trait. A higher coefficient indicates larger divergence between the Amazon and Cerrado. To test for morphological differences between differences between means of morphological traits from Cerrado and Amazon populations, I performed a t-test in the R ‘Permute’ package (Simpson 2012) with 100,000 permutations. This test requires equality of group variance, which I confirmed using a Bartlett test.

Environmental predictors

To evaluate the relationship between morphological traits and the environmental predictors, I used Pearson correlation and tree regression in R (Liaw and Wiener 2002). The tree regression was used to assess the relative importance of each environmental predictor in explaining the amount of variation in the response variables. Regression trees recursively partition the data into increasingly homogeneous groups (Liaw and Wiener 2002). The importance of each predictor variable is assessed using a technique called bagging, which consists of constructing a regression tree using a randomly selected subset of the data and then assessing the accuracy of the model on the remaining data. If the accuracy of the model decreases when a variable is left out of the model, then the variable is kept as a predictor. I ran this tree regression model with 2000 iterations to obtain the relative variable importance of the four environmental predictors.

Results

Morphological traits of *D. minutus* showed significant differentiation between the Amazon and the Cerrado (Fig. 1-2, Table 1-1). Seasonality of precipitation explained the highest percentage of body size variation, followed by annual precipitation, temperature, and temperature seasonality (Fig. 1-3). Body size was found to be larger in a more seasonal, colder climate (Fig. 1-3, Table 1-2). These results support the water availability and heat balance hypotheses. Interestingly, head characters (head width and head length, Fig. 1-4) showed a stronger association with environmental predictors than body size (Fig. 1-4). This suggests that, in addition to the effect of climate on body *size*, climate also affects anuran body shape. In addition, body size showed a negative correlation with vocalization traits (Fig. 1-5), indicating that variation in body size might lead to vocal differentiation.

Biome based divergence

PCA of the morphological data showed clear differences between the Cerrado and Amazon specimens (Fig. 1-2). The first two axes explain 73.6 % of intraspecific variation in morphology. Based on factor loading PC1 was interpreted as a size component and explained 56% and PC2, interpreted as a shape component with heavy loadings on the head characters (Table 1-3S), explained 17.6% of the variation (Fig. 1-2).

Discriminant analysis showed a significant difference between specimens from the Amazon and Cerrado, producing one significantly different canonical dimension (MANOVA Wilks statistic = 0.373, $p < 0.0001$). The most divergent trait between the Amazon and Cerrado was head width, followed by tibia, head length, hand length, forearm, foot length, and snout vent length (Table 1-4S). A permutation t-test indicated a significant difference between Cerrado and Amazon specimens in all morphological traits (Table 1-1) except for snout-vent length, forearm, and foot length (Table 1-1).

Environmental drivers of morphology variation

Body size increased with precipitation seasonality (Fig. 1-3, r = 0.503, $p < 0.001$, Table 1-2) and decreased with annual mean temperature (r = -0.427, $p < 0.001$, Table 1-2). The hind-limb characters followed the same trend as body size and were correlated with precipitation seasonality and negatively correlated with annual mean temperature (Table 1-2). These results support that water availability and thermoregulation are important factors in determining body size.

Head characters were uncorrelated with precipitation seasonality (Table 1-2). The head characters (head length and head width) were positively correlated with annual precipitation (HL: r = 0.485, p < 0.001; HW: r = 0.680, $p < 0.001$, Table 1-2), with annual mean temperature (HL: r = 0.282, $p < 0.001$; HW: r = 0.586, $p < 0.001$, Table 1-2), and negatively correlated with temperature seasonality (HL: -0.451, $p < 0.001$; HW: -0.737, $p < 0.001$, Table 1-2). PC2 represented heavy loading on head characters (Table 1-3S). Hereafter, I will refer to PC2 as representing head shape (Fig. 1-4).

Hand length also followed the same trend as head shape showing a positive correlation with annual precipitation (r = 0.278, $p < 0.001$, Table 1-2), annual mean temperature (r = 0.354, $p < 0.001$, Table 1-2), and a negative correlation with temperature seasonality (r = -0.284, $p < 0.001$, Table 1-2). In addition, hand length was negatively correlated with annual precipitation (r = -0.227, $p < 0.01$, Table 1-2). Forearm length showed negative correlation with temperature seasonality (r = -0.312, $p < 0.001$, Table 1-2) and a positive correlation with annual precipitation (r = 0.405, $p < 0.001$).

Relative importance of environmental predictors

Tree regression analysis

Tree regression analysis showed that the most important predictor of body size was precipitation seasonality followed by annual precipitation, annual mean temperature, and annual seasonality (Fig. 1-3). These results support the hypothesis that water availability and heat balance are important in determining body size, but water availability is more important. The total variance explained by the four predictors of body size was 13.72%. The tree regression model performed poorly for the hind limb characters except tibia length (Table 1-5S). For tibia length, the most important predictors were precipitation seasonality and annual precipitation with very similar scores followed by temperature seasonality and annual temperature. Together, these variables explained 20.17% of the variance in this trait (Table 1-5S). Annual mean precipitation was the most important predictor for head shape (head length, and head width), followed by temperature seasonality, and then annual mean temperature. All three predictors resulted in a large decrease in accuracy, indicating that they are important in explaining variation in the head characters (Table 1-5S). Precipitation seasonality was not an important predictor of head characters (Table 1-5S). The tree regression model explained 33.13% of the variance in head length and 59.61% in head width.

For the hand length, the most important predictors were annual precipitation and temperature seasonality. These two variables resulted in similar decrease in accuracy. Annual mean temperature and precipitation seasonality were less important for explaining hand length.

Together, these four variables explained 25.62% of variation in hand length (Table 1-5S). Finally, for forearm length, the most important predictors were annual mean temperature, followed by annual mean precipitation, and precipitation seasonality explaining 32.92% of variation (Table 1-5S).

Morphology and vocalization relationship

All spectral traits of note A (DF, FF, MiF, MaF) showed significant negative correlations with snout vent length of *D. minutus* (DF: r = -0.57, p = 0.013; FF: r = -0.57, p = 0.013; MF: r = -0.57, p = 0.014, MiF: r = -0.57, p = 0.014; MaF: r = -0.57, p = 0.014, Fig. 1-5, Table 1-6S). In addition, all spectral traits for note B and C showed significant correlations with head width (Note B: DF: r = -0.57, p = 0.011; FF: r = -0.57, p = 0.011, MiF: r = -0.57, p = 0.01; MaF: r = -0.57, p = 0.01; Note C: DF: r = -0.56, p = 0.019; FF: r = -0.56, p = 0.019; MiF: r = -0.500, p = 0.041; MaF: r = -0.59, p = 0.012, Table 1-7S). No correlation was found between spectral traits and head length (Table 1-8S). No relationship between head shape and spectral traits was found after removing the effect of body size on head width using residuals from a linear regression.

Discussion

Findings provide compelling evidence showing how environmental variation influences evolutionary processes in the lesser tree frog in a manner that can lead to divergence in phenotypic traits (Smith et al. 1997; Schneider et al. 1999; Slabbekoorn and Smith 2002; Thomassen et al. 2010). Environmental differences between the Amazon and Cerrado biomes result in significant morphological differences in anuran body size and shape (Fig. 1-2). This study supports both the water availability (Nevo 1973; Ashton 2002; Olalla-Tarraga et al. 2009) and heat balance (Olalla-Tarraga and Rodriguez 2007) hypotheses, according to which differences in precipitation and temperature explain body size variation. These environmental variables impact traits important for sexual selection (body size and vocalization) and might cause reproductive divergence between populations. In addition, vocalization traits associated with advertisement calls were correlated with variation in body size (Fig. 1-5, Table 1-6S and 7S). Thus, if environmental differences between habitats can be used as proxy for the presence of divergent selection (Kozak et al. 2008; Thomassen et al. 2010; Smith et al. 2013), then differentiation in morphology that results in vocalization divergence might result in greater reproductive incompatibility between ecologically divergent populations.

Environmental drivers of morphological variation

Overall body size increases in a drier, more seasonal (i.e. higher temporal variation of precipitation) and colder environment. Precipitation seasonality (Bio 15) was the most important variable in explaining body size variation (Fig. 1-3B), followed by annual mean precipitation (Bio 12), annual mean temperature (Bio 1) and temperature seasonality (Bio 4) (Fig 4B). Therefore, these results support the hypotheses that water availability and heat balance play an important role in determining body size. These results are consistent with studies of other amphibian species that showed larger size in drier areas such as the cricket frog *Acris creptitans* (Nevo 1973) and rough-skinned newt *Taricha granulosa* (Nussbaum 1971), and an interspecific comparison of the Cerrado anurans (Olalla-Tarraga et al. 2009). A mechanistic explanation for this finding is that after metamorphosis, the increase of surface volume ratio can minimize desiccation effects (Farrell and Macmahon 1969).

Precipitation variables were more important than temperature variables in determining body size providing confirmation of the hypothesis that water availability controls anuran body size variation (Olalla-Tarraga et al. 2009). Compared to Holoartic anurans where heat balance is the major determinant of body size (Olalla-Tarraga et al. 2006; Olalla-Tarraga and Rodriguez 2007), in the tropics, water availability seems to be more critical in seasonal areas than energy availability for heat balance (Olalla-Tarraga et al. 2009).

Although temperature was not the most important determinant of morphological variation in *D. minutus*, I found evidence of an effect of temperature on adult body size. This could be due to the relationship between temperature, surface volume ratio, and metabolism, a principle known as Bergmann’s rule. Bergmann (1847) proposed that larger size is associated with higher latitudes and lower temperatures to minimize heat loss. Although Bergmann's rule was originally proposed for endotherms, a number of studies in ectotherms, such as insects (Blanckenhorn 2004), amphibians, and reptiles (Ashton 2002; Ashton and Feldman 2003; Olalla-Tarraga et al. 2006) also investigated the applicability of this rule to these groups. In accordance to the Bergamann’s rule (Bergmann, 1847) proposed for endotherms, the heat balance hypothesis (Olalla-Tarraga et al. 2009) was proposed for ecthoterms, which has the same predictions that species will display larger body size in cooler temperatures. The extent to which the heat balance hypothesis applies to ectotherms remains unclear (Olalla-Tarraga et al. 2006; Adams and Church 2008). In this study, temperature variables indicate to have effect on *D. minutus* body size, in which larger bodies in lower temperatures might minimize heat loss (Olalla-Tarraga et al. 2009).

I also detected a signal of climate on head shape. In *D. minutus*, variation in head shape may reflect larval developmental length, as was found in *Hyla cinerea* (Blouin 1991; Blouin 1992) and Spadefoot toads (Gomez-Mestre and Buchholz 2006). Larval developmental length depends on water temperature (Blouin 1992; Gomez-Mestre and Buchholz 2006) and hydroperiod (Blouin 1991; Székely et al. 2010), which affect adult body size and shape (Blouin 1992; Gomez-Mestre and Buchholz 2006; Blouin 1991; Székely et al. 2010). An alternative explanation is that differences in head shape are related to feeding behavior in adults (Emerson 1985). Experiments raising tadpoles under different temperature and hydroperiod regimes will likely be necessary to fully understand the importance of these variables in explaining head shape variation.

The relationship between body size and vocalization

As in studies of other anurans, I found a negative correlation between body size and spectral traits (Gerhardt 1994; McClelland et al. 1996). In many frog species, larger males emit lower call frequencies, which influences mate choice (Ryan 1992; Gerhardt and Huber 2002). As a consequence, reproductive isolation may evolve as a by-product of divergent selection on traits important for assortative mating (Schluter 2000; Rundle and Nosil 2005; Servedio 2011; Nosil 2012). Therefore, if environmental differences in water availability and temperature between the Amazon and the Cerrado affect body size in *D. minutus* and this variation is causing changes in vocal frequencies, then divergent selection might be causing reproductive divergence between populations of *D. minutus*. Future work using mate choice experiments hopes to elucidate the importance of vocalization differentiation in population divergence in *D. minutus.*

Conclusion

This study provides evidence that environmental differences in precipitation and temperature between the Cerrado and the Amazon biome influence morphological traits such as body size and head shape. I also found that differences in body size affects spectral traits of *D. minutus* male calls that are important in mate choice (Haddad, 1987). Thus, greater reproductive incompatibility is expected between ecologically divergent populations and might indicate the initial stages of ecological speciation (Nosil 2009).

This type of study facilitates identification of possible adaptive traits that are important to non-random mating and might facilitate speciation events. These 'magic traits' (Gavrilets 2004), are receiving increased attention because they can facilitate reproductive isolation even with moderate levels of gene flow (Servedio 2011; Nosil 2012). Understanding how environmental variables affect these traits can provide important information about the processes that generate population divergence across a heterogeneous landscape.

Figures

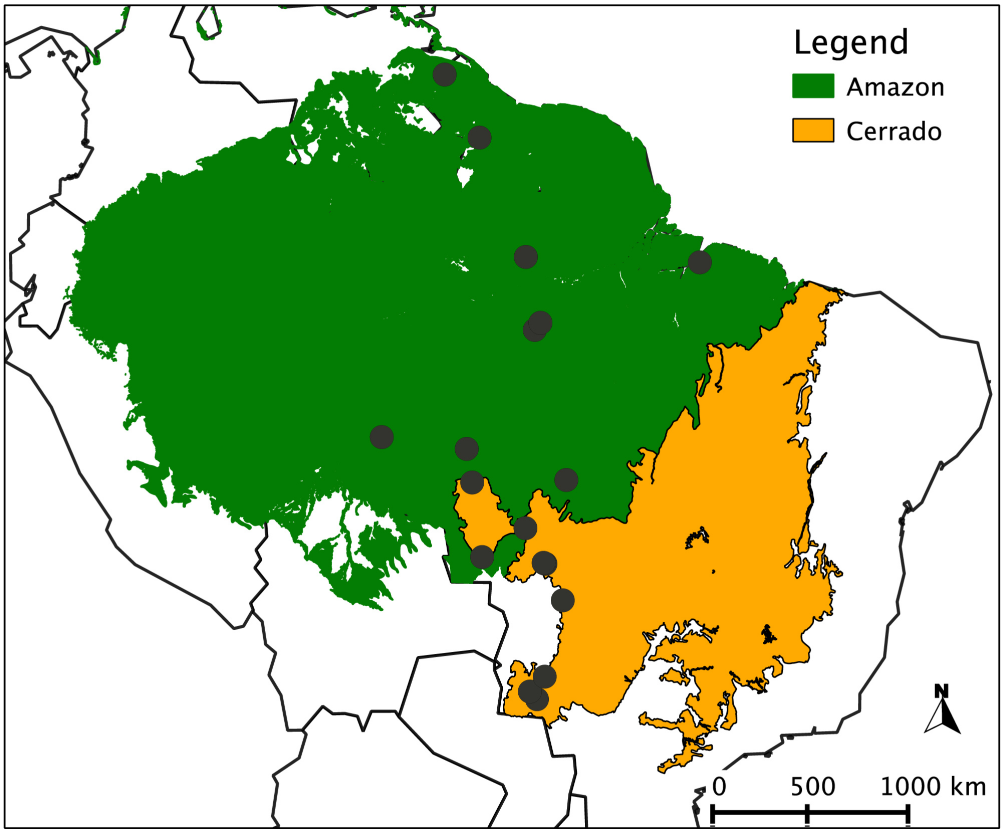

Fig. 1-1. Sampling localities (Table 1-1S) from museum specimens for *Dendropsophus minutus* in the Cerrado and the Amazon biomes.

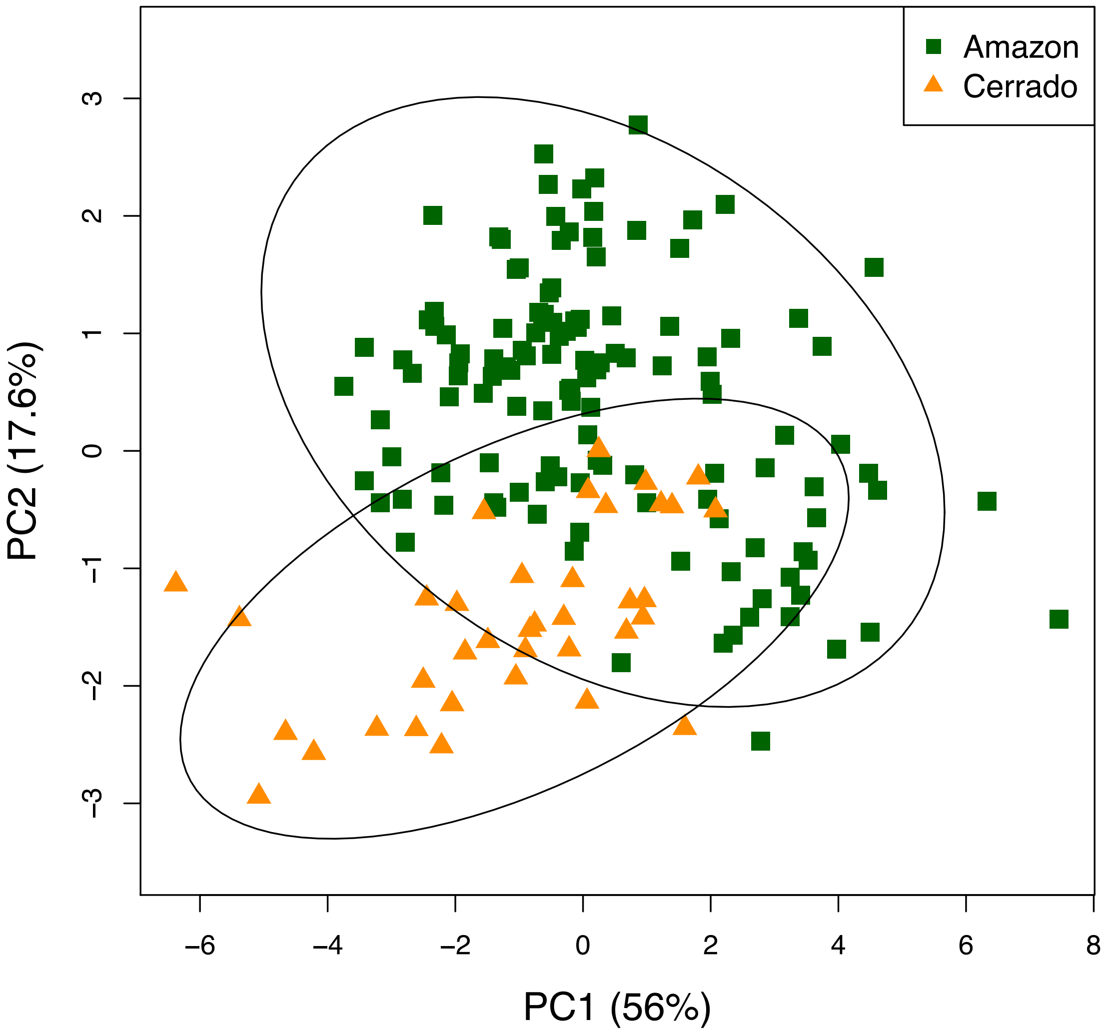

Fig. 1-2. Scatterplots of principal component axis 1 vs. 2 with group centroids (95% confidence interval) for *D. minutus* based on nine morphological traits of individuals from the Cerrado and the Amazon (n=148) (MANOVA Wilks statistic = 0.373, P<0.0001).

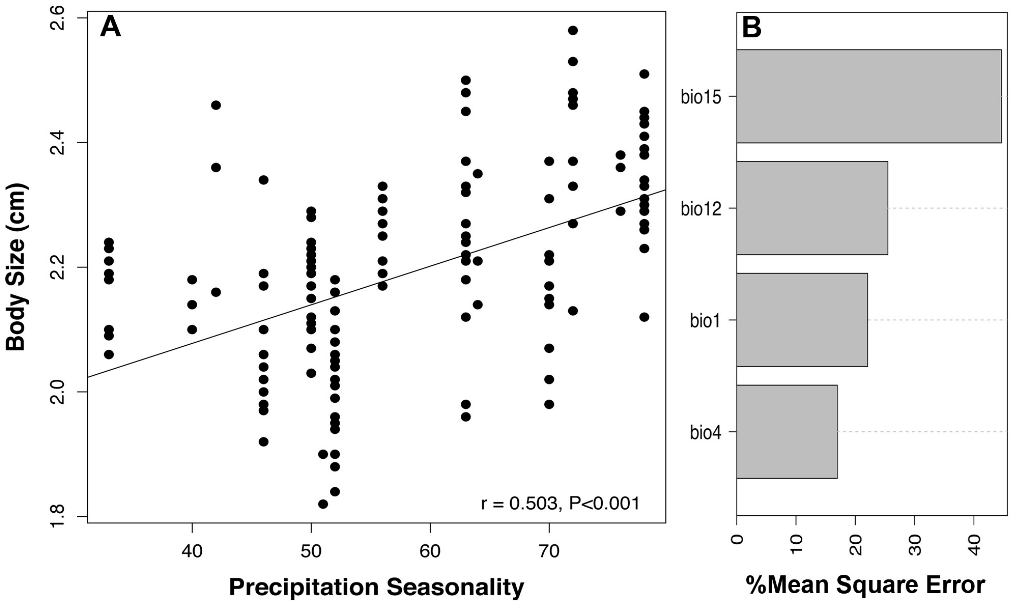

Fig. 1-3. A) Plot of *D. minutus* body size and precipitation seasonality (coefficient of variation). B) Environmental variables predictors (Bio1: annual mean temperature, Bio 4: temperature seasonality, Bio 12: annual precipitation, Bio 15: precipitation seasonality) for body size variation in the conditional tree regression model. Higher increase in mean square errors indicates a more important variable explaining variation in body size.

