

Environmental predictors for annual fish assemblages in subtropical grasslands of South America: the role of landscape and habitat characteristics

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Received: 17 March 2017 / Accepted: 20 March 2018
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Abstract Annual fishes (AF) are ecologically unique assemblage with major conservation concern. Several AF species are found in temporary waters of South American subtropical grasslands, an ecoregion largely neglected by conservation policies. We applied a broad-scale approach to understand distribution pattern of the AF and tested the effects of environmental predictors on occurrence, abundance and assemblage structure of this diverse Neotropical group of threatened endemic aquatic

fauna. We recorded 23 AF species. AF assemblage was strongly spatially structured and many individual species formed discrete units. AF presence was negatively associated with altitude at the landscape scale, and negatively associated with maximum pool depth and presence of predatory fish at the local scale. We found negative association between AF abundance and water depth. Assemblage composition was spatially structured, differed among hydrographic regions and was influenced by altitude, temperature and monthly precipitation. AF inhabits highly vulnerable habitats currently located in livestock and agricultural landscapes. Habitat maintenance through wildlife-friendly practices should be considered as an alternative to promote AF conservation.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10641-018-0751-1>) contains supplementary material, which is available to authorized users.

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Keywords *Austrolebias* · *Cynopoecilus* · Killifish · Temporary waters · Pampas

Introduction

An important goal of ecology is to understand species distribution patterns and major environmental and spatial factors that shape biologic populations and communities (Anderson and Millar 2004; Peres-Neto and Legendre 2010). This knowledge is essential for basic research and has important applied implications to develop biomonitoring, predictive models and conservation programs (Soinin 2015; Vasconcelos et al. 2015).

AF are ecologically unique fishes; require special habitat conditions and feature specialised life cycle (Berois et al. 2012), surviving dry phase of their habitat as

diapausing, desiccation-tolerant embryos (Wourms 1972). They are untypical small-sized and short-lived teleost fishes (Blažek et al. 2013; Berois et al. 2014) and live exclusively in seasonal intermittent pools in Africa (family Nothobranchiidae) and the Neotropics (family Rivulidae) (Furness et al. 2015). AF are unable to perform long-distance dispersal, and their species distribution is often restricted (Watters 2009; Costa 2010; Reichard 2015; Bartáková et al. 2015). A combination of their low dispersal ability and highly specialised life cycle on one hand, and intensive natural habitat loss on the other hand, has led several AF species to be considered threatened with extinction (ICMBio 2013; IUCN 2015).

AF species generally inhabit small and discrete pools, individually isolated or spatially clustered in a set of permanent and semi-permanent water bodies (Costa 2010; Reichard 2015; Volcan et al. 2015), with wide-ranging environmental conditions (Errea and Danulat 2001; Reichard et al. 2014; Polačik and Podrabsky 2015). AF populations comprise generally a single age cohort (Polačik et al. 2011; Lanés et al. 2014a, 2016) and several AF species often coexist in the same pool (Polačik and Reichard 2010; Canavero et al. 2014; Loureiro et al. 2015). While landscape and habitat characteristics on fish community structure are well documented in streams, lakes and large wetlands (Matthews 1998; Lowe-McConnel 1999), the effects of environmental factors on AF assemblages are still poorly understood (Reichard et al. 2009; Lanés et al. 2014a; Polačik and Podrabsky 2015).

Fish community structure is often tightly linked to the environmental variables (Morán-López et al. 2006). For example, water depth influences species composition by filtering species based in their association with littoral or deeper habitats (Fernandes et al. 2010; Teresa et al. 2016). In temporary aquatic habitats, the effects of climatic conditions, geomorphology, altitude, relief and distance from permanent waters are recognized as drivers influencing communities at a landscape scale (Stenert and Maltchik 2007; Maltchik et al. 2014) and water depth, macrophyte coverage, habitat heterogeneity and presence of predators at a local scale (Reichard et al. 2009; Pazin et al. 2006; Escalera-Vazquez and Zambrano 2010; Reichard et al. 2017). On the other hand, unlike in permanent waters, the relationship between species richness and area is not supported in intermittent wetlands (Batzer et al. 2004; Reichard et al. 2017) and it is suggested that assemblages are not clearly related to abiotic conditions (Schwartz and

Jenkins 2000). In addition to their seasonal desiccation, temporary pools are highly space-limited when compared to larger rivers and lakes, hindering the access of not-specialised fishes (Williams 2006). Yet, they often comprise abundant and relatively diversified assemblage of non-annual teleost fishes co-occurring with AF, including their potential predators (Vaz-Ferreira et al. 1966; Lanés et al. 2014a, b; Maltchik et al. 2014; Lanés et al. 2015, 2016).