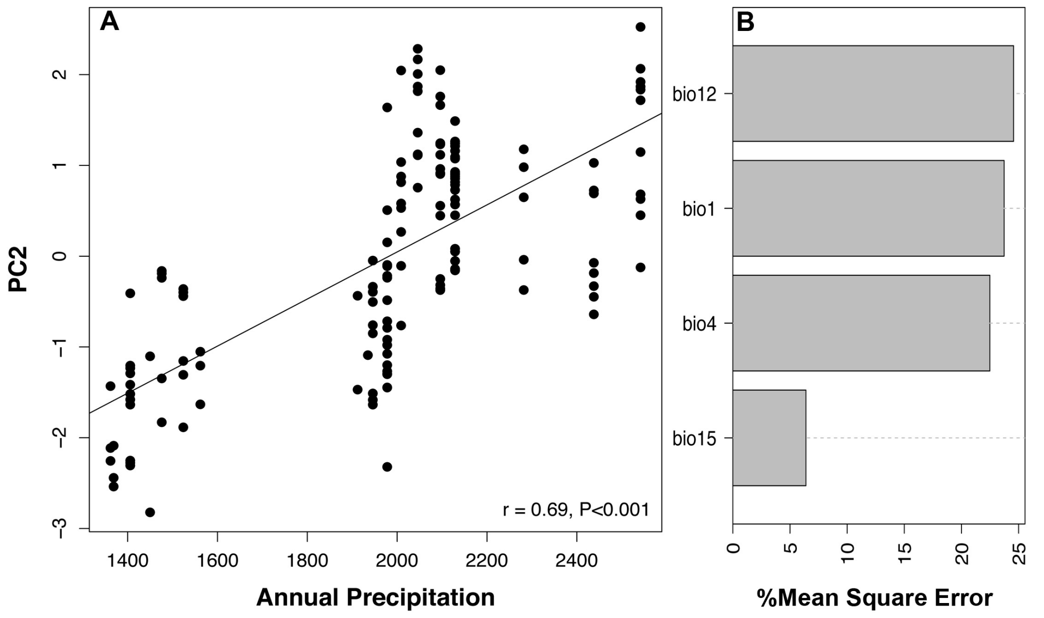

Fig. 1-4. A) Pearson correlation between *D. minutus* PC2 scores (representing head shape) and precipitation seasonality. B) Environmental predictors (Bio1: annual mean temperature, Bio 4: temperature seasonality, Bio 12: annual precipitation, Bio 15: precipitation seasonality) of body shape variation in the conditional tree regression model. Higher increase in mean square error indicates a variable that is more important for explaining variation in body shape.

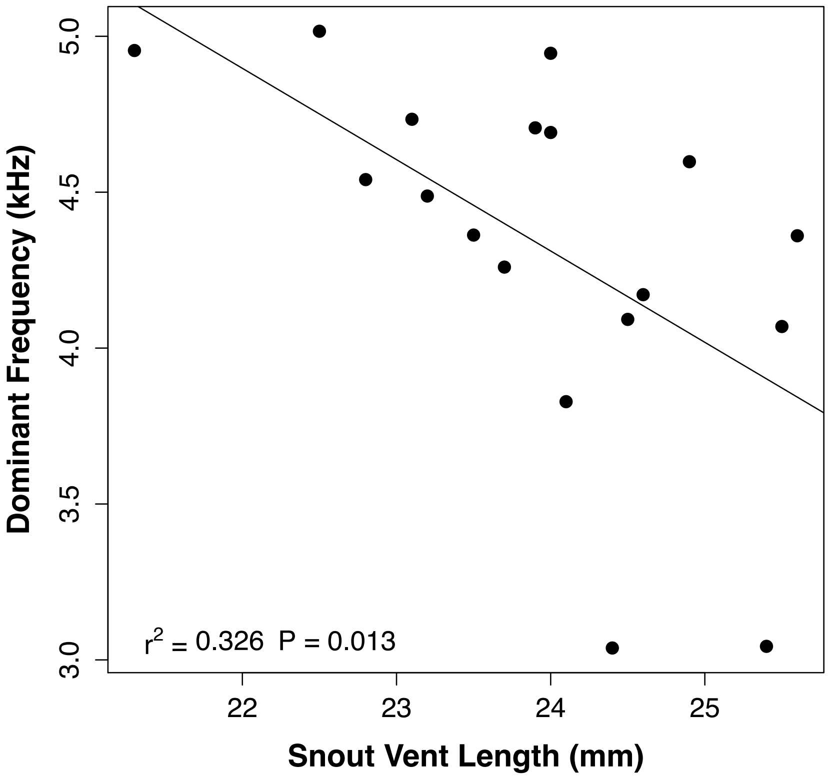

Fig. 1-5. Dominant frequency of the advertisement call plotted against snout vent length of *D. minutus* (n = 18).

Tables

Table 1-1. Mean, standard deviation (SD), and p values from permutation test showing difference in morphological traits in *Dendropsophus minutus* between the Amazon and the Cerrado. Numbers in bold show significant differences ($p < 0.05$).

	Cerrado	(n = 37)	Amazon	(n = 127)	t-test	
	Mean	SD	Mean	SD		*p*
Snout Vent Length	2.19	0.15	2.19	0.16	-0.09	0.932
Head Length	0.62	0.05	0.68	0.04	7.894	**0.000**
Head Width	0.67	0.04	0.77	0.04	13.321	**0.000**
Thigh	1.15	0.08	1.20	0.08	2.968	**0.003**
Tibia	1.21	0.07	1.25	0.09	2.579	**0.010**
Forearm	0.80	0.07	0.80	0.07	0.575	0.582
Foot Length	1.68	0.11	1.72	0.13	1.936	0.053
Hand Length	0.63	0.05	0.68	0.06	4.715	**0.000**
Forearm	0.38	0.05	0.40	0.05	2.089	**0.040**

Table 1-2. Correlations between morphological traits and environmental predictors (Bio 1: Mean annual temperature, Bio 4: Temperature seasonality, Bio 12: Annual precipitation, Bio 15: Precipitation seasonality). Numbers in bold show significant differences ($p < 0.05$).

	Bio 1		Bio 4		Bio 12		Bio 15	
	p	r	*p*	r	*p*	r	*p*	r
Snout Vent Length	**0.000**	-0.427	0.725	0.029	0.349	-0.077	**0.000**	0.503
Head Length	**0.001**	0.282	**0.000**	-0.451	**0.000**	0.485	0.209	-0.104
Head Width	**0.000**	0.586	**0.000**	-0.737	**0.000**	0.68	0.090	-0.14
Tight	**0.006**	-0.225	0.088	-0.14	0.682	0.034	**0.000**	0.391
Tibia	**0.000**	-0.293	0.057	-0.156	0.820	0.019	**0.000**	0.535
Tarsus	**0.000**	-0.416	0.848	0.016	0.113	-0.131	**0.000**	0.528
Foot Length	**0.000**	-0.341	0.225	-0.1	0.771	-0.024	**0.000**	0.553
Hand Length	0.351	-0.077	**0.000**	-0.312	0.050	0.161	**0.000**	0.405
Forearm	**0.000**	0.354	**0.000**	-0.284	**0.001**	0.278	**0.005**	-0.227
PC1	**0.003**	0.241	**0.009**	0.212	0.260	-0.093	**0.000**	-0.489
PC2	**0.000**	0.719	**0.000**	-0.665	**0.000**	0.690	**0.000**	-0.423
PC3	**0.008**	-0.217	**0.019**	0.192	**0.010**	-0.210	0.099	0.136

Appendix

Table 1-1S. Localities from museum specimens for *Dendropsophus minutus* in Cerrado and Amazon biomes. NA: Museum specimens with only municipality locality without geographic coordinates.

Localities	Latitude	Longitude	Biome
Jardim, MS	-21.473	-56.148	Cerrado
Bonito, MS	-21.136	-56.482	Cerrado
Aquidauana, MS	-20.481	-55.783	Cerrado
Itiquina, MS	-17.083	-54.933	Cerrado
Chapada dos Guimaraes, MT	-15.433	-55.75	Cerrado
Escola Evangelista, MT	-15.416	-55.805	Cerrado
Morro do Chapeu APM Manso, MT	-15.390	-55.838	Cerrado
Araputanga, MT	-15.126	-58.726	Cerrado
Sao Jose do Rio Claro, MT	-13.812	-56.69	Cerrado
Juina, MT	-11.717	-59.196	Cerrado
Claudia Fazenda Iracema, MT	-11.600	-54.767	Amazon
Aripuana, MT	-10.165	-59.452	Amazon
Alto Paraiso, RO	-9.617	-63.45	Amazon
Pq Nacional da Amazonia, PA	-4.617	-56.25	Amazon
Pq Nacional da Amazonia Rio Tapajos, PA	-4.283	-55.983	Amazon
Ipean Belem, PA	-1.443	-48.486	Amazon
Reserva Biologica Rio Trombetas, PA	-1.189	-56.671	Amazon
Rupununi Iwokrama Forest Reserve, Guyana	4.420	-58.849	Amazon
Baramita River, Guyana	7.371	-60.491	Amazon
Serra do Navio, Amapa	NA	NA	Amazon
Iwokrama, Guyana	NA	NA	Amazon
Fazenda Sao Franscico, AM	NA	NA	Amazon

Table 1-2S. Sampling localities for vocalization data of *Dendropsophus minutus*

Localities	Latitude	Longitude
Barra do Garcas	-15.8499	-52.1260
Campo Verde	-15.5473	-55.1283
Chapada dos Guimaraes - Buriti	-15.4160	-55.8047
Chapada dos Guimaraes - Peba	-15.3841	-55.7098
Chapada dos Guimaraes – Agua Fria	-15.3148	-55.7316
Nobres	-14.6944	-56.3230

Table 1-3S. Principal component analyses summarizing variation in morphological traits measured from the *Dendropsophus minutus* males from 23 localities.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Snout Vent Length	-0.364	-0.113	0.252	0.302	0.695	-0.371	-0.147	0.239	0.005
Head Length	-0.222	0.506	-0.296	0.743	-0.191	0.086	0.096	0.022	0.040
Head Width	-0.188	0.621	-0.256	-0.467	0.066	-0.335	-0.409	-0.092	-0.054
Tight	-0.396	-0.028	0.028	-0.214	-0.361	-0.427	0.584	0.371	-0.063
Tibia	-0.415	-0.067	0.180	-0.046	-0.056	-0.025	0.100	-0.624	0.621
Tarsus	-0.379	-0.225	0.128	0.014	-0.439	0.213	-0.616	0.388	0.144
Foot Length	-0.420	-0.091	0.159	0.020	-0.064	0.167	-0.018	-0.426	-0.759
Hand Length	-0.362	0.008	-0.386	-0.282	0.381	0.622	0.233	0.216	0.089
Forearm	0.053	0.530	0.748	-0.10	0.024	0.315	0.131	0.168	0.031
Standard deviation	2.258	1.241	0.96	0.685	0.57	0.488	0.449	0.391	0.227
Proportion of Variance	0.567	0.171	0.103	0.052	0.036	0.026	0.022	0.017	0.006
Cumulative Proportion	0.567	0.738	0.84	0.892	0.929	0.955	0.977	0.994	1

Table 1-4S. Coefficients of the first canonical discriminant function that predicts differentiation in males of *Dendropsophus minutus* between Amazon and Cerrado.

Traits	Discriminant Function 1
Snout Vent Length	-4.331
Head Length	9.141
Head Width	19.512
Tight	-1.413
Tibia	9.574
Tarsus	-1.266
Foot Length	-3.598
Hand Length	5.592
Forearm	0.735

Table 1-5S. Tree regression analysis with the mean square error scores for morphological traits of *Dendropsophus minutus.* Higher mean scores indicates a more important predictor variable. Negative scores suggest that random permutations of out of bag samples were performing better than the actual value. Negative % variance explained for thight and tarsus also showed poor performance of tree regression model. Bio 1: Mean annual temperature, Bio 4: Temperature seasonality, Bio 12: Annual precipitation, Bio 15: Precipitation seasonality.

	Snout Vent Length	Head Length	Head Width	Tight	Tibia	Tarsus	Foot Length	Hand Length	Forearm
% Variance	13.72	33.13	59.61	-44.14	20.17	-51.12	16.38	25.62	32.93
bio1	22.75	37.47	47.43	29.07	25.86	11.15	24.65	29.47	36.30
bio4	17.17	44.50	49.48	8.29	28.87	-20.86	23.01	40.80	40.21
bio12	27.71	51.78	54.41	7.70	36.91	-15.51	31.30	40.50	34.62
bio15	45.35	-15.08	5.12	-21.21	36.48	-5.56	37.98	25.95	21.92

Table 1-6S. Linear regression between snout vent length and spectral traits of *Dendropsophus minutus*. Numbers in bold show significant differences ($p < 0.05$).

SVL	Estimate	Std. Error	t-value	*p*	r^2
Type A (n = 18)					
Dominant Frequency	-0.111	0.040	-2.782	**0.013**	0.326
Fundamental Frequency	-0.223	0.080	-2.782	**0.013**	0.326
Minimum Frequency	-0.112	0.040	-2.774	**0.014**	0.325
Maximum Frequency	-0.110	0.040	-2.753	**0.014**	0.321
Type B (n = 19)					
Dominant Frequency	0.000	0.000	-2.003	0.061	0.191
Fundamental Frequency	0.000	0.000	-2.003	0.061	0.191
Minimum Frequency	0.000	0.000	-1.994	0.062	0.19
Maximum Frequency	0.000	0.000	-2.051	0.056	0.198
Type C (n = 17)					
Dominant Frequency	0.000	0.000	-1.243	0.233	0.093
Fundamental Frequency	0.000	0.000	-1.243	0.233	0.093
Minimum Frequency	0.000	0.000	-0.756	0.461	0.037
Maximum Frequency	0.000	0.000	-1.552	0.142	0.138

Table 1-7S. Linear regression between head length and spectral traits of *Dendropsophus minutus.*

Head Length	Estimate	Std. Error	t-value	*p*	r^2
Type A (n = 18)					
Dominant Frequency	-4.027	3.537	-1.139	0.272	0.075
Fundamental Frequency	-2.013	1.768	-1.139	0.272	0.075
Minimum Frequency	-3.894	3.523	-1.105	0.285	0.071
Maximum Frequency	-4.000	3.556	-1.125	0.277	0.073
Type B (n = 19)					
Dominant Frequency	-3635.982	5122.19	-0.710	0.487	0.029
Fundamental Frequency	-1817.991	2561.095	-0.710	0.487	0.029
Minimum Frequency	-3535.035	5123.600	-0.690	0.500	0.027
Maximum Frequency	-3703.685	5176.071	-0.716	0.484	0.029
Type C					
Dominant Frequency	-4109.935	2405.757	-1.708	0.108	0.163
Fundamental Frequency	-2054.968	1202.879	-1.708	0.108	0.163
Minimum Frequency	-1998.893	2165.51	-0.923	0.371	0.054
Maximum Frequency	-4771.376	2306.818	-2.068	0.056	0.222

Table 1-8S. Linear regression between head width and spectral traits of *Dendropsophus minutus*. Numbers in bold show significant differences ($p < 0.05$).

Head Width	Estimate	Std. Error	t-value	*p*	r^2
Type A (n = 18)					
Dominant Frequency	-0.028	0.018	-1.504	0.152	0.124
Fundamental Frequency	-0.056	0.037	-1.504	0.152	0.124
Minimum Frequency	-0.028	0.019	-1.498	0.154	0.123
Maximum Frequency	-0.028	0.018	-1.517	0.149	0.126
Type B (n = 19)					
Dominant Frequency	0.000	0.000	-2.851	**0.011**	0.324
Fundamental Frequency	0.000	0.000	-2.851	**0.011**	0.324
Minimum Frequency	0.000	0.000	-2.911	**0.010**	0.333
Maximum Frequency	0.000	0.000	-2.892	**0.010**	0.330
Type C (n = 17)					
Dominant Frequency	0.000	0.000	-2.628	**0.019**	0.315
Fundamental Frequency	0.000	0.000	-2.628	**0.019**	0.315
Minimum Frequency	0.000	0.000	-2.235	**0.041**	0.250
Maximum Frequency	0.000	0.000	-2.869	**0.012**	0.354

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CHAPTER 2

Testing alternative mechanisms of divergence and speciation along the Amazon-Cerrado gradient in the lesser tree frog (*Dendropsophus minutus*).

Abstract

Evaluating the relative roles of genetic drift and divergent selection is fundamental to understanding the mechanisms that shape the patterns of biodiversity observed in rainforests. Despite a growing number of studies showing that divergent selection is important for speciation, studies in the Amazon rainforest have emphasized the role of vicariance in driving diversification. Often, genetic drift is assumed to play a major role without testing the relative contributions of natural selection and neutral processes. Here I test the relative roles of genetic drift and natural selection in generating intraspecific divergence in phenotypic and genotypic traits between populations of a frog (*Dendropsophus minutus*) along the Amazon-Cerrado gradient. To test the hypothesis that divergent selection caused by the environmental gradient is more important than geographical distance by itself in the divergence between populations, I collected *D. minutus* vocalization (n = 228 recordings), morphology (n = 761) and DNA (n = 262) data along three transects: one control transect in the Amazon region (350km, n = 6 sites), another control transect in the savanna (450km, n = 12 sites), along the gradient between the Amazon and Cerrado (~ 350km, n = 22 sites). I show that environmental variables are more important than geographical distance in explaining divergence in reproductive and morphological traits. Traits important in reproduction, including spectral characters of advertisement calls, were found correlated with genetic distance along but not perpendicular to the gradient suggesting the importance of the Amazon-Cerrado gradient in driving adaptive divergence. In addition, environmental variation along the transition between can be used as proxy for phenotypic and genetic variation indicating the dominant role of natural selection over neutral processes in the speciation process in this region. Collectively, the results of this study suggest a role for the Amazon-Cerrado gradient in driving speciation.

Introduction

Understanding the causes of species formation has been a major goal for biologists ((Coyne and Orr 2004; Schluter 2009; Nosil 2012). Natural selection and genetic drift are both important evolutionary processes in intraspecific divergence (Butlin et al. 2012). However, which process is more important in generating new species has been a subject of debates for decades (Endler 1982; Mayr and Ohara 1986; Smith et al. 1997; Moritz et al. 2000; Smith et al. 2005b). A number of studies provide evidence for the role of natural selection in speciation events (Coyne and Orr 2004; Schluter 2009; Nosil 2012), and recent research provides additional analysis of the relative contributions of drift and selection (Smith et al. 2005a; Mila et al. 2009; Freedman et al. 2010; Thomassen et al. 2010; Nosil 2012). Nevertheless, such studies are fundamental to understanding the mechanisms that result in the patterns of biodiversity.

Many studies of the Amazon region show that historical and vicariance events have shaped the biogeographical patterns of diversity (Haffer 1969; Bush 1994; Haffer 1997; Moritz et al. 2000; Hoorn et al. 2010; Ribas et al. 2012). The Pleistocene refugia (Haffer 1969), riverine barrier (Wallace 1954), and marine incursions (Webb 1995; Lovejoy et al. 2006) hypotheses are often cited as examples of allopatric speciation resulting from vicariance events. The intensely debated refugia hypothesis (Haffer 1969) has been largely refuted by phylogenetic studies and fossil records showing that most of the Amazonian diversity is older than the Pleistocene period (Colinvaux et al. 1996; Jaramillo et al. 2006; Hoorn et al. 2010; Ribas et al. 2012). Thus, we now need studies that test alternative hypotheses to identify the mechanisms of diversification in the Amazon (Bush 1994; Hoorn et al. 2010; Ribas et al. 2012).

Geological physical barriers such as rivers might not be the main cause of the Amazonian diversification, because environmental differences on either side of rivers, may also play an important role in driving divergence between populations (Tuomisto and Ruokolainen 1997; Tuomisto 2007). In addition, speciation may occur without barriers (de Aguiar et al. 2009; Feder et al. 2012) across a range of spatial contexts, from allopatry to sympatry (Nosil 2008). Independent of the geographical context, recent studies have also shown that ecology plays an important role in driving divergence between populations (Nosil et al. 2008; Schluter 2009; Funk et al. 2011; Smith et al. 2011; Feder et al. 2012). To better understand the evolutionary mechanisms driving diversification in the Amazon rainforest, we need to focus not only on the historical perspective, but on how heterogeneous Amazon landscapes may play a role in the divergence process (Tuomisto and Ruokolainen 1997; Tobias et al. 2010).

Environmental gradients in tropical rainforests have shown that natural selection is an important driver in the speciation process (Smith et al. 1997; Schneider et al. 1999; Ogden and Thorpe 2002; Smith et al. 2005a; Smith et al. 2011). These studies support the gradient hypothesis, which assumes that divergent selective pressures in different habitats can be strong enough to create locally adapted populations and lead to parapatric speciation events despite ongoing gene flow between those populations (Endler 1982; Rice and Hostert 1993).

The gradient between the Amazon and the Cerrado provides an exceptional study system to test mechanisms of diversification in the tropics. The Amazon rainforest and Cerrado are the largest biomes in South America, and both encompass heterogeneous landscapes (Da Silva and Bates 2002; Silva et al. 2006). The Amazon basin forms a broad contact zone with the Cerrado, presenting an extensive ecological gradient between both biomes. The Amazon rainforest is the largest (>6 million km^2) and most diverse rainforest in the world (Da Silva et al. 2005), and presents a highly heterogeneous landscapes (Tuomisto et al. 1995; Tuomisto et al. 2003). The Cerrado is the largest and richest tropical savanna in the world (1.86 million km^2) and is mainly comprised of continuous savanna vegetation (~72%), but also contains a mosaic of dry forest and savanna (~24%) and continuous dry forest (4%) (Da Silva and Bates 2002). The Cerrado also presents a heterogeneous landscape, with a diversity of soil types, geomorphology and vegetation types (Silva et al. 2006), and seasonal climates (Klink and Machado 2005). From northwest of the Amazon to southeast in the Cerrado, the climate varies from continuously rainy to wet/dry and to long dry season (Davidson et al. 2012). These differences, along with other environmental conditions, may result in contrasting selection pressures when compared to adjacent savanna and rainforest habitat and, as a consequence, may be important in the speciation process.

Frogs species are an ideal model to study divergence processes along this Amazon-Cerrado gradient, particularly because of their susceptibility to precipitation and humidity variation. For example, frog populations living in drier environments may display a larger body size, likely due to the reduction in desiccation risk (Nevo and Capranica 1985). Body size and call frequency are negatively correlated (Ryan 1980), and consequently, frogs in drier environments typically call at lower frequencies (Nevo 1973). In addition, environmental factors such as vegetation structure and noise (Slabbekoorn and Smith 2002) may act on communication signals, resulting in geographical variation in male calls (Wilczynski et al. 1999). Consequently, investigating phenotypic traits important to fitness, such as morphology and vocalization, can help to elucidate the role of the Amazon-Cerrado gradient in species formation.

In order to understand the mechanisms driving divergence in frog populations across the Amazon-Cerrado gradient, I tested the relative roles of genetic drift and natural selection in driving divergence in vocalization, morphology and genetics in the lesser tree frog (*Dendropsophus minutus*). The lesser tree frog is widely distributed in South America, occurring in the lowlands east of the Andes, Brazil, Paraguay, and Uruguay (Frost 2013). This species is found in both open and forest areas, at the edge of forests and in clearings (Lima et al. 2006). *D. minutus* shows intra-specific variation in vocal traits and morphology in different habitats (Cardoso and Haddad, 1984, Kaplan, 1994), suggesting that ecological differences may shape these characteristics.

The broad distribution pattern of *D. minutus* provides an exceptional opportunity to contrast the differentiation caused by geographical distance (isolation-by-distance: IBD, Wright 1943) and via environmental differences along the Amazon-Cerrado ecotone (gradient hypothesis, Endler 1977). This environmental difference would act as divergent selection factor on phenotypes. In this context, I aim to investigate the relative roles of geographical distance and ecological factors in driving phenotypic and genetic differentiation in *D. minutus* by testing if: 1) divergence in phenotypic and genetic traits across the Amazon-Cerrado ecotone (Transition) is higher than within habitat comparison (Smith et al. 1997; Orr and Smith 1998); 2) environmental variables are more important than geographical distance in explaining vocalization and morphological differentiation, and 3) traits important in reproduction are correlated with genetic distance along but not perpendicular to the gradient. If natural selection is more important than drift, then ecological differences along the gradient should be more important than distance in driving divergence among populations.

Methods

Field sampling and habitat classification

I conducted fieldwork in the Amazon, Cerrado, and along the transition between these biomes in Mato Grosso State, Brazil at 37 locations (Fig. 2-1, Table 2-1S). In the sampling area, two rivers (Arinos and Juruena) traverse the transition, running from south to north. I collected vocal, morphological and DNA sequence data of *D. minutus* within the Amazon biome (~500km, n = 6 sites) and within the Cerrado biome (~500km, n = 12 sites), and along the gradient on each side of the Arinos and Juruena rivers (~350km, n = 22).

Habitat classification

I used altitude and 19 bioclimatic variables from the WorldClim database (Hijmans et al. 2005) based on 50 years of climatology (1950-2000) and satellite remote-sensing data from passive optical sensors (MODIS; https://lpdaac.usgs.gov/lpdaac/products/modis_overview). From the MODIS archive, I used the Leaf Area Index (LAI), Normalized Vegetation Index (NDVI), and Enhanced Vegetation Index (EVI) as measures of vegetation cover. For each sampling locality, I extracted the 23 environmental and geographical variables using Quantum GIS software (Quantum 2010), Table 2-2S). I used a classification tree classification model (Liaw and Wiener 2002) in R (R-Development-Core-Team 2008) to test whether environmental variables could be used to classify the habitat types (Transition, Cerrado, Amazon). I built a classification tree based on 23 bioclimatic predictors extracted from each sampling site and used habitat type as a response variable. Under this model, I accurately classified 94.6% (35/37) sites as Transition, Cerrado, or Amazon habitat types. The most important variables in determining the differences between habitats were annual precipitation (BIO 12) and precipitation seasonality (BIO 15; Table 2-1S).

Morphology and vocalization data

To assess morphological variation within and between sites, I measured nine traits with plastic dial calipers to the nearest 0.01mm. Measured traits included: snout-vent length (SVL), head length (HDL), head width (HW), thigh length (THL), tibia length (TL), metatarso length (ML), foot length (FL), forearm length (FA), and hand length (HL). All morphological measurements followed Heyer et al. (1994). Only adult males (n = 761) were measured to avoid confounding effects of sexual dimorphism.

D. minutus vocalization presents a complex vocal repertoire that includes three types of notes, A, B, and C, which are combined depending on the social context (Haddad 1987). The emission of notes A and B are associated with an advertisement call and C with a territorial call (Haddad 1987). I recorded *D. minutus* vocalizations at about 1 m from the calling frog with a Marantz digital recorder (PDM660) coupled to a Sennheiser ME66 shotgun microphone at a sampling frequency of 44.1 kHz and 16 bits resolution. After each recording, I measured the air temperature with a digital thermometer to the nearest 0.5° C. The following vocal traits were measured using spectrogram and waveform in the Sound Ruler software (Gridi-Papp 2007): dominant frequency (DM), fundamental frequency (FF), minimum frequency (MIF), maximum frequency (MAF), number of pulses (NP), pulse duration (PD), and pulse interval (PI). The definitions of vocalization traits followed (Ryan and Rand 2003). Since temperature has an effect on spectral (DM, FF, MIF, MAF) and temporal traits (NP, ND, PI; (Gerhardt 1994), I removed the effect of temperature on each trait using the residual values from the linear regressions for those traits that showed significant correlation with temperature. In total, I recorded 231 individuals, which included note types A (n = 228), B (n = 231), and C (n = 179). For each recorded individual, I analyzed three to five note types and used the average of each measured trait.

Genetic data

Tissue samples were collected from liver and leg muscle from all sampling sites in the study area and preserved in 99% ethanol. Genomic DNA was extracted using Qiagen DNA extraction kit (QIAGEN Inc., Valencia, CA) following the standard protocol for animal tissue. I amplified the genomic DNA of seven microsatellite loci and labeled forward primers with 5'-fluorescent tags (6-FAM) for visualization. PCR amplifications were performed under the following reaction condition: 5-50 ng DNA, 0.2 mM reverse primer, 0.01mM forward primer, 0.01 mM dye labeled M13 primer, 0.4 mg/mL BSA, and 5.0 ml of Qiagen Multiplex Mastermix (Qiagen, Valencia, USA). Initial denaturation of 95° C for 15 min, 25 cycles of denaturation (94° C for 30 s), annealing (53° C for 90 s), and extension (72° C for 60 s), and a final extension at 60° C for 30 min. Microsatellite fragments were sized with ABI37370xl DNA analyzer and results were scored using GeneMapper version 3.7 (Applied Biosystems).

Genetic analysis

I used MICROCHECKER to investigate potential existence of null alleles, large allele dropout, and scoring errors due to stuttering (Van Oosterhout et al. 2004). Deviations from Hardy-Weinberg equilibrium were tested using Adgenenet R package (Jombart 2008) and presence of linkage disequilibrium was tested using the Genetics R packages (Warnes and Leisch 2006). I used pairwise F_{st} (Weir and Cockerham 1984) values to estimate genetic differentiation between populations in the Adegenet R package (Jombart 2008).

Relative Importance of Ecological and Geographical Distance

I performed partial mantel test (Manly 1997) to estimate the effects of geographical and environmental distance on morphology, vocalization and genetic divergence of *D. minutus* within the Amazon transect (n = 5), the Cerrado transect (n = 10), and along the gradient (n = 22).

The partial mantel test compares the partial correlation coefficient of dissimilarity matrices with the coefficients of a null distribution generated by randomization of rows and columns of a focal matrix while maintaining the other two matrices constant. Specifically, I estimated vocalization divergence based on Euclidean distances of the spectral and temporal traits measured for notes A, B, and C. I estimated the morphological divergence between sites using Euclidean distance of the average of each phenotypic trait from each site and I also used principal component 1, 2 and 3 scores from Principal Components Analysis (PCA) based on nine morphological traits. Genetic divergence was based on F_{st} pairwise differences between sites. Environmental dissimilarity was calculated base on the Euclidean distances of PC1 scores from a Principal Component Analysis of 23 environmental variables. I calculated the geographical Euclidean distance between locations and environmental dissimilarity using Ecodist R package (Goslee and Urban 2007).

To evaluate the gradient hypothesis, I tested correlation between environmental distance and: 1) vocalization divergence; 2) morphological divergence; and 3) genetic divergence. Since geographical and environmental distances are correlated ($r = 0.434$, $P=0.002$), I controlled for geographical distance in the partial mantel test. In order to analyze the effect of geographical distance, I tested for correlation between geographical distance and: 1) vocalization divergence; 2) morphological divergence; and 3) genetic divergence (F_{st}). Because geographical distance was correlated with the responses variables, I controlled for ecological distance.

To test the ‘relative importance’ of 23 bioclimatic variables and geographic distance on phenotypic variation, I used morphological and vocalization traits as response variables in the tree regression technique in the Random Forest R package (Liaw and Wiener 2002). I ran a tree regression model with 2000 iterations to obtain the relative variable importance of 25 environmental predictors (Table 2S) and geographic distance predictors. I used the mean decrease accuracy index to measure the relative variable importance of each predictor on the response phenotypic traits. The relative importance of the predictors are the average of 2000 trees and is estimated by the increase of prediction error that is calculated with the difference between the subset (bagging procedure) and the out of bag samples (not used to predict) used to test the model prediction (Hothorn et al. 2010).

Association between phenotypic traits and genetic distance analysis

To test for evidence of adaptive divergence in the phenotypic traits, I performed a mantel test (Manly 1997) between Euclidean distances of phenotypic traits (morphology and vocalization) against genetic distance in Ecodist R package (Goslee and Urban 2007) within the Cerrado, the Amazon, and the Transition transects.

Results

Ecological and geographical distance analysis

The gradient hypothesis is supported by a strong correlation between genetic divergence and geographical distance along the Transition transect ($r = 0.8474$, $P = 0.001$), as opposed to the Cerrado ($r = 0.329$, $P = 0.296$) and Amazon ($r = 0.156$, $P = 0.375$) transects, which did not show significant correlation (Fig. 2-2). The genetic divergence across the transition, but not within biomes, suggests that the ecological variation along the transition between the Amazon and the Cerrado might play an important role in the divergence process.

In addition, correlation between phenotypic divergence and ecological distance along the Transition corroborate with the gradient hypothesis (Fig. 2-3). After correction for the auto spatial correlation effect, I found a significant correlation in 20 out of 28 traits in the Transition transect, but just one out of 28 traits in the Amazon, and none in the Cerrado transect (Fig. 2-3, Table 2-3S). Contrasting with ecological distance, correlation between phenotypic divergence and geographical distance was found significant in only two traits in the Transition, one in the Amazon, and none in the Cerrado transect (Fig. 2-3, Table 2-3S).

Tree regression analysis also showed environmental variables to be more important predictors than geographical distance in explaining variation in vocalization and morphological traits (Fig. 2-4, Table 2-3S). Out of 28 phenotypic traits, 21 traits showed that environmental variables related to precipitation, temperature and vegetation cover predictors were more important than geographical distance (longitude, latitude) in explaining phenotypic variation. In only one trait, number of pulses for note A, was distance the most important predictor, but the tree regression model only explained 15.2% of variation in this trait (Fig. 2-2S).

Across the Transition transect, the reproductive traits (all spectral variables for note A and B) showed significant correlations with genetic distance (Fig. 2-5).

Vocal analysis

Specifically for vocalization traits, the gradient hypothesis is supported by significant association between all spectral traits for notes A, B, and C (DM, FF, MIF, MAF) and ecological distance in the transition transect (Fig 3D, Table 2-3S). None of the temporal traits showed correlation with ecological distance (Table 2-3S) most likely because temporal traits are highly dependent on the social context of the chorus. Within the Amazon and the Cerrado transects, ecological distance was not correlated with any of the vocalization traits (Table 2-3S) most likely because of little ecological divergence between sites within the same habitat types and because of high levels of gene flow (F_{ST}= 0.027-0.036) in the Cerrado region. Contrasting with ecological distance, geographical distance was supported only in pulse duration (r = 0.313, P=0.005) and in dominant frequency (r=0.185, P=0.05) for note B in the transition transect (Table 2-3S).

Environmental variables were more important than geographical distance in predicting variation for all spectral and temporal traits for notes A, B, and C, except for number of pulses for note A (Fig. 2-4, Fig. 2-2S Fig. 2-3S, Fig. 2-4S, Fig. 2-5S). A Principal Component Analysis (PC1) for spectral traits for notes A, B, and C, was used to summarize the data (Fig. 2-4). The random forest model explained 46.54% of the variation in spectral traits of note A and the most important predictor was annual precipitation (Bio 12). For note B, the model explained 48.0% of spectral variation, and the most important predictor was precipitation of the driest quarter (Bio 17). For note C, the model explained 49.4% and the most important predictor was temperature seasonality (Bio 4) (Fig. 2-4). For the temporal traits in note A, the model explained: 11.5% of variation for pulse interval and the most important variable was Leaf Area Index (LAI); 15% for the pulses number and the most important variable was latitude; and 34.7% for pulse duration and the most important variable was NDVI. The tree regression model performed very poorly in predicting variation in four temporal traits (Pulse Duration for Notes B and C; Pulse interval for note B, and number of pulses for note B (<1.5%), possibly due to the fact that temporal traits are highly dependent of social context. This result is concordant with the results obtained from the partial mantel test for temporal traits and geographical and ecological distances.

The spectral traits for notes A and B (DM, FF, MIF, MAF) also showed a significant correlation with genetic distance in the Transition transect (Fig. 2-5). In the Amazon transect, only note A dominant frequency presented correlation with genetic distance (r = 0.772, P = 0.049, Table 2-4S). In the Cerrado transect only maximum frequency of note B (Table 2-4S) showed correlation with genetic distance. These results suggest that the variation in these traits as a function of ecological distance has a genetic basis.

Morphological analysis

There is a significant association between morphological divergence and ecological distance for four out of nine traits in the transition transect (HW: r=0.66, P=0.001, TL: r=0.460, P=0.004, TB: r=0.23, P=0.035, HaL: r = 0.660, P=0.001, Table 2-3S). In addition, using the PC scores from a principal component analysis, size and shape showed significant correlation with ecological distance in the Transition transect (PC1: r = 0.389, P=0.007, PC2: r=0.564, P=0.002). None of the traits showed correlation with ecological distance in the Amazon transect except head length (r = 0.61, P = 0.029). Only two traits showed correlation with ecological distance in the Cerrado transect (Femur: r=0.342, P=0.014; Tibia: r=0.33, P=0.034).

Contrasting with ecological distance, geographical distance was only significantly correlated with PC2 (r=0.184, P=0.032) and PC3 (r=0.29, P=0.003) in the Transition transect. In the Amazon transect, only head width (r=0.615, P=0.029) was correlated with distance, and in the Cerrado PC1 (r=0.313, P=0.037), Femur(r=0.342, P=0.014), and Tibia (r=0.33, P=0.034) was significantly correlated with distance. Overall, morphology showed fewer traits correlated with ecological and geographical distance compared to vocalization traits, however ecological distance was overall more important than geographical distance in explaining divergence in morphology along the Transition transect.

In the tree regression analysis, environmental variables were also more important than distance in predicting variation in all morphological traits (Fig. 2-4). I used nine morphological traits as a response variable and the model using 23 environmental and geographical predictors (Table 2S) explained 52% of variation of head length (most important: Bio18), 36.21% of hand Length (most important: Bio15), 33.09% of head width (most important: Bio7), 16.92% of foot length (most important: Bio15), 14.62% of tibia (most important: NDVI), 11.77% of Femur (most important: Bio15), 7.69% of snout vent length (most important: BIO6) (Fig. 2-5S). The only response trait that the random forest performed poorly was for forearm (<0.01%). Overall, morphological traits showed little association with genetic distance (Table 2-4S). None of the traits showed significant correlation with genetic distance, but after collapsing the nine traits into PC scores, genetic distance was correlated with PC2 (r = 0.508, P=0.002), and PC3 (r = 0.897, P=0.008) in the Transition transect.

Genetics analysis

All population pairwise F_{ST} comparisons ranged between 0.027 to 0.138 with genetic divergence lower in the Cerrado transect (F_{ST} = 0.027-0.036) than in the Transition transect (F_{ST} =0.029-0.063) and in the Amazon transect (F_{ST} =0.059-0.138)(Fig. 2-2, Table 2-4S). Genetic distance (F_{ST}) was correlated with distance along the gradient (r = 0.8474, P = 0.001), but no correlation was found in the Cerrado (r = 0.329, P = 0.296) and the Amazon (r = 0.156, P = 0.375) transects. After correcting for the spatial autocorrelation effect genetic distance was not correlated with ecological distance in the Cerrado (r=0.75, P=0.25), in the Transition (r=0.628, P=0.99), and in the Amazon (r=0.201, P= 0.34) (Table 3S) most likely due to high levels of gene flow.

No evidence for large allele dropout and scoring errors due to stuttering was detected, but MICROCHECKER (Van Oosterhout et al. 2004) suggested the existence of null alleles, and I used this software to correct for this issue before performing further analyses. After Bonferroni corrections, most of loci were in Hardy-Weinberg equilibrium and no evidence for linkage disequilibrium was found. Heterozygous deficiency was detected for sites A1 (one loci), A2 (three loci), C1 (two loci), C4 (three loci), C6 (two loci) (Table 2-1S).

Discussion

This study indicates that selection on phenotypic traits is a major driver in the divergence between the Amazon-Cerrado transition. The results showed an association of phenotypic traits important to fitness with environmental variables along the transition (Fig. 2-3). In addition, this association showed to be more related with ecological divergence than geographical distance (Fig. 2-3 and Fig. 2-4). These results support that ecological differences along the gradient play a more important role in the divergence process than geographical distance. Furthermore, genetic divergence in neutral loci is highly correlated with distance in the Transition transect but not in the Cerrado and the Amazon transects. Notably, reproductive traits also showed a correlation with genetic distance (Fig. 2-5) along the transition. Thus, divergence in vocalization traits appears to be causing adaptive evolution.