Basic data on life history and ecology of wild populations of AF are very scarce, frequently based on circumstantial evidence. At the same time, precise information are highly demanded by researchers, environmental conservation agencies, and are essential to apply environmental licensing and conservation strategies and management. Subtropical grasslands of South America are located in developing countries that play a key role in international crop production (Vega et al. 2009) and contain temporary pools that harbour a high diversity of endangered AF species (Baigún et al. 2012; FZBRS 2014; Loureiro et al. 2013). Environmental conservation in this ecoregion is considered as generally neglected, not being adequately protected under current conservation efforts (Overbeck et al. 2007).

In this study, we applied a broad-scale approach to analyse distribution pattern and tested the effects of environmental predictors on assemblage structure (occurrence, abundance and composition) of endemic AF in subtropical grasslands to answer the following questions: (i) Which are the environmental variables that better explaining the variation in the occurrence, abundance and composition in this assemblage? (ii) Is the distribution of individual AF species spatially autocorrelated? (iii) Is the variation in the assemblage composition correlated with hydrographic regions?

Material and methods

Study area

Rio Grande do Sul (RS) is the southernmost state of Brazil and harbours 75% of grassland area of South Brazilian grassland region (*Campos Sulinos* in Portuguese) (Overbeck et al. 2007). These grasslands, where herb, shrub and treelet species co-occur within the grass matrix, are considered relicts from cooler and drier climatic conditions during glacial and post-glacial periods, maintained until today through the action of

grazing and fire (Behling and Pillar 2007). Grassland vegetation in southern Brazil was separated into two biomes according IBGE (2004) classification: Pampa biome, which occur exclusively in the southern half of Rio Grande do Sul state, and the Atlantic forest biome. The Pampa biome of Brazil, which constitutes our study area, belongs to the biogeographical domain of Pampean province (sensu Cabrera and Willink 1980) and is also referred in the literature as Río de la Plata grasslands (Bilenca and Miñarro 2004) and Uruguayan savanna ecoregion (sensu Olson et al. 2001) which extends into Argentina and Uruguay.

The climate is humid subtropical (Peel et al. 2007) with warm summers and cool winters without a distinct dry season. There is 1200–1600 mm of annual precipitation and mean annual temperature vary between 13 and 17 °C. Although this climate classification indicates a well-distributed rainfall throughout the year, there are frequent shortages of soil moisture depending on the drought, coupled with higher evapotranspiration during late spring and summer. During winter and spring, slightly higher precipitations, mainly between June to October, result in flooding of lower areas and depressions (Klein 1998). Presence of water in temporary pools in South America grasslands is mediated by the balance between precipitation, temperature and evaporation (Lanés et al. 2014a, b; Loureiro et al. 2015). Our study area covers three hydrographic regions (sensu SEMA 1994): Littoral, Guaíba and Uruguay (Fig. 1). Land use in the region is represented by cattle and sheep grazing, agriculture, urban and infrastructure developments, and afforestation with exotic trees (*Acacia*, *Eucalyptus*, *Pinus*).

Samplings

AF are considered rare and/or difficult to record. Because their peculiar life cycle, they are normally difficult to find during most of the year and are usually recorded only during a short period of time in temporary aquatic environments. Samplings were intensively concentrated between June and October 2010. This is the most suitable period to AF sampling in the study area, because temporary pools contain water due to low evapotranspiration rate.

Natural pools in studied region have been altered or destroyed, mainly by agriculture. They have been altered and barred to provide extra water supply to farms and livestock watering, or deepened to supply non-annual

fish (small fish farms). As our focus was directed exclusively to potential habitats of AF, we directed our effort on natural areas, and used four criteria to select our sample units: (1) distance between pools, (2) absence of dams, dykes or other structures modifying the natural hydroperiod, (3) size of the pools, and (4) distance from the nearest road. Thus, we selected distant sampling units at least 15 km from each other; without the presence of modifying structures of natural hydroperiod; with size less than 2 ha and distant about 200 m from the nearest road.

Applying the criterion mentioned above, we selected 82 sites. Fish samples were taken with a dip-net (D-shaped hand net, 60 cm × 40 cm, 2 mm mesh size). Sampling effort was standardised regardless of pool size, and twenty-five hauls (corresponding each to approx. 0.6 m², totalling 15 m² sampled per site) were carried out per sampling site, sweeping the net parallel to the pool bottom, exploring all available habitats. The chosen sampler mesh-size was fine enough to capture all AF sizes present at a sampling site. We adopted this simple sample design, without considering the temporal dynamics, due to the wide range of our study (approximately 170,000 km²) and particular characteristics of our model (short life in time-constrained habitat). Collected fish were euthanized with clove oil, fixed in situ with 10% formalin, and later transferred into 70% ethanol. AF specimens were identified and sexed based on Costa (2002, 2006). The material was collected under IBAMA/ICMBio authorization (process number 18334–1 and 18,334–2).