Vocalization as a proxy for reproductive isolation

Reproductive traits showing correlation with environmental variables and with genetic differentiation in neutral loci in the Transition suggests that natural selection might be leading to reproductive divergence. These correlations between ecological variables and sexual selected signals might lead to reproductive isolation via assortative mating (Orr and Smith 1998; Coyne and Orr 2004; Nosil 2012), such as in walking sticks (Nosil and Crespi 2004) and the frog *Acris crepitans* (Ryan and Wilczynski 1991). In most anuran species mate choice is based solely on the male call (Gerhardt and Huber 2002), which has been shown to be good indicator of male fitness (Wells 1988). Different habitats may result in differentiation in advertisement call traits (Nevo 1973; Narins and Smith 1986), which can influence in sexual selection (Wilczynski et al. 1999). This variation can also arise as a result of divergent selection on sensory system by pleiotropic effect (Ryan and Wilczynski 1988) and differences in mating signal transmission in different environments (Schluter 2001; Boughman 2002; Seehausen et al. 2008; Nosil 2012).

Therefore, divergence in vocalization traits between the Amazon and the Cerrado biome suggests that reproductive isolation may evolve as a by-product of divergent selection on traits important in sexual selection (Schluter 2001; McKinnon et al. 2004; Rundle and Nosil 2005). Adaptive divergence in those reproductive traits along the gradient would restrict gene flow between populations, and would result in neutral evolution of those neutral loci (Michel et al. 2010; Funk et al. 2011; Butlin et al. 2012). If adaptive ecological divergence is likely to occur in those vocalization traits, this study would support the isolation by adaptation mechanism of genetic differentiation (Funk et al. 2011; Nosil 2012). Adaptive differentiation to different environments can result population divergence, causing reduced gene flow and increasing genetic drift in neutral loci (Funk et al. 2011). In addition, linkage of neutral loci with selected loci could also lead to greater differentiation of neutral loci than expected under drift (Funk et al. 2011). Future work on female preference experiment will help to elucidate if divergence across the gradient may result in assortative mating. This type of study can help to link ecological processes and the development of reproductive isolation mechanisms (Orr and Smith 1998; Coyne and Orr 2004).

Morphological variation

Morphological characters also showed association with environmental variables in which precipitation variables in general were more important predictors than distance in explaining morphological variation. This result also supports that ecological differences along the gradient can be used as a proxy for phenotypic divergence. Precipitation variables as important predictors in morphology variation support the water availability hypothesis in which the reduced surface-to-volume ratio of larger body size minimizes water loss, especially in drier areas (Nevo 1973). In temperate regions, Holoartic anurans tend to have larger body size towards colder macroclimates and is mainly associated with thermoregulation (Nevo 1973; Ashton 2002; Olalla-Tarraga et al. 2009). Results suggest that water conservation may be more important issue in the savanna region due to seasonal climate. These results are consistent with previous studies in the equator region where desiccation seems to play a major role in determining body size (Olalla-Tarraga et al. 2006; Olalla-Tarraga and Rodriguez 2007).

Many studies investigating phenotypic variation along environmental clines make conclusions regarding adaptive variation, but lack data on levels of heritability and plasticity of the traits in question (Stillwell 2010). Determining the genetic and environmental basis of morphological variation along the gradient is essential to understand about adaptability of those traits. By examining these traits in a common environment, I have evidence that phenotypic variation between the Aman and the Cerrado has a genetic basis (Oyamaguchi et al., Ms in prep).

Conclusion

Environmental gradients can shape divergence among subpopulations within a species (Smith et al. 2005a; Mila et al. 2009; Smith et al. 2011). The Amazon-Cerrado gradient provides an ideal setting to explore the relative importance of neutral and adaptive processes in driving divergence (Da Silva and Bates 2002; Bates et al. 2003; Seddon and Tobias 2007). This study shows that ecological factors are important to the evolution of Amazonian biodiversity (Tuomisto 2007; Tobias and Seddon 2009; Tobias et al. 2010). Using environmental variables to understand phenotypic and genotypic divergence processes, I identify first step evidence that traits are under natural selection by showing an association between divergence in reproductive traits with ecological factors and the significant correlation of this divergence with genetic distance. These correlations suggest that natural selection plays a dominant role over genetic drift along the transition and may play an important role in the speciation process. Further experimentations testing female preference for those divergent calls, the heritability of these traits and the effects of environmental factors on vocalization are necessary to confirm that adaptive evolution is occurring along the gradient.

Figures

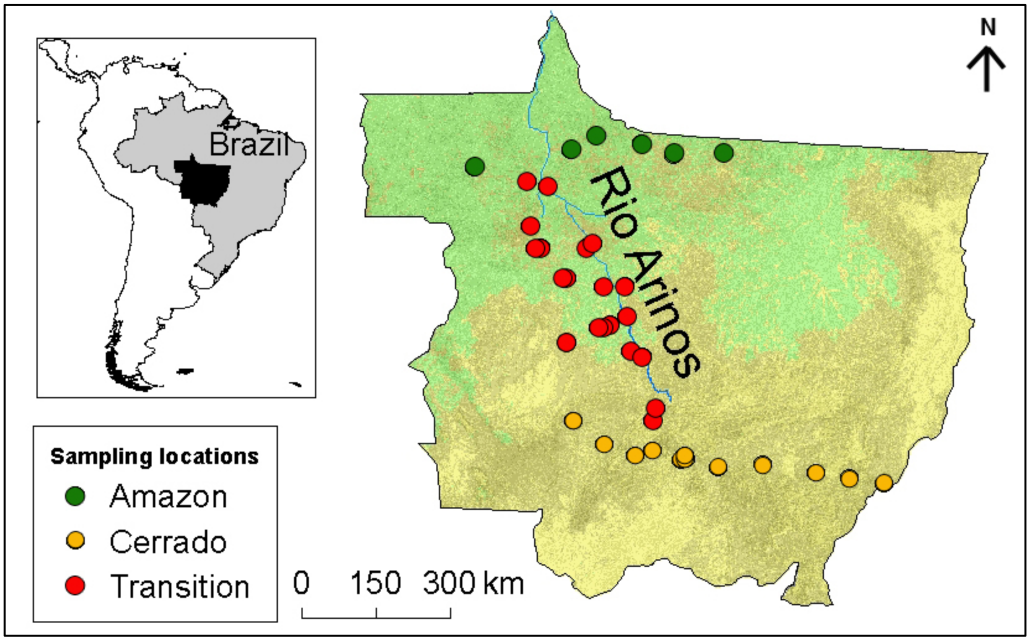

Fig. 2-1. Sampling sites in the Amazon (n = 6), the Cerrado (n = 12), and along the transition (n = 22) between the Amazon and the Cerrado Biomes in Mato Grosso State, Brazil.

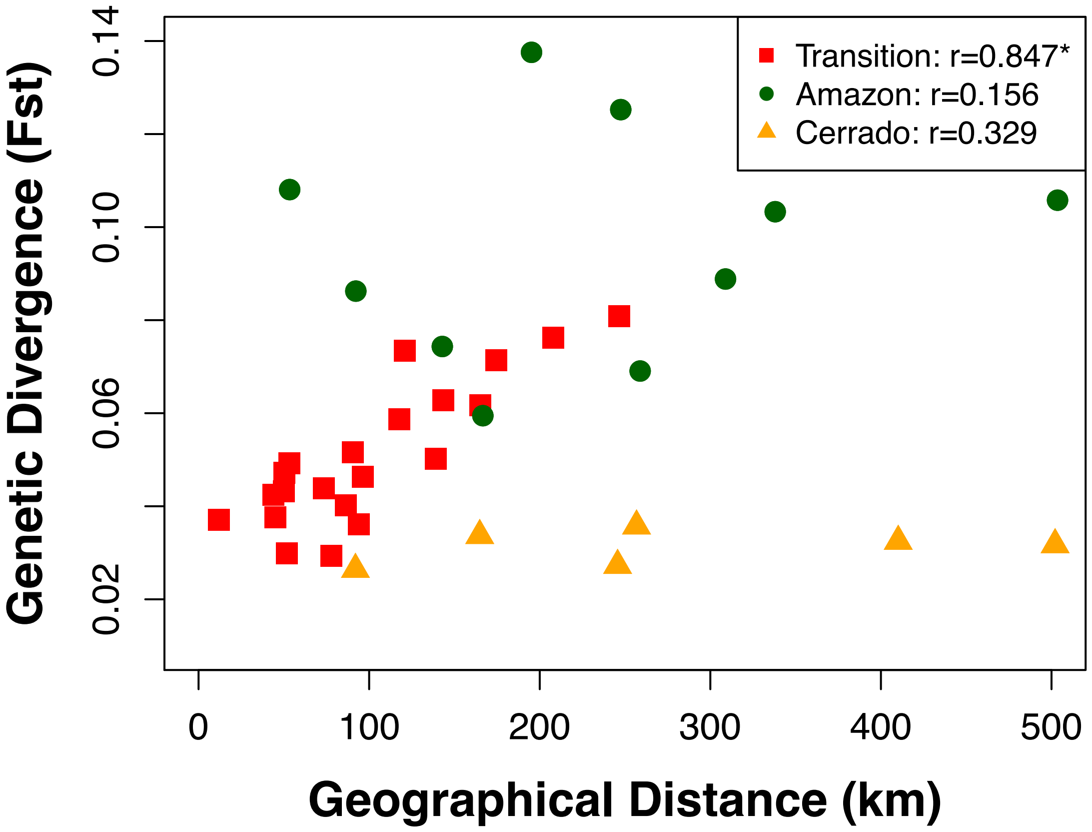

Fig. 2-2. Plot of genetic divergence against geographical distance within the Amazon transect (P=0.375), within the Cerrado transect (P=0.296), and within the Transition transect (*P=0.001).

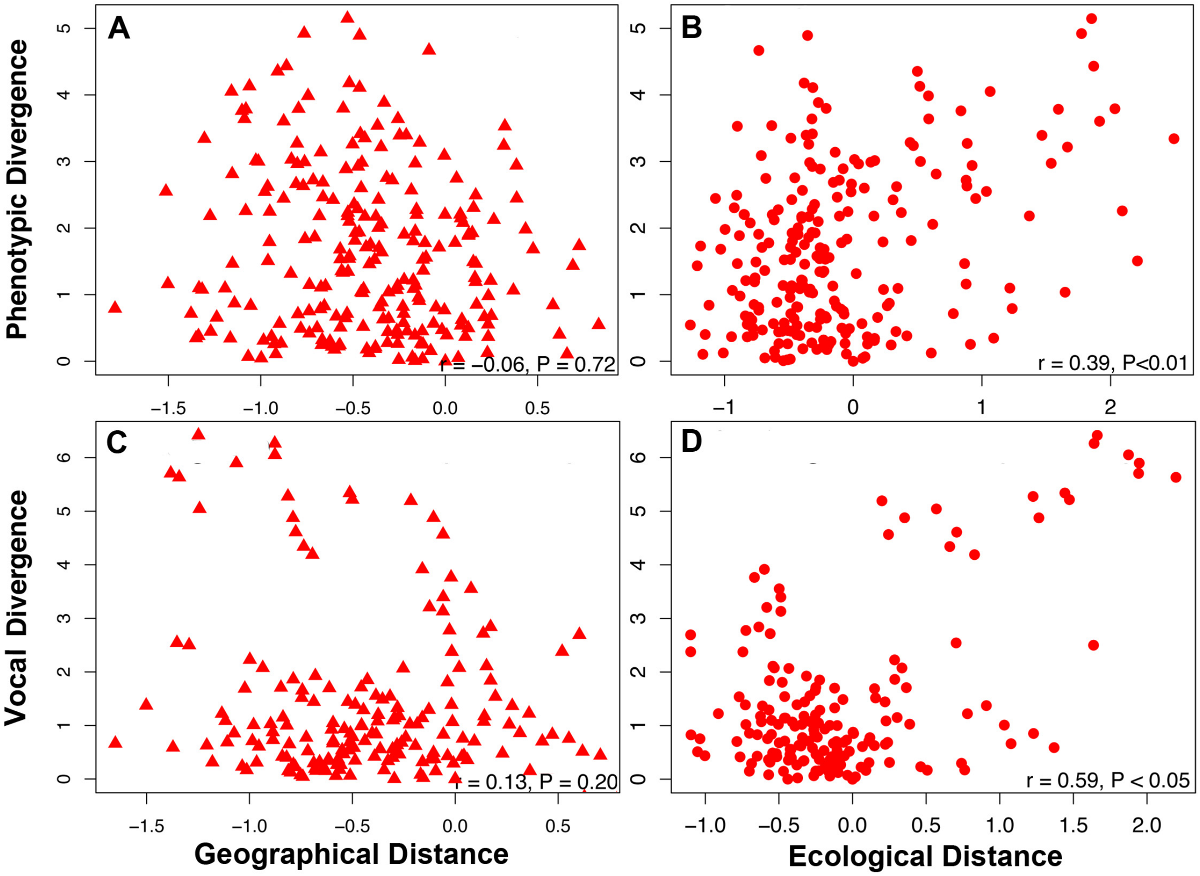

Fig. 2-3. Plot of Euclidean distance of morphological characters against A) residuals of geographical distance and, B) residuals of ecological distance within the Transition transect. Plot of Euclidean distance of vocalization characters (Note A) against C) residuals of geographical distance, and D) residuals of ecological distance.

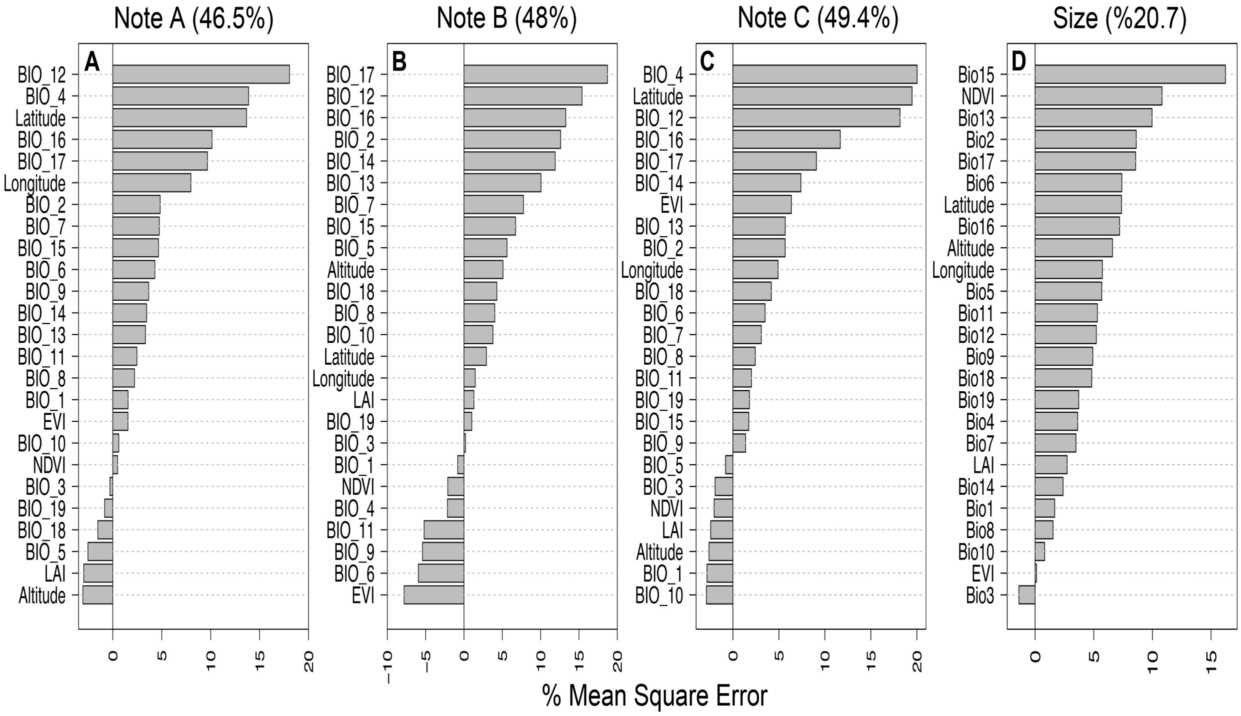

Fig. 2-4. Variable importance scores in the conditional tree regression model for environmental variables and distance (latitude and longitude) predictors of phenotypic variation (table 2S appendix). Higher increase in mean square errors indicates a more important predictor variable. Percentage of variance explained by the predictors in the responsible variable is in parenthesis besides of title for each graph. A) Variable importance of conditional regression tree for frequencies trait of note A. B) Variable importance of conditional regression tree for frequencies trait of note B. C) Variable importance of conditional regression tree for frequencies trait of note C. D) Variable importance of conditional regression tree for morphological traits of *D. minutus*.

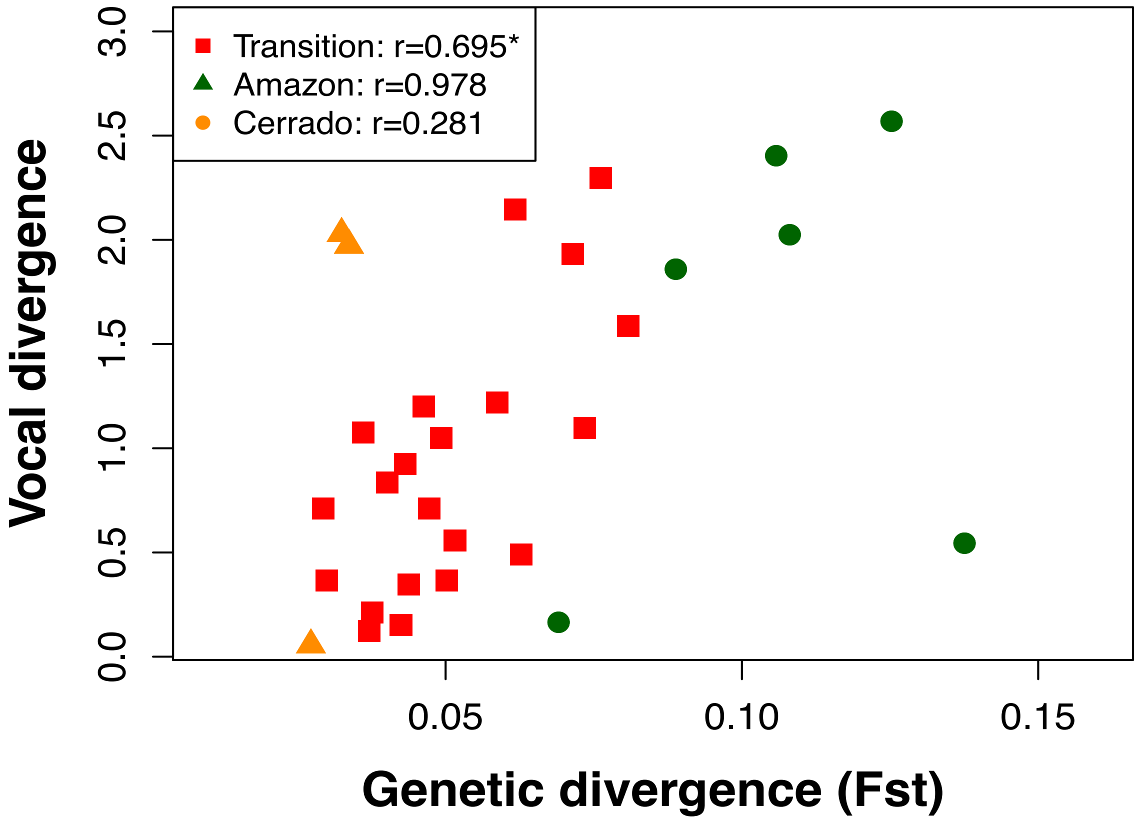

Fig. 2-5. Plot of Euclidean distance of vocalization frequencies residuals (note A) against genetic divergence within the Cerrado (P=0.340), the Amazon (P=0.048), and the Transition (*P=0.040) transects.

Appendix

Supplemental figures

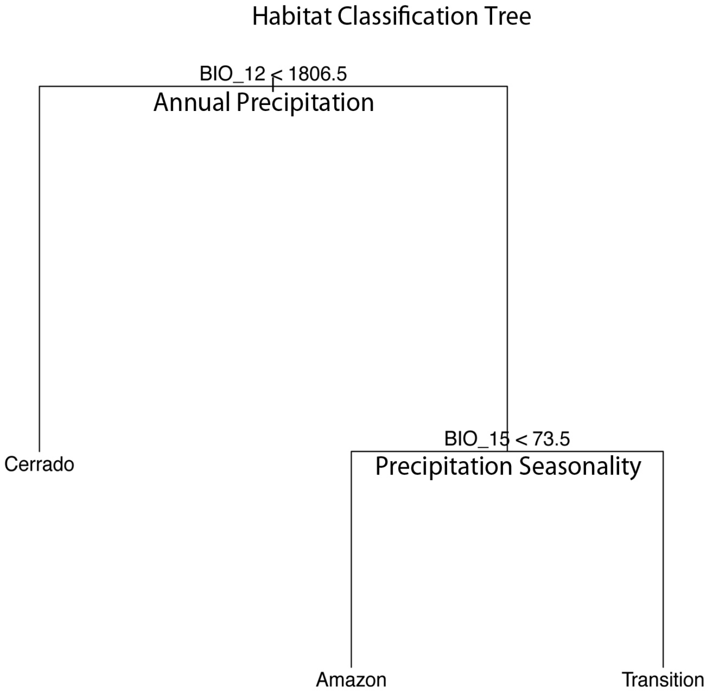

Fig. 2-1S. Habitat classification tree results for Amazon, Cerrado, and Transition sites. At each node, the indicated splitting variable is the predictor for habitat type classification. The left branch of each node represents the lower value for each variable and the right branch represents higher values.

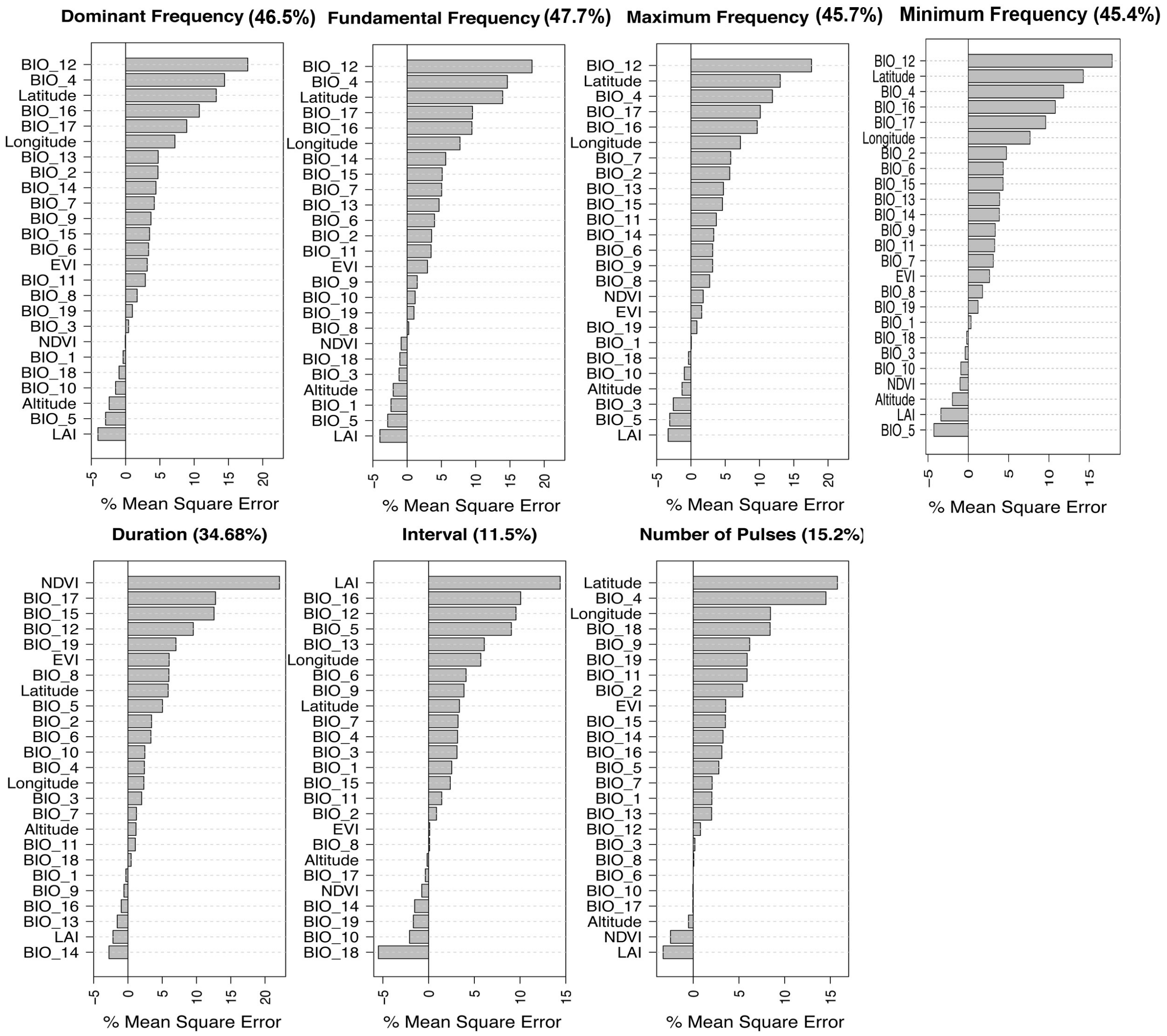

Fig. 2-2S. Variable importance scores in the conditional tree regression model for environmental variables and distance (latitude and longitude) predictors of vocalization variation for note A. Higher increase in mean square errors indicates a more importance of predictor variable.

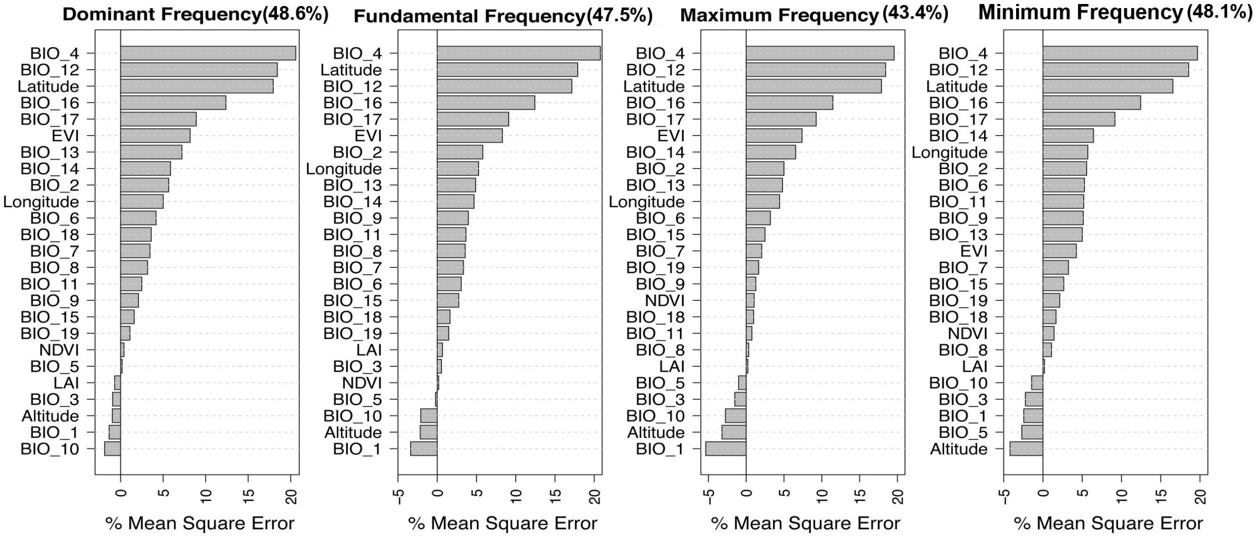

Fig. 2-3S. Variable importance scores in the conditional tree regression model for environmental variables and distance (latitude and longitude) predictors of vocalization characters variation for note B. Higher increase in mean square errors indicates a more importance of predictor variable.

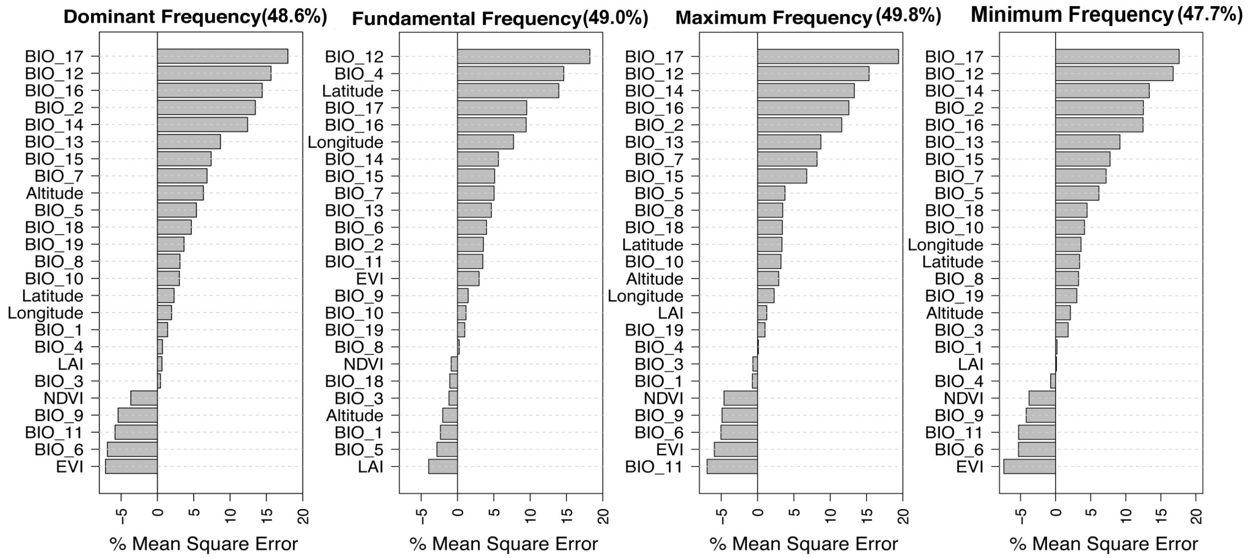

Fig. 2-4S. Variable importance scores in the conditional tree regression model for environmental variables and distance (latitude and longitude) predictors of vocalization characters variation for note C. Higher increase in mean square errors indicates a more importance of predictor variable.

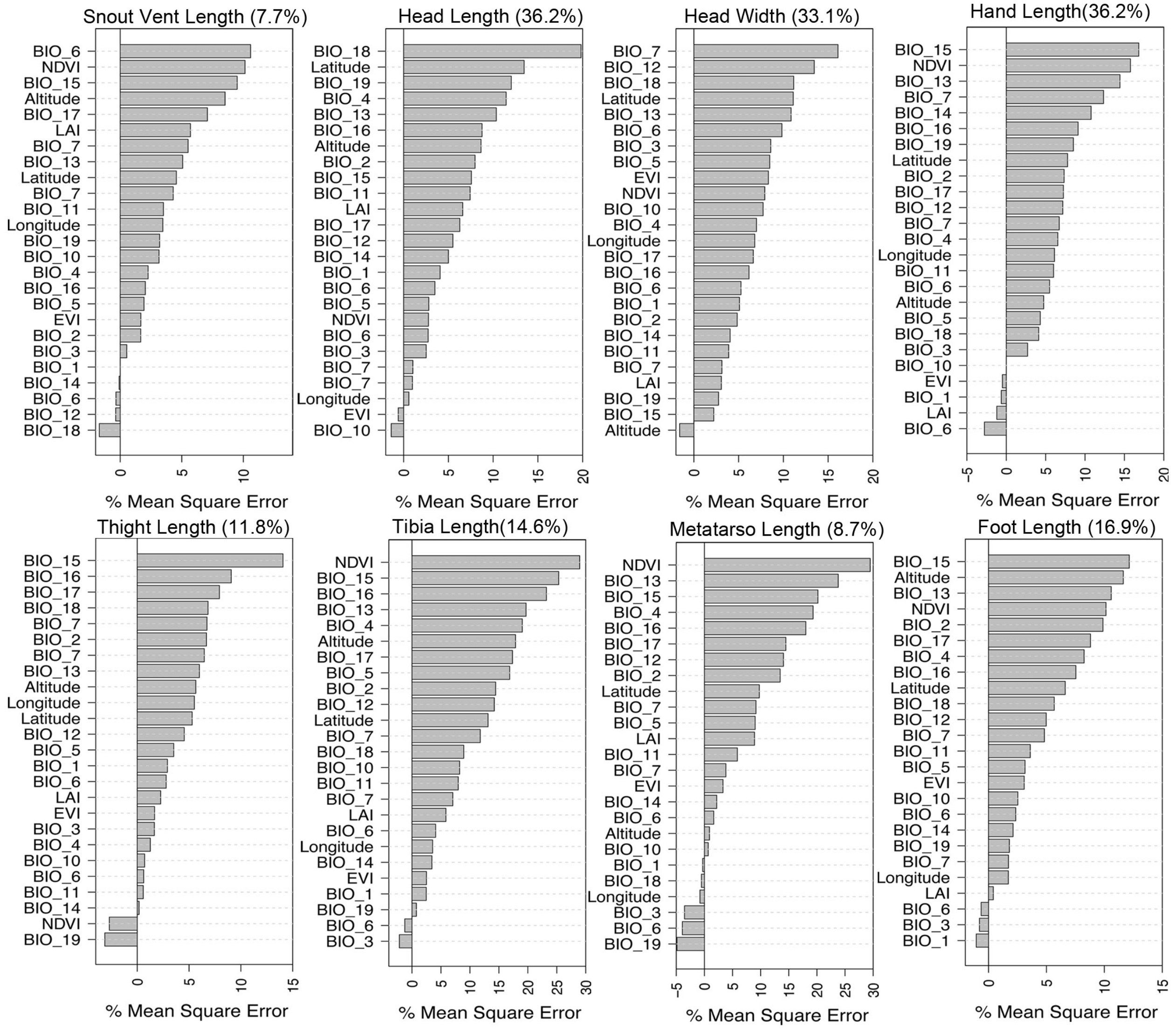

Fig. 2-5S. Variable importance scores in the conditional tree regression model for environmental variables and distance (latitude and longitude) predictors of morphology characters variation. Higher increase in mean square errors indicates a more importance of predictor variable.

Supplemental tables

Table 2-1S. Sampling localities and habitat type for *Dendropsophus minutus* collected in Mato Grosso State, Brazil (2008-2010).

Habitat	Locality	Latitude	Longitude
A1	Guaranta do Norte	-9.8377	-55.0199
A2	Paranaita	-9.6579	-56.515
A3	Apiacas	-9.5258	-57.3408
A4	Nova Bandeirantes	-9.8053	-57.7972
A5	Aripuana	-10.1124	-59.5448
A6	Alta Floresta	-9.8858	-55.9487
C1	Barra do Garcas	-15.8499	-52.126
C2	General Carneiro	-15.7184	-52.7583
C3	Vila Paredao	-15.6358	-53.3512
C4	Primavera do Leste	-15.4773	-54.3232
C5	Campo Verde	-15.5473	-55.1283
C6	Chapada dos Guimaraes - Buriti	-15.416	-55.8047
C7	Jangada	-15.319	-56.6298
C8	Barra dos Bugres	-15.1293	-57.1911
C9	Chapada dos Guimaraes - Peba	-15.3841	-55.7098
C10	Chapada Guimaraes – Peba2	-15.3148	-55.7316
TT-1	Acorizal	-15.2268	-56.3124
T1	Nobres	-14.6944	-56.323
T2	Diamantino	-14.4664	-56.2498
T3	Sao Jose de Rio Claro	-13.4415	-56.7307
T4	Nova Maringa	-12.9468	-57.0922
T5	Brianorte	-12.264	-57.2288
T6	Porto dos Gauchos - P1	-11.5248	-57.4939
T7	Porto dos Gauchos - P2	-11.4863	-57.3894
T8	Itanhanga	-12.2498	-56.8241
T9	Tapurah	-12.8082	-56.7866
T10	Nova Mutum	-13.5341	-56.5016
T11	Juruena - P1	-10.3631	-58.5949
T12	Juruena - P2	-10.4552	-58.2272
T13	Castanheira	-11.1771	-58.5456
T14	Juina - P2	-11.5554	-58.3453
T15	Juina - P1	-11.5669	-58.4565
T16	Brasnorte - P2	-12.1047	-57.863
T17	Brasnorte - P1	-12.1397	-57.9239
T18	Campo Novo dos Parecis	-13.2787	-57.8817
T19	Nova Maringa - Rio do Sangue - P1	-13.0095	-57.205
T20	Nova Maringa - Rio do Sangue - P2	-13.0188	-57.3172
T21	Tangara da Serra	-14.6971	-57.7605

Table 2-2S. Environmental data used from the WorldClim database (Hijmans et al. 2005) and MODIS data archive from 2012 as predictors variables for phenotypic variation and to classify habitat types for sampling localities.

BIO1 = Annual Mean Temperature
BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3 = Isothermality (BIO2/BIO7) (* 100)
BIO4 = Temperature Seasonality (standard deviation *100)
BIO5 = Max Temperature of Warmest Month
BIO6 = Min Temperature of Coldest Month
BIO7 = Temperature Annual Range (BIO5-BIO6)
BIO8 = Mean Temperature of Wettest Quarter
BIO9 = Mean Temperature of Driest Quarter
BIO10 = Mean Temperature of Warmest Quarter
BIO11 = Mean Temperature of Coldest Quarter
BIO12 = Annual Precipitation
BIO13 = Precipitation of Wettest Month
BIO14 = Precipitation of Driest Month
BIO15 = Precipitation Seasonality (Coefficient of Variation)
BIO16 = Precipitation of Wettest Quarter
BIO17 = Precipitation of Driest Quarter
BIO18 = Precipitation of Warmest Quarter
BIO19 = Precipitation of Coldest Quarter
ALT = Altitude
NDVI = Normalized Vegetation Index
EVI = Enhanced Vegetation Index
LAI = Leaf Area Index

Table 2-3S. Results of partial mantel test from Euclidean pairwise comparison of phenotypic (vocalization and morphology) against 1) residuals of ecological distance, 2) geographical distance in the Transition, the Amazon, and the Cerrado transects.