Local habitat variables

Pool size and boundaries were measured in situ based on (1) visual observations of the watermarks and (2) vegetation indicators (e.g., plants with morphological, physiological or reproductive adaptations to prolonged saturation/inundation and the proportion between aquatic and terrestrial species in the plant community). The maximum pool water depth (cm) was obtained using a ruler. Aquatic vegetation cover was visually classified into two classes: above and below 50% of area covered by macrophytes. Each different type of vegetation (emergent, floating and submerged) was classified into five classes: 0 = absence of the specific vegetation type; 1 = <5%; 2 = 5–25%; 3 = 25–75%; 4 = > 75%. Habitat diversity in each wetland was quantified by counting the number of distinct habitats of dominant vegetation. The

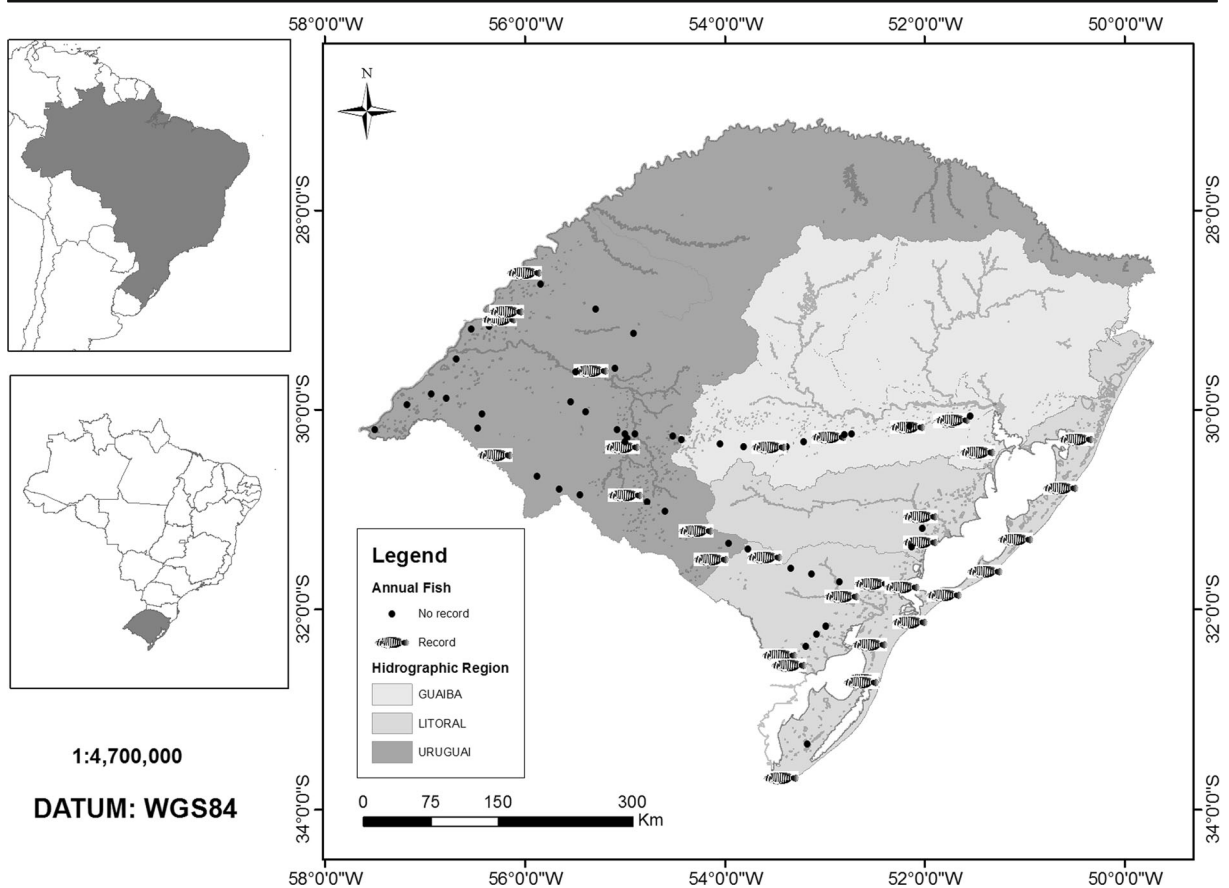


Fig. 1 Sampling sites and annual fish records in southern Brazil grasslands of Pampa Biome distributed across hydrographic regions

dominant vegetation in each site was classified into five habitat types: woody vegetation (tree/shrub); floating-leaf stands; emergent vegetation; submerged vegetation and open water. The habitat diversity was the cumulative number of vegetation and open water habitats in the wetland systems. The minimum size to consider a habitat was 10% of the total area of each studied site. The presence