	Transition				Amazon				Cerrado			
	Geography		Ecology		Geography		Ecology		Geography		Ecology	
Note A	r	P	r	P	r	P	r	P	r	P	r	P
Dominant Frequency	0.133	0.100	0.585	**0.030**	0.354	0.100	-0.298	0.800	-0.150	0.700	-0.179	0.800
Maximum Frequency	0.133	0.100	0.585	**0.040**	0.379	0.100	-0.318	0.800	-0.139	0.700	-0.183	0.700
Minimum Frequency	0.126	0.100	0.540	**0.040**	0.347	0.100	-0.272	0.800	-0.145	0.700	-0.175	0.800
Fundamental Freq.	0.133	0.100	0.585	**0.040**	0.354	0.100	-0.298	0.800	-0.150	0.700	-0.179	0.800
PC1	0.132	0.200	0.586	**0.040**	0.359	0.100	-0.297	0.800	-0.146	0.700	-0.179	0.700
Pulse Duration	-0.036	0.600	-0.046	0.500	0.393	0.100	-0.100	0.600	0.614	0.030	-0.469	1.000
Pulse Interval	0.038	0.300	0.244	0.090	-0.143	0.600	0.130	0.300	-0.051	0.600	0.039	0.400
Pulse Number	0.030	0.300	-0.055	0.500	-0.158	0.600	0.021	0.300	-0.245	0.900	0.087	0.030
Note B												
Dominant Frequency	0.124	0.100	0.555	**0.008**	0.574	0.200	-0.543	1.000	0.099	0.200	-0.176	0.800
Maximum Frequency	0.127	0.100	0.561	**0.010**	0.564	0.200	-0.568	1.000	0.097	0.200	-0.175	0.800
Minimum Frequency	0.119	0.100	0.532	**0.020**	-0.555	0.200	-0.515	1.000	0.106	0.200	-0.183	0.800
Fundamental Freq.	0.124	0.200	0.555	**0.010**	0.575	0.200	-0.544	1.000	0.099	0.200	-0.175	0.800
PC1	0.123	0.100	0.552	**0.010**	0.569	0.200	-0.545	1.000	0.100	0.200	-0.177	0.800
Pulse Duration	0.313	**0.005**	-0.208	1.000	-0.413	1.000	0.264	0.200	-0.148	0.700	-0.167	0.700
Pulse Interval	-0.059	0.700	0.190	0.100	-0.012	0.400	0.202	0.200	-0.008	0.400	-0.119	0.700
Pulse Number	0.144	0.070	-0.190	1.000	0.449	0.200	-0.534	1.000	0.273	0.100	-0.374	1.000
Note C												
Dominant Frequency	0.185	**0.050**	0.429	**0.020**	0.551	0.200	-0.586	1.000	-0.203	0.800	-0.051	0.400
Maximum Frequency	0.159	0.090	0.416	**0.020**	0.560	0.100	-0.606	1.000	-0.209	0.800	-0.029	0.300
Minimum Frequency	0.137	0.100	0.396	**0.040**	0.581	0.200	-0.580	1.000	-0.206	0.800	-0.116	0.600
Fundamental Freq.	0.185	0.060	0.429	**0.020**	0.551	0.200	-0.586	1.000	-0.203	0.800	-0.051	0.400
PC1	0.170	0.007	0.421	**0.020**	-0.565	0.100	-0.597	1.000	-0.217	0.900	-0.036	0.400
Pulse Duration	-0.107	0.850	0.357	**0.024**	-0.596	0.907	0.690	**0.012**	-0.114	0.698	-0.212	0.803

Table 2-3S. continuation

	Transition				Amazon				Cerrado			
	Geography		Ecology		Geography		Ecology		Geography		Ecology	
Morphology	r	P	r	P	r	P	r	P	r	P	r	P
Snout Vent Length	-0.003	0.486	0.175	0.116	0.044	0.443	-0.036	0.457	0.139	0.181	0.047	0.333
Head Length	-0.101	0.857	0.236	0.089	-0.284	0.919	0.281	0.099	0.074	0.316	0.042	0.331
Head Width	0.151	0.078	0.659	**0.001**	0.610	**0.029**	-0.568	1.000	0.151	0.074	-0.082	0.619
Femur	-0.025	0.549	0.460	**0.004**	-0.217	0.729	0.163	0.199	0.342	**0.014**	0.293	0.158
Tibia	-0.051	0.718	0.300	**0.035**	0.175	0.327	-0.226	0.772	0.333	**0.034**	0.153	0.137
Metatarso	-0.063	0.761	0.032	0.354	0.374	0.112	-0.455	0.998	0.199	0.100	0.199	0.202
Foot length	-0.086	0.824	0.278	0.054	-0.105	0.562	0.044	0.291	0.185	0.115	0.011	0.393
Hand length	0.008	0.383	0.660	**0.001**	-0.540	0.994	0.511	0.036	0.204	0.114	-0.113	0.733
Forearm	0.736	-0.057	-0.092	0.796	-0.140	0.622	0.036	0.349	0.282	0.560	-0.261	0.995
PC1	-0.056	0.722	0.389	**0.007**	-0.097	0.560	0.049	0.294	0.313	0.037	0.142	0.247
PC2	0.185	0.049	0.564	**0.002**	0.077	0.394	0.038	0.415	-0.084	0.698	-0.187	0.807
PC3	0.290	**0.003**	-0.033	0.507	0.003	0.517	-0.031	0.418	-0.155	0.808	-0.060	0.443
Genetics Distance	0.635	**0.034**	-0.628	0.990	-0.171	0.081	0.201	0.340	0.750	0.250	-0.743	0.900

Table 2-4S. Pairwise *Fst* of *Dendropsophus minutus* microsatellites between sites in the Amazon (“A”), in the Cerrado (“C”), and in the Transtion (“T”).

	A1	A2	A3	A4	A5	C1	C4	C6	C7	T3	T4	T5	T6	T7
A2	0.059													
A3	0.069	0.086												
A4	0.089	0.074	0.108											
A5	0.106	0.103	0.125	0.138										
C1	0.047	0.047	0.076	0.054	0.097									
C4	0.055	0.044	0.070	0.070	0.089	0.027								
C6	0.038	0.043	0.054	0.061	0.074	0.032	0.034							
C7	0.045	0.052	0.060	0.057	0.091	0.032	0.036	0.026						
T3	0.068	0.057	0.081	0.080	0.086	0.055	0.053	0.045	0.054					
T4	0.062	0.055	0.076	0.065	0.105	0.054	0.060	0.042	0.046	0.047				
T5	0.051	0.046	0.062	0.067	0.095	0.049	0.054	0.036	0.042	0.052	0.043			
T6	0.051	0.046	0.073	0.058	0.099	0.036	0.046	0.036	0.039	0.063	0.046	0.049		
T7	0.064	0.055	0.059	0.070	0.082	0.054	0.059	0.044	0.044	0.050	0.036	0.043	0.037	
T8	0.051	0.040	0.071	0.067	0.088	0.042	0.045	0.032	0.042	0.044	0.030	0.038	0.040	0.029

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CHAPTER 3

Experimental evidence for local adaptation to seasonal variation in the larval development of the lesser tree frog (*Dendropsophus minutus*)

Abstract

Comprehending the sources of phenotypic responses to environmental variation is fundamental to predict the effects of human-induced climate change. Under climate change, the Amazon rainforest will be converted into a more savanna like habitat. Hence, investigating geographical variation in phenotypes between the Cerrado and the Amazon can provide insights about the temporal climate change effects in the Amazon. The goal of this study was to understand the environmental and genetic contribution to *Dendropsophus minutus* tadpole variation between the Amazon and the Cerrado biomes. I executed a common garden experiment to test three specific hypotheses: 1) the geographic pattern of phenotypic variation has a genetic component; 2) the development of tadpoles from the Cerrado is faster than in the Amazon; and 3) *D. minutus* shows plastic responses to different temperature conditions. This experiment comprised of tadpoles from two Cerrado sites (n=5 egg masses) and from one Amazon site (n=3 egg masses). These eggs were exposed to four temperature conditions: 1) at constant 26.5^{o}C (T1); 2) at constant 32^{o}C (T2); and two fluctuating temperature: 3) 26.5-29^{o}C (T3) and 4) 26.5-32^{o}C (T4). Treatment T2 showed high mortality rate indicating that 32^{o}C is the upper temperature threshold. Due to differential mortality in each treatment, I used Skilling-Mack analysis, which is applied for an unbalanced block design. Results showed that larval development differentiation was a result of both genetic and environmentally induced differentiation. Under a common garden experiment, larval development from the Cerrado was on average four days faster than the Amazon showing the genetic basis of this variation. Under fluctuating thermal conditions, *D. minutus* accelerated development showing a plastic response to environmental variation. These traits might contribute to this species to tolerate and adapt to climate change as long as the maximum temperature fluctuation is not above the thermal tolerance (32^{o}C) of *D. minutus* larvae.

Introduction

Understanding phenotypic responses of organisms to environmental variation is a central focus of evolutionary biology (Via and Lande 1985; West-Eberhard 1989; Thompson 1991; Thorpe et al. 2005; Pfennig et al. 2010), and is taking on new urgency in the face of human-induced climate change (Bradshaw 2006; Parmesan 2006; Hoffman and Sgro 2011; Berllard 2012). Global mean surface temperatures are projected to continue increasing, and precipitation differences between wet and dry seasons are likely to become more pronounced (Stocker et al. 2013). Amphibians are particularly sensitive to these changes because they are physiologically constrained by their water-permeable skin, ectothermic life history, and biphasic life cycle (see review, Blaustein et al. 2010). Survival and reproduction of most amphibians are also fundamentally linked to freshwater ecosystems (Duellman 1994), which are also being altered by climate change (Parmesan 2006; Todd et al. 2011). Whether amphibians can metabolically cope with ongoing climate change will depend on the ability of species to either tolerate, or adapt to new environmental conditions.

Changes in temperature and precipitation regimes are likely to affect numerous aspects of amphibian reproduction (Beebee 1995; Newman 1992; Blaustein et al. 2001; Blaustein et al. 2010). In ectotherms, one of the most important environmental cues for larval development is temperature (Berven, 1982; Smith-Gill and Berven 1979; Atkinson and Sibly 1997; Angilletta et al. 2004). Higher temperatures can increase metabolic rate and accelerate developmental rates (Angilletta et al. 2004). Water availability also influences developmental rate that is under strong selective pressures from pond desiccation (Newman 1988, 1992; Denver 1997; Denver 1998; Laurila et al. 2002; Gomez-Mestre and Buchholz 2006; Székely et al. 2010). Amphibians that reproduce in ephemeral ponds are especially vulnerable to changes in temperature and precipitation (Newman 1989, 1992). In these temporary water bodies, shorter larval periods increase the chance of larval survival by ensuring an earlier emergence from a drying pond (Newman 1992; Laurila et al. 2002; Ryan and Winne 2001; Márquez-García et al. 2010). However, a shorter larval period also results in metamorphosis at a smaller size (Wilbur and Collins 1973; Werner 1986; Zuo et al. 2012) that ultimately may influences adult morphology (Gomez-Mestre 2006), and male reproductive success (Beck and Congdon 2000; Gervasi and Foufopoulos 2007; John-alder and Morin 2014). If temperatures become too high they may lower the larval survival and may cross the thermal tolerance thresholds, which varies between species (Blaustein et al. 2010). Further, shorter hydroperiods (the length of time in a location with standing water, Gaff et al. 2000) may result in reproductive failure for species (Dodd 1993; Semlitsch and Wilbur 1998; Richter et al. 2003) and contribute to population decline (Blaustein et al. 2010).

Phenotypic response of local populations to environmental variation is a key mechanism for amphibian species to tolerate or adapt to new environmental conditions. Populations in different microhabitats show intraspecific variation that can result in differential responses to novel conditions (Semlitsch et al. 1990; Sork et al. 2010). This phenotypic variation pattern can be produced by a combination of natural selection (Schluter 2009), random variation (Butlin et al. 2012), and phenotypic plasticity (Via and Lande 1985; Losos et al. 2000). Numerous studies have shown that natural selection plays a dominant role in the divergence process (Endler1980; Rice 1993; Schluter 2001, 2009; Rundle 2000; Coyne and Orr, 2004; Nosil, 2008, 2012; Ogden & Thorpe, 2002; Smith *et al.* 2005; Schneider *et al.*, 1999) indicating that ecological factors are important drivers of intraspecific variation. Phenotypic variation between populations often co-varies with environmental variables (Oyamaguchi et al., Ms. in prep, Thomassen 2010; Smith et al. 2011; Smith et al. 2013). These empirical studies on ecological differentiation often presume a dominant role of natural selection on the divergence of phenotypic traits. However, differentiation between populations may also be a byproduct of only phenotypic plasticity (Losos et al. 2000). Phenotypic plasticity is the capacity of a single genotype to produce different phenotypes in response to environmental conditions (West-Eberhard 1989). Very few studies offer experimental evidence showing the genetic and environmental contributions to this variation (e.g. Malhotra 1991; Schluter 1994; Losos 1997; Thorpe et al. 2005) due to the challenges in maintaining all the ecotypes in a common garden (Thorpe et al. 2005), especially for non-model organisms. Yet this type of study is fundamental to understanding the source of phenotypic variation and also to elucidate how amphibians will respond to the rapid climate change. Investigating the genetic and environmental contributions to phenotypic variation provides important information about the evolutionary potential of populations to selection pressures associated to climate change (Hoffmann and Sgro 2011).

To understand the genetic and environmental contribution to phenotypic variation, the goal of this study was to experimentally investigate larval development variation in the lesser tree frog (*Dendropsophus minutus*) between the Amazon and the Cerrado biomes. These two biomes present contrasting environmental differences in temperature and precipitation regimes. The Amazon rainforest is the largest biome (>6 millions km^2) in South America followed by the Cerrado biome (1.86 million km^2) (Da Silva and Bates 2002; Da Silva et al. 2005). Moving from the northwest of Amazon to the southeast of Cerrado biome, the climate varies from continuously rainy to wet/dry season (Davidson et al. 2012). Pronounced differentiation in vegetation from a dense rainforest to an open grassland matches with variation in temperature and precipitation (Da Silva et al. 2005; Da Silva et al. 2006). These contrasting environments are thought to drive morphological and vocal differentiation between *D. minutus* populations (Oyamaguchi et al., Ms. in prep). The source of these differences between populations may be due to genetic, environmental, or gene-environment interactions. In this study, I executed a common garden experiment to test three specific hypotheses: 1) the geographic pattern of phenotypic variation has a genetic component; 2) the development of tadpoles from the Cerrado is faster than in the Amazon suggesting directional selection; and 3) *D. minutus* shows plastic responses to different temperature conditions.

Target species

Dendropsophus minutus is a small frog broadly distributed in South America (Frost 2013) and is found in wide different types of habitats (e.g. open and forested areas, at the edges of forests, in clearings, gallery forests; Lima et al. 2006; Silvano 2010). This species reproduces during rainy season (November to May, Lima et al. 2006) and females deposit eggs in lentic water (Haddad and Prado 2005). This frog shows intraspecific variation in morphology and vocalization between populations across its distribution range (Cardoso and Haddad 1984). This geographic pattern co-varies with environmental variables associated with precipitation and temperature, suggesting that ecological factors are important in shaping this differentiation (Oyamaguchi et al. Ms in prep).

Methods

Data collection and husbandry

I collected live males (n = 10) and gravid females (n = 8) of *D. minutus* from the Amazon and Cerrado biomes (n = 3 sites, Fig. 3-1, Table 3-1S) between December 16 and 20, 2011. After each sampling night, I paired males and gravid females (n = 8) from each location (Amazon: n =

3; Cerrado: n = 5) in a dark room for them to mate and to collect fertilized eggs. Since these eggs were collected under very similar condition, locality effect was not an issue in experiment. These eggs were stored in a thermal insulated box and transported to an environmental room at 18°C at the University of Sao Paulo. On December 23, 2011, fertilized eggs from each location were divided into four experimental treatments.

Experimental temperatures treatments were assigned based on pond temperatures recorded hourly using data loggers (Thermochron Ibutton, Maxim Integrated Products). The temperature was monitored for eight days in the Cerrado ponds (n = 2) and for four days in the Amazon pond (n = 1) during summer 2011 (Fig. 3-1S). I used the average temperature in each habitat to determine the control temperature. The average was very similar between ponds from different habitats ranging between 27.3°C and 27.8 °C. However, the variance was higher in the Cerrado (σ^2=4.6) than the Amazon (σ^2=2.47) (Fig. 3-1S).

Tadpoles were housed under four temperature treatments; 1) at constant 26.5°C (T1); 2) at constant 32°C (T2); and two fluctuating temperature: 3) 26.5-29°C (T3) and 4) 26.5-32°C (T4). Treatment T1 (26.5 ° C) is considered the control, which simulates the average temperature found in each habitat (experimental design illustrated in Table 3-2S). Although I found averages ranging between 27.3°C and 27.8 ° C, due to technical issue, T1 was executed under constant 26.5 °C. The four temperature treatments were elements of a 2 x 4 unbalanced un-replicated factorial design (Logan 2010). One factor consisted of tadpoles from the Cerrado and the Amazon location, a second factor was temperature with four levels: at constant 26.5°C, and 32°C, fluctuating temperature between 26.5-29°C and between 26.5-32°C. Treatment T1 (constant 26.5°C) was intended to simulate the average temperature observed in the collected ponds. Treatment T2 (constant 32°C) simulated a high temperature in which tadpoles were exposed in ephemeral ponds. The two fluctuating temperatures regimes were intended to include the range of temperature recorded in the field sites. Treatment T3 (26.5-29°C) simulated low fluctuation regime observed in the Amazon (Fig. 3-1S). The treatment T4 (26.5-32°C) simulated high fluctuation regime in the Cerrado (Fig. 3-1S). In the Cerrado ponds I found temperature up to 34°C. However, the maximum temperature used in the treatments was 32°C due to the limitation of aquatic heaters (75W Eihem, within ± 0.5°C variation).

Treatments took place in plastic tanks (26L) with two aquatic heaters (75W Eihem, within ± 0.5°C variation). For fluctuating temperatures I controlled one of the heaters with an electronic timer that was turned on at 8:00hrs and turned off at 17:00hrs. The water temperature was monitored hourly during the whole experiment using data loggers (Thermochron Ibutton, Maxim Integrated Products). Aquatic aerators ensured constant water circulation, which eliminates the effects of spatial variation in temperature. Every other day each water tank was sterilized with a UV filter for approximately 24hrs. During the experiment the photoperiod was set 12L/12D. In each treatment, 60 tadpoles (Amazon n = 30, Cerrado n = 30) were maintained individually separated in ~0.2L plastic conteiners with holes. Each individual was fed the same amount of fish food (Tetraveggie) every day.

Development and growth

I monitored the metamorphic stages of each individual after hatching using the Gosner table (Gosner 1960). I also measured the total body length (TBL), tail length (TL), inter-orbital distance (IOD), tail muscle width (TMW), eye diameter (ED), maximum tail height (MTH) and tail muscle height (TMH) from the dorsal and lateral pictures using a millimeter paper scale in the background. All the measurements were made using the Image J software (Abramoff 2004) and followed Altig (2007). I started registering metamorphic stages and measurements after tadpoles reached stage 25 (Gosner 1960). Each tadpole was photographed approximately every three days. I calculated developmental time (the length of tadpole development up to stage 41), developmental rate (stages/day), and growth rate (mm/day) after they reached to stages 40 and 41 (Gosner 1960). I also monitored the survival of tadpoles every 24-48 hrs.

Maternal effect

In addition to genetic differences between populations, differential addition of energy resources to the eggs (maternal effect) could influence populations' phenotype (Kaplan 1992). Females laying larges eggs increases provisioning and confers larger tadpoles in the initial stages, affecting morphology in the subsequent stages (Relyea, 2002). To address this maternal effect, I measured initial larvae morphology (stage 25, Gosner 1960) using the same measurements for development and growth. I compared tadpole morphology in the stage 25 (Gosner 1960) between the Amazon and the Cerrado for each treatment using a permutation T-test in the R (R Development Core, 2011)'Permute' package (Simpson 2012) with 100,000 iterations. This analysis showed no significant differences between the Amazon and the Cerrado in each treatment (Fig 3-2S). While I cannot directly assess the maternal effect, this result suggests that this effect was negligible on subsequent tadpole morphology. To properly address the maternal effects in *D. minutus,* multigenerational common-garden experiment would be ideal.

Developmental comparison between and within treatments

To test if tadpole variation in *D. minutus* was the result of a genetic differentiation, I compared developmental time, the seven morphological traits, developmental rate (stages/day), and growth rate (mm/day) between the Amazon and the Cerrado tadpoles. If the geographic variation pattern in the phenotypic traits is due to only plasticity, no significant differences in morphology is expected between the Amazon and the Cerrado. I compared the Amazon and the Cerrado tadpoles using a permutation T-test in the R (R Development Core, 2011)'Permute' package (Simpson 2012) with 100,000 iterations. This test requires equality of group variance, which I confirmed using a Bartlett test (R Development Core, 2011). I used the Wilcoxon rank test (R Development Core, 2011) for the groups in which the variances were unequal.

To test differences between treatments, I applied the Skillings-Mack statistics (Skillings and Mack 1981) followed by post hoc comparisons with Wilcoxon pairwise test using Holm correction. Skilling-Mack is a nonparametric test frequently used for unbalanced block designs with missing data (Chatfield and Mander 2009). This test was used because of differential mortality rate between treatments and no homogeneity of variances. Significant differences between treatments indicate the plastic component of phenotypic variation.

Comparisons of survivorship between the Amazon and the Cerrado and among treatments were analyzed using Kaplan-Meier survivorship formula followed by the log-rank test in the R package 'survival' (Diez 2013).

Results

Results from the common garden experiment suggest that the pattern of geographic variation in *D. minutus* has a genetic basis. Tadpoles from the Cerrado developed faster (Fig. 3-2), and showed a higher developmental rate (Fig. 3-3) and growth rate compared to the tadpoles from the Amazon (Fig. 3-4) when raised in the same conditions. Comparisons between treatments showed that phenotypic plasticity also contributes to phenotypic variation (Fig. 3-2B,

Fig. 3-3B, Fig. 3-4B, and 5). Although *D. minutus* showed phenotypic plasticity under different thermal conditions, mortality rate was higher under treatments with higher temperatures (Fig. 3-6). Thus, this study supports the idea that both genetic variance and phenotypic plasticity factors are likely to contribute to within and among population variation between the Cerrado and the Amazon biomes.

Genetic basis of phenotypic variation

Tadpoles took on average 53 days to develop, until stage 41 (Gosner 1960). In each treatment, tadpoles from the Cerrado completed metamorphosis earlier than tadpoles did from the Amazon (x= 4 days, Fig. 3-2A, Fig. 3-2B, Table 3-1) except for treatment T2 (32^{o}C). In this study I was not able to compare the Amazon and Cerrado under T2 treatment conditions due to high mortality rate (Fig. 3-6). This result for treatment T2 is most likely due to 32^{o}C temperature being above the thermal tolerance threshold for *D. minutus*. For treatment T1, in addition to a faster development, tadpoles from the Cerrado showed a narrower variance in developmental time indicating the presence of directional selection (Fig. 3-2A).

Faster developmental time was a consequence of higher developmental rate of the Cerrado tadpoles for all treatments (Fig. 3-3, Table 3-1), except for treatment T2. In addition, the Cerrado tadpoles showed a faster growth rate in the T3 treatment (Fig. 3-3, Table 3-1). Tadpoles from the Cerrado and the Amazon grew up to 40.6mm and the tail started being absorbed after they reach this size (Fig. 3-2S). None of morphological traits showed significant differences between habitats (Table 3-1, Fig. 3-2S).

Tadpole survivorship was not significantly different between the Amazon and the Cerrado (Fig. 3-6, Table 3-3S). Although survivorship between the Amazon and the Cerrado in treatment T2 was not different (Fig. 3-6, $\chi^2 = 2.3$, $p = 0.133$), tadpoles from the Cerrado were more resilient surviving longer period than the individuals from Amazon (Fig. 6).

Phenotypic plasticity

Environmental plasticity also contributed to phenotypic variation in *D. minutus*. Comparisons between treatments showed significant differences in developmental time ($\chi^2 = 9.94$, $p = 0.019$) and developmental rate ($\chi^2 = 12.53$, $p < 0.006$). Within the Cerrado comparisons, *D. minutus* developed faster in treatment T3 and T4 than treatment T1 (x = 5 days faster, Fig. 3-5, Table 3-6S). Within the Amazon comparisons, tadpoles under treatments T3 and T4 also developed faster than T1 treatment (T3: x = 4.6 days faster, T4: x = 5.7 days faster, Fig. 3-5B, Table 3-7S). Developmental rate was significantly different between treatments T1 and T3, and between treatments T1 and T4 within the same habitat comparisons (Table 3-8S and Table 3-9S). None of the seven morphological traits or growth rate showed significant differences among treatments (Table 3-2).

Varying temperature conditions resulted in differential mortality rate among treatments (Fig. 3-6, Table 3-4S and Table 3-5S). Treatment T1 and T3 showed the highest survivorship with no significant difference between them. Treatment T2 showed the lowest survival rate (Fig. 3-6). Although tadpoles were able to develop under the last treatment condition, they showed a longer developmental time, with slower developmental rate (Fig. 3-3), and growth rate (Fig. 3-4). None of the tadpoles from treatment T2 was able to complete metamorphosis. Treatment T4 had about 40% of survivorship (Fig. 3-6). Interestingly, although the oscillating temperature treatment T4 had an upper range of 32°C (held constant in T2), tadpoles were able to finish metamorphosis and showed a shorter developmental time than T1 and T2 (Fig. 3-2) with higher developmental rate (Fig. 3-3), and growth rate (Fig. 3-4). This result showed that *D. minutus* tadpoles were able to tolerate this high temperature for short periods of time.

Discussion

Assessing the environmental and genetic effects on phenotypic variation helps us to elucidate the evolutionary processes that led to this variation (Via and Lande 1985; West-Eberhard 1989; Thompson 1991; Scheiner 1993; Price et al. 2003; Pfenning 2010) and to predict how organisms will respond to climate change (Bradshaw 2006; Hoffman and Sgro 2011). The experimental work described here provides evidence that larval developmental differences between habitats has a genetic component and is faster in the Cerrado than in the Amazon. In addition, *D. minutus* accelerates its development under oscillating temperature conditions, also showing a plastic response to different thermal conditions. Genetic differences in development time between biomes suggest directional selection caused by a more seasonal condition in the Cerrado resulting in this differentiation. In addition, the plastic response to temperature variation may have contributed to climatic adaptation under a more unpredictable condition (Newman 1992; Scheiner 1993; Pfennig et al. 2010). Thus, this plastic response can also be considered as an adaptive trait. Under climate change scenario, forests in southern Amazon will be converted into more savanna-like habitat (Salazar et al. 2007; Saatchi et al. 2013). Understanding the sources of phenotypic responses in these two contrasting habitats provides insights about the temporal effects of climate change in this species.

Evolutionary responses of *D. minutus* larval development

A previous study examining divergence of *D. minutus* between the Amazon and the Cerrado found that phenotypic traits co-vary with environmental differences in precipitation and temperature (Oyamaguchi, et al., Ms in prep.). This phenotypic differentiation is suggestive of being a byproduct of divergent selection from these two contrasting habitats (Oyamaguchi, et al., Ms in prep.). However, to support that natural selection plays a dominant role in the divergence process, this differentiation requires a genetic basis (Rundle and Nosil 2005; Butlin et al. 2012; Nosil 2012). This study extends the previous findings showing that this phenotypic variation is a result of gene-environment interaction. Using a common garden experiment, I found that the Cerrado tadpoles developed faster than individuals from the Amazon by an average of four days, showing a genetic basis for this difference between habitats. Thus, I can reject the null hypothesis that the pattern of phenotypic variation found in natural populations is only due to plasticity, suggesting that natural selection may have played a role in shaping this pattern.

Accelerated development has been observed for many species as a consequence of higher temperatures (Angilletta and Dunham 2003; Angilletta et al. 2004; Berven, 1982; Smith-Gill and Berven 1979; Atkinson and Sibly 1997), shorter hydroperiod (Wilbur and Collins 1973; Newman 1988, 1992; Laurila et al. 2002; Merilä et al. 2004), and predation (Werner 1986; Relyea 2002). Shorter larval periods can increase chances of survival in ephemeral habitats (Semlitsch and Wilbur 1998; Newman 1992; Ryan and Winne 2001). A younger age at metamorphosis may be favored when the risk of mortality increases (Márquez-García et al. 2010). Thus, the faster development of tadpoles from the Cerrado is likely to be a product of natural selection from this more seasonal habitat.

For many species, younger age at metamorphosis results in a smaller body size (Newman 1989; Morey et al. 2004; Gomez-Mestre and Buchholz 2006; Laurila et al. 2002; Merilä et al. 2004). As a result of this trade-off, changes in adult morphology, post-metamorphic performance, and immune function have been observed (Blaustein et al. 2010). Experimental results showed that although the tadpoles of *D. minutus* reached metamorphosis at an earlier age, their size was similar to the tadpoles that underwent metamorphosis later. Thus, different development rates did not affect the size at metamorphosis. The results are in accordance with the threshold model proposed by others (Wilbur and Collins 1973; Rowe and Ludwig 2002) that predicts that organisms must reach a minimum size threshold to be able to metamorphose. The same results were found in *Rhinella spinulosa* (Márquez-García et al. 2010), and *Limnodynastes peronii* (Niehaus et al. 2012). Hence, different life history characteristics might result in different developmental patterns in amphibians. Further research on other species from the study region will contribute to understanding the generalities of the observed development pattern in *D. minutus*.

Plastic response to fluctuating temperature

Understanding the effects of fluctuating environment is fundamental to the prediction of phenotypic responses to climate change. Temporary ponds have dramatic fluctuation in daily temperatures. Despite the fact that temperature fluctuations are common in many habitats (Niehaus et al. 2006), most experimental studies are designed with constant temperature (Qualls and Shine 2006; Niehaus et al. 2011). In this study, tadpoles from the Amazon and the Cerrado also show a plastic response to fluctuating thermal conditions. It is possible that fluctuating temperature could be a signal of a drying pond in which individuals that are able to accelerate their development are favored (Newman 1989, 1992). The results suggest that in fact fluctuating temperature stimulates faster development, as has been observed in striped marsh frogs (*Limnodynastes peronii*) (Niehaus et al. 2012). This plastic response may allow individuals to persist under more unpredictable environmental conditions (Newman 1988, 1989; Laurila et al.

2002; Pfennig et al. 2010), such as a more seasonal climate in the Cerrado. However, high mortality in high temperatures (T2 & T4) also indicates a temperature threshold tolerance for *D. minutus* development (32^{o}C), which may restrict their distribution.

Developmental plasticity is common in amphibians (Semlitsch et al. 1990; Stearns et al. 1991; Newman 1988, 1989, 1992; Laurila et al. 2002; Denver et al. 1998) and may be important in the diversification process by allowing populations to persist in novel environments (West-Eberhard 1989; Gomez-Mestre and Buchholz 2006; Pfennig et al. 2010). Considering an adaptive landscape scenario, phenotypic plasticity facilitates peak shift or valley crossing, which promotes population divergence (see review Pfennig et al. 2010). If environmental conditions change, a population will no longer reside at an optimum adaptive peak. Thus, for this population to reach an alternative peak, plastic traits would facilitate crossing maladaptive valleys (Pfennig et al. 2010). If a selective pressure persists for long enough, then genetic changes favoring lineages adapted to new environmental conditions will result in genetic accommodation (West-Eberhard 2005). Thus, this developmental plasticity in *D. minutus* may have contributed to its persistence in a more seasonal habitat in which they were able to tolerate and to adapt to more unpredictable climate conditions.

Effects of climate change on tadpole development

Under future climate-change models, the Southern Amazon is predicted to transition from a rainforest to a savanna like habitat (Salazar et al. 2007; Saatchi et al. 2013). The dry season length in southern Amazon has already increased since 1979 (Fu et al. 2013). In addition, the southern Amazon region has seen precipitation declines by about 32% per decade between 1970 and 1999 (Li et al. 2008). The southern and western regions of the Amazon forest are currently affected by changes in precipitation regimes, evidence of the first consequences of the global climate change (Fu et al. 2013; Saatchi et al. 2013). Persistence of species facing rapid climate change will depend on the ability of local populations to tolerate, migrate or adapt (Sork et al. 2010).

As the forest converts into more savanna-like habitat, populations of *D. minutus* from the Cerrado are likely to expand their distribution and may displace individuals from the southern Amazon region due to their higher survivorship in cases of shorter hydroperiod. *Dendropsophus minutus* from both habitats also showed plastic responses to temperature oscillation indicating certain tolerance to environmental changes in temperature. If temperature continues to increase and seasonality becomes more pronounced in the southern Amazon with more frequent droughts events (Saatchi et al. 2013), then populations will likely decline in areas where the duration of temporary ponds falls below the minimum developmental time (~48 days). In addition, populations in areas where the maximum oscillation temperature is above their thermal tolerance will also suffer higher mortality rates. Further research on the sources of phenotypic variation and thermal tolerance for other amphibian species in this area is fundamental for comprehension of the generalities and capabilities of species to persist under the drastic effects of climate change in the southern Amazon.

Conclusions

In conclusion, phenotypic variation in *D. minutus* larval development across a heterogeneous landscape indicates to be a result of gene-environment interaction. Furthermore, oscillating temperatures triggering faster development responses suggest that phenotypic plasticity might be an adaptive trait as a response to a more seasonal or unpredictable climate.

Since climate change is predicted to convert southern Amazon forest into savanna-like habitat, we can use this experimental work with populations from different climatic conditions varying in space to extrapolate the temporal effects of climate change. Local adaptation from the Cerrado populations and plasticity might contribute to this species adapting to future climate change in the Amazon. Future work transplanting Amazon populations to Cerrado conditions will provide insights about whether Amazon populations will tolerate or adapt to climate change.

Figures

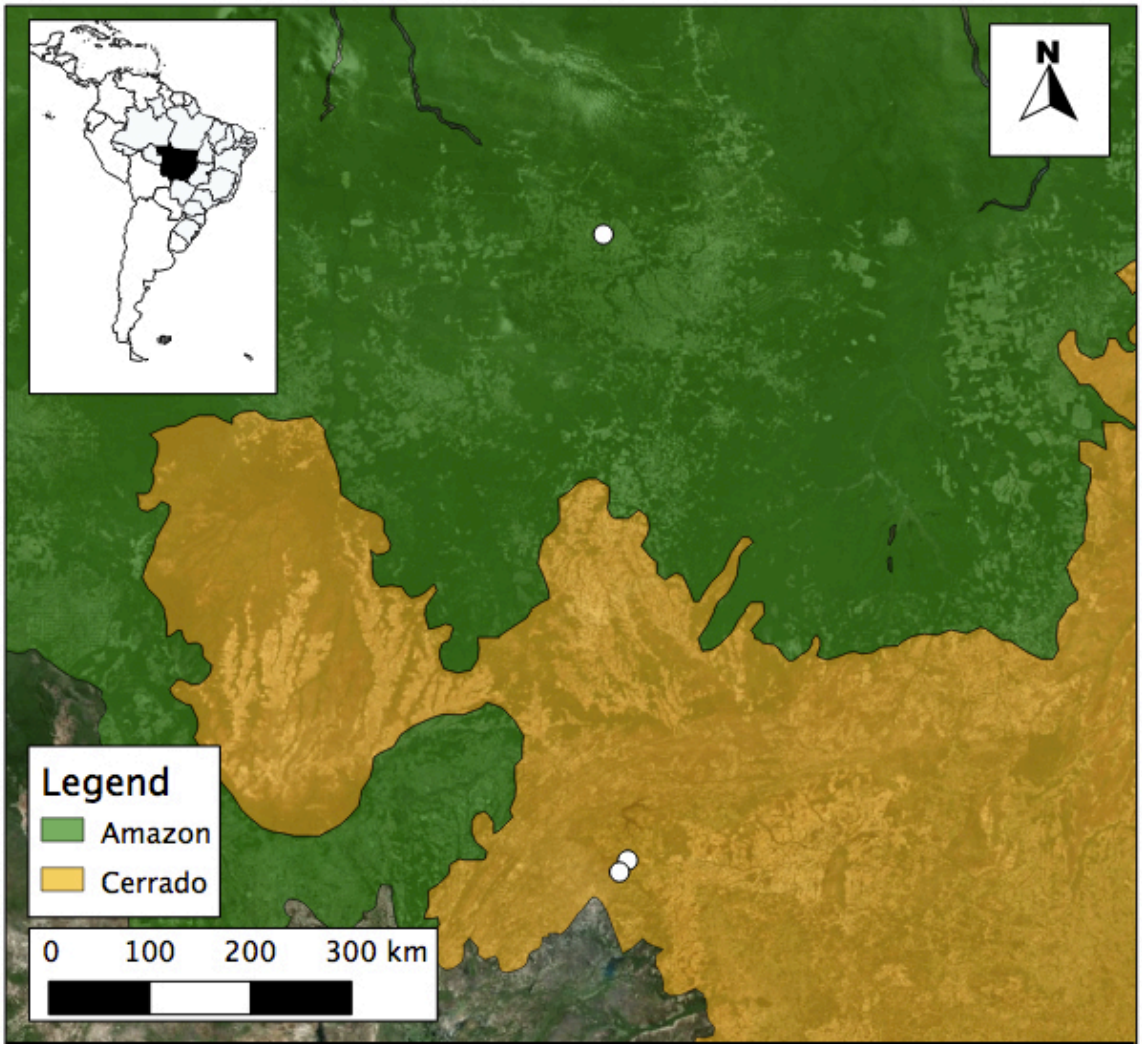

Fig 3-1. Sampling localities of *D. minutus* in the Cerrado (n = 2) and in the Amazon (n = 1).

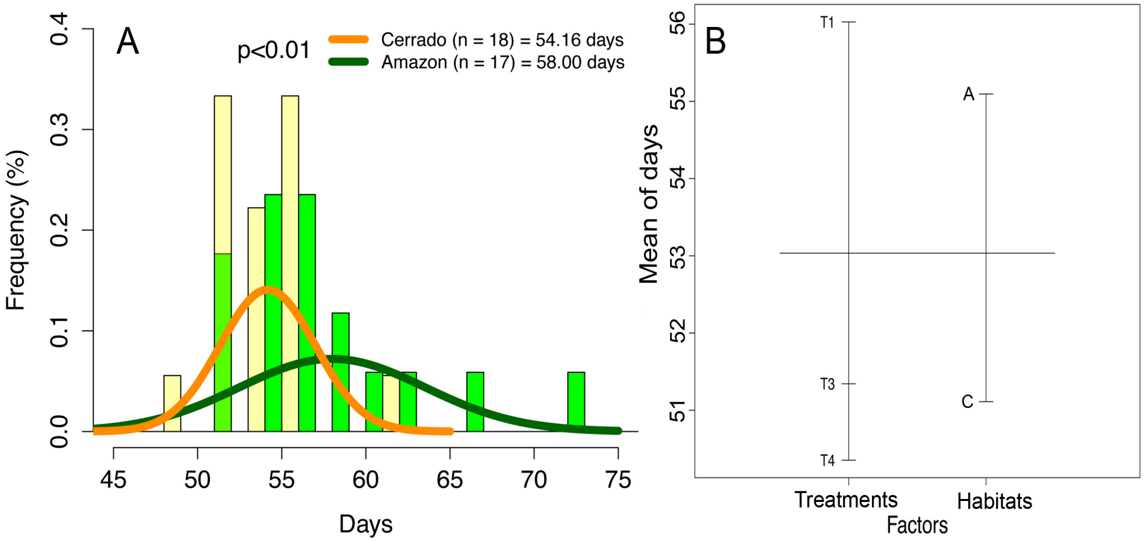

Fig. 3-2. **A**: Development time of *D. minutus* for tadpoles from the Amazon and the Cerrado from a common garden experiment with a constant temperature of 26°C (t = 2.6014, P = 0.0073). **B**: Mean of days for *D. minutus* development time under different treatments. Horizontal bar represents the mean development time for all tadpoles. Vertical lines represent the different development time responses for four temperature treatments (T1: constant 26°C, T2: constant 32°C, T3: fluctuating temperature between 26-29°C, and T4: fluctuating temperature between 26-32°C), and for tadpoles from the Cerrado (C) and Amazon (A).

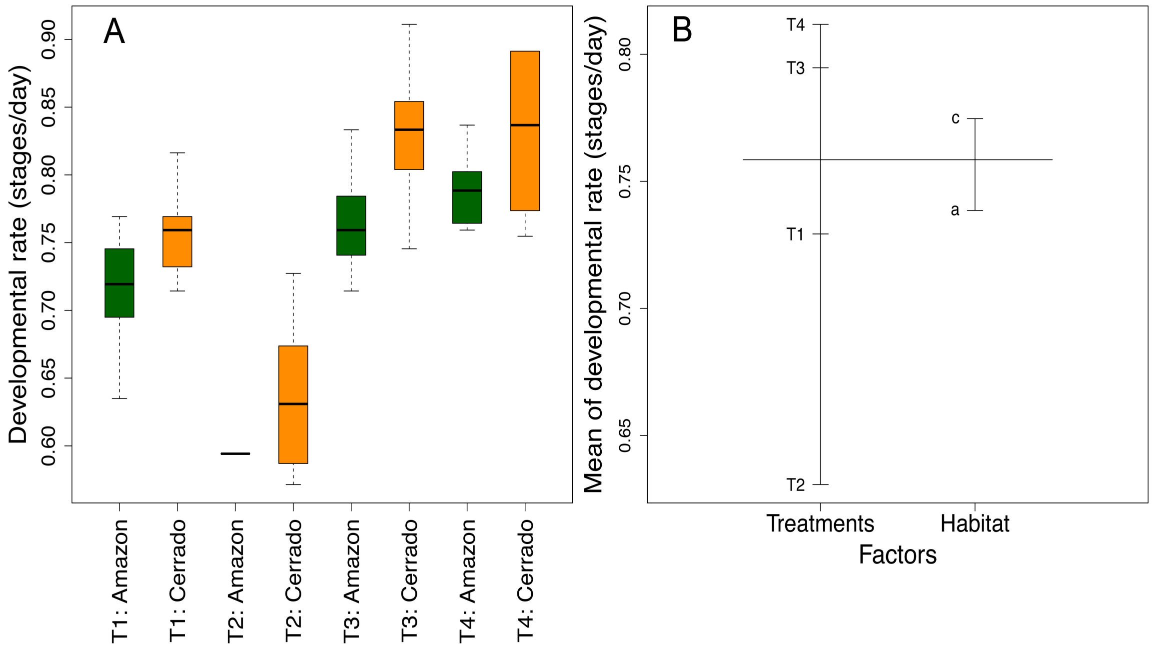

Fig. 3-3. **A**: Boxplots of development rate for *D. minutus* from the Cerrado and the Amazon under four different temperatures treatments (T1: constant 26°C ($t = -2.85$, $P < 0.01$), T2: constant 32°C, T3: fluctuating temperature between 26-29°C ($t = -5.19$, $P < 0.0001$), and T4: fluctuating temperature between 26-32°C ($t = -2.20$, $P < 0.05$). **B**: Mean of development rate for *D. minutus* under different treatments. Horizontal bar represents the mean development rate for all tadpoles. Vertical lines represent the different development rate responses for four temperature treatments (T1, T2, T3, T4), and for tadpoles from the Cerrado (C) and Amazon (A).

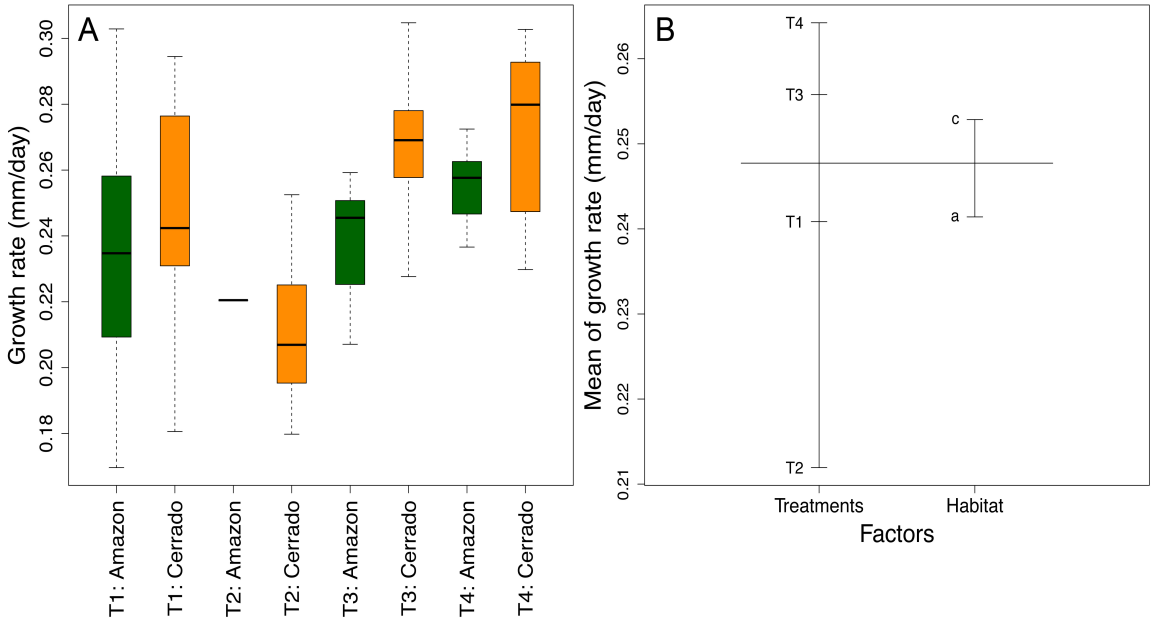

Fig. 3-4. **A**: Boxplots of growth rate for tadpoles of *D. minutus* from the Cerrado and the Amazon under four different temperatures treatments (T1: constant 26°C ($t = -1.14$, $P = 0.26$), T2: constant 32°C, T3: fluctuating temperature between 26-29°C ($t = -3.18$, $P<0.01$), and T4: fluctuating temperature between 26-32°C ($t = -1.76$, $P = 0.09$)). **B**: Mean of growth rate for *D. minutus* under different treatments. Horizontal bar represents the mean development rate for all tadpoles. Vertical lines represent the different growth rate responses for four temperature treatments (T1, T2, T3, T4), and for tadpoles from the Cerrado (C) and Amazon (A).

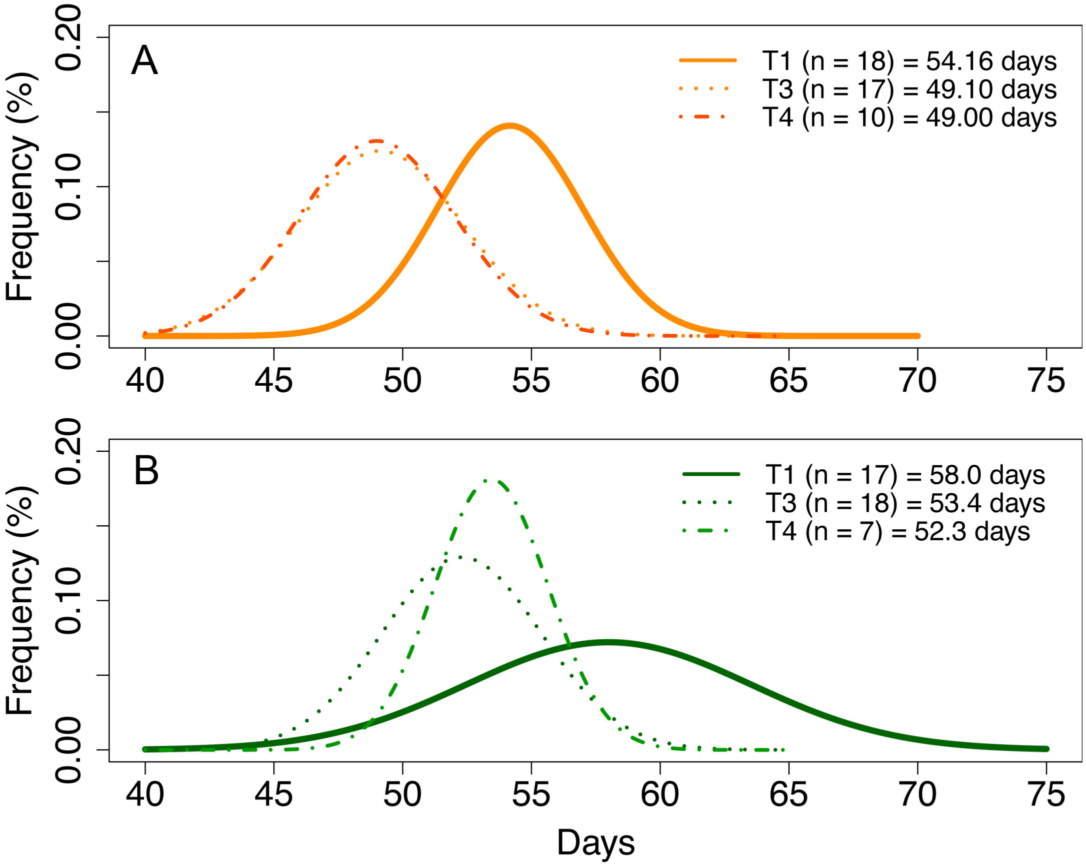

Fig. 3-5. Comparison of *D. minutus* development time for **A**: Cerrado and **B**: Amazon for three temperature treatments (T1: constant 26^{o}C, T3: fluctuating temperature between 26-29^{o}C, and T4: fluctuating temperature between 26-32^{o}C) within same habitat.

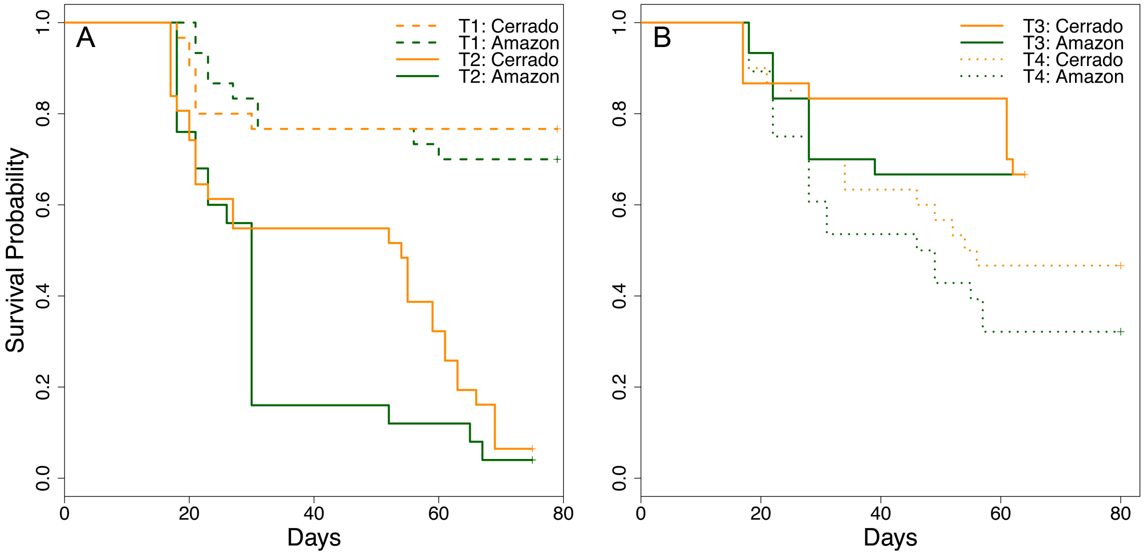

Fig. 3-6. Kaplan-Meier survival probability of *D. minutus* tadpoles from the Cerrado and the Amazon under four temperature treatments (**A:** constant 26^{o}C (T1), constant 32^{o}C (T2); **B:** fluctuating temperature between 26-29^{o}C (T3), and fluctuating temperature between 26-32^{o}C (T4))

Tables

Table 3-1. Results of permutation T-test comparing phenotypic traits of *D. minutus* between the Cerrado and the Amazon within three temperature treatments (T1: constant 26°C, T3: fluctuating temperature between 26-29°C, and T4: fluctuating temperature between 26-32°C). Numbers in bold show significant differences ($p < 0.05$).

Traits	T1		T3		T4	
	t	P	t	P	t	P
Body length	0.3366	0.7396	0.1303	0.9032	0.2721	0.7871
Tail length	0.5644	0.574	-0.5243	0.6036	0.0146	0.9877
Inter-orbital distance	1.2896	0.2084	0.6402	0.5322	0.499	0.6207
Tail muscle width	1.2498	0.215	0.777	0.4447	-0.7457	0.474
Eye diameter	0.1342	0.8913	-0.2974	0.7781	0.3027	0.7638
Maximum tail height	0.965	0.344	0.4914	0.6185	-0.0625	0.9512
Tail muscle height	0.3764	0.7011	0.4613	0.6539	-0.4304	0.6677
Days	2.6014	**0.0073**	4.6649	**0.0001**	2.1714	**0.0494**
Developmental rate	-2.8467	**0.0073**	-5.1927	**0.0001**	-2.1973	**0.0471**
Growth rate	-1.1393	0.2617	-3.1802	**0.0029**	-1.7586	0.0924

Table 3-2. Results from Skilling-Mack test comparing phenotypic traits of *D. minutus* under four temperature treatments (T1: constant 26°C, T2: constant 32°C, T3: fluctuating temperature between 26-29°C, and T4: fluctuating temperature between 26-32°C). Numbers in bold show significant differences ($p < 0.05$).

Traits	χ^2	P
Body length	0.90450	0.824343
Tail length	0.28374	0.963057
Inter-orbital distance	0.05016	0.997056
Tail muscle width	0.38581	0.943158
Eye diameter	0.48235	0.922752
Maximum tail height	0.58779	0.899221
Tail muscle height	1.76818	0.621883
Days	9.93376	**0.019138**
Developmental rate	12.5272	**0.005779**
Growth rate	4.83552	0.184245

Appendix

Supplemental figures

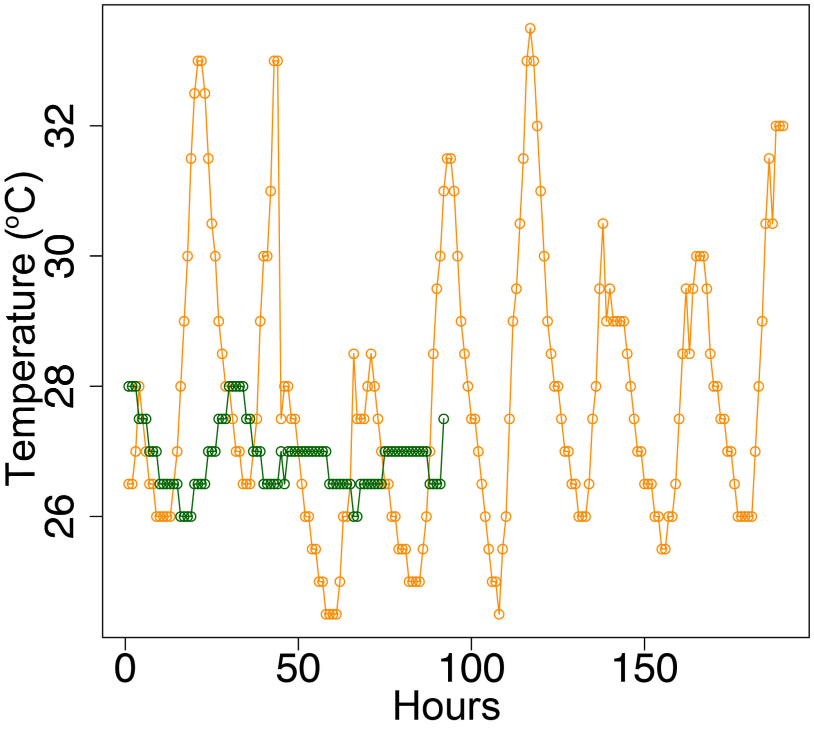

Fig. 3-1S. Hourly registered temperature in the Cerrado pond (yellow line) and in the Amazon pond (green line) showing fluctuating temperature.

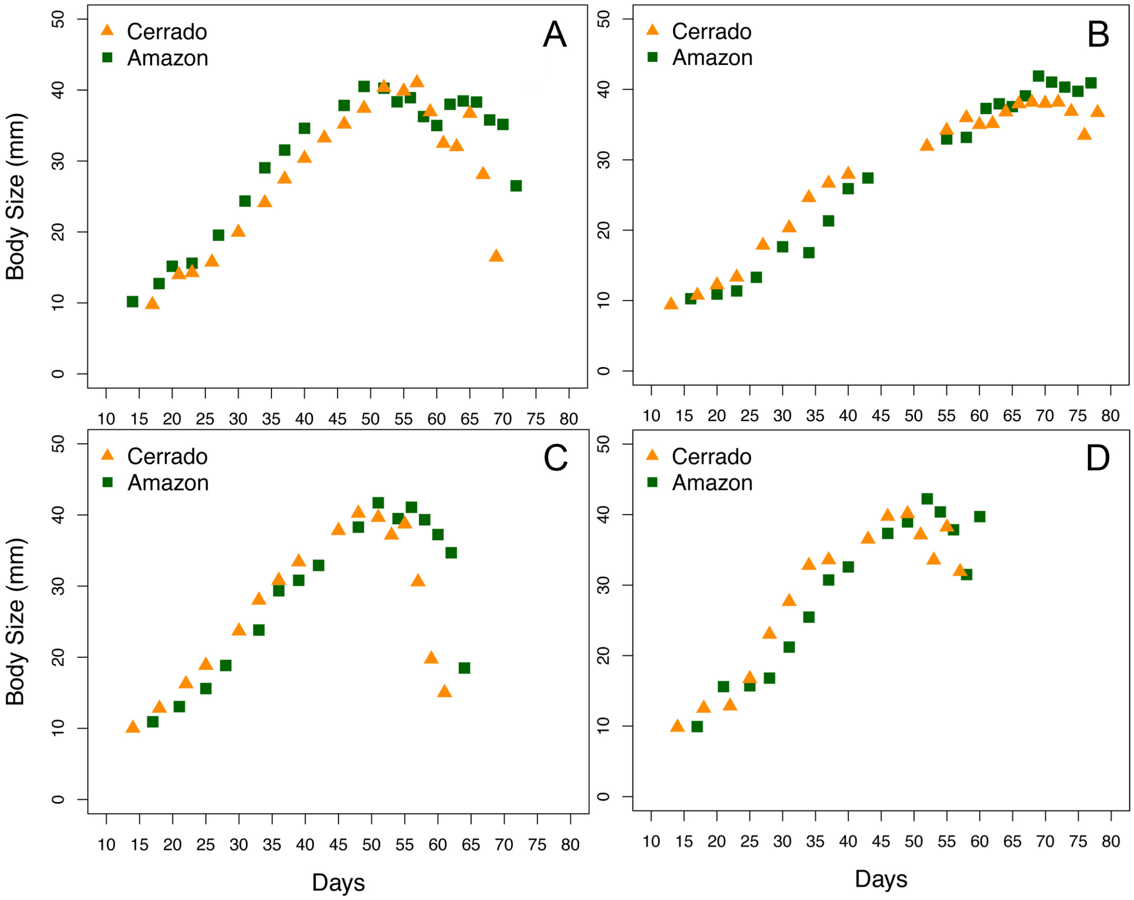

Fig. 3-2S. Average of body size as a function of number of days in four temperature treatments. A: constant 26^{o}C treatment (T1); B: constant 32^{o}C (T2); C: fluctuating temperature between 26-29^{o}C (T3); D: fluctuating temperature between 26-32^{o}C (T4).

Supplemental tables

Table 3-1S. Sampling localities and habitat type for *Dendropsophus minutus* collected in Mato Grosso State, Brazil (2011).

Habitat	Locality	Latitude	Longitude
Amazon	Alta Floresta	9.8858 S	55.9487 W
Cerrado	Chapada dos Guimaraes - Buriti	15.416 S	55.8047 W
Cerrado	Chapada dos Guimaraes - Peba	15.3148 S	55.7316 W

Table 3-2S. Common garden 2 X 4 factorial design. One factor consisted of tadpoles from the Cerrado and the Amazon location, a second factor was temperature with four levels: at constant 26.5°C, and 32°C, fluctuating temperature between 26.5-29°C and between 26.5-32°C. Factor 1 DF = 1, Factor 2 DF = 3, Factor 1 X Factor 2 DF = 5.

	Biomes	
Temperature	**Cerrado**	**Amazon**
26.5°C (Control)	Egg mass 1 (n = 6 tadpoles) Egg mass 2 (n = 6 tadpoles) Egg mass 3 (n = 6 tadpoles) Egg mass 4 (n = 6 tadpoles) Egg mass 5 (n = 6 tadpoles)	Egg mass 6 (n = 15 tadpoles) Egg mass 7 (n = 3 tadpoles) Egg mass 8 (n = 12 tadpoles)
32°C	Egg mass 1 (n = 6 tadpoles) Egg mass 2 (n = 6 tadpoles) Egg mass 3 (n = 6 tadpoles) Egg mass 4 (n = 6 tadpoles) Egg mass 5 (n = 6 tadpoles)	Egg mass 6 (n = 15 tadpoles) Egg mass 7 (n = 3 tadpoles) Egg mass 8 (n = 12 tadpoles)
26.5 °C - 29 °C	Egg mass 1 (n = 6 tadpoles) Egg mass 2 (n = 6 tadpoles) Egg mass 3 (n = 6 tadpoles) Egg mass 4 (n = 6 tadpoles) Egg mass 5 (n = 6 tadpoles)	Egg mass 6 (n = 15 tadpoles) Egg mass 7 (n = 3 tadpoles) Egg mass 8 (n = 12 tadpoles)
26.5 °C - 32 °C	Egg mass 1 (n = 6 tadpoles) Egg mass 2 (n = 6 tadpoles) Egg mass 3 (n = 6 tadpoles) Egg mass 4 (n = 6 tadpoles) Egg mass 5 (n = 6 tadpoles)	Egg mass 6 (n = 15 tadpoles) Egg mass 7 (n = 3 tadpoles) Egg mass 8 (n = 12 tadpoles)

Table 3-3S. Kaplan-Meier survivorship results from comparison between the Amazon and the Cerrado within each treatment.

	χ^2	P-values
T1	0.0000	0.99500
T2	0.6992	0.40300
T3	0.0152	0.90200
T4	0.7700	0.38000

Table 3-4S. Kaplan-Meier survivorship results from comparisons between treatments within *D. minutus* tadpoles from the Amazon. Numbers in bold show significant differences ($p < 0.05$).

Amazon	T1	T2	T3
T2	**23.5200**		
T3	**11.6500**	**8.1110**	
T4	1.8060	**8.3250**	0.4000

Table 3-5S. Kaplan-Meier survivorship results from comparisons between treatments within *D. minutus* tadpoles from the Cerrado. Numbers in bold show significant differences ($p < 0.05$).

Cerrado	T1	T2	T3
T2	**17.8400**		
T3	**15.2900**	**6.7160**	
T4	0.3432	**4.0610**	0.1000

Table 3-6S. Pairwise comparisons of development time between treatments within the Cerrado tadpoles. Numbers in bold show significant differences ($p < 0.05$).

Cerrado	T1	T2	T3
T2	NA		
T3	**0.0091**	NA	
T4	**0.0274**	NA	0.4359

Table 3-7S. Pairwise comparisons of development time between treatments within the Amazon tadpoles. Numbers in bold show significant differences ($p < 0.05$).

Amazon	T1	T2	T3
T2	NA		
T3	**0.0068**	NA	
T4	**0.0459**	NA	0.5148

Table 3-8S. Pairwise comparisons of development rate between treatments within the Cerrado tadpoles. Numbers in bold show significant differences ($p < 0.05$).

Cerrado	T1	T2	T3
T2	**0.0007**		
T3	**0.0068**	0.3901	
T4	**0.0459**	0.5148	0.5148

Table 3-9S. Pairwise comparisons of development rate between treatments within the Amazon tadpoles. Numbers in bold show significant differences ($p < 0.05$).

Amazon	T1	T2	T3
T2	NA		
T3	**0.0001**	NA	
T4	**0.0013**	NA	0.6676

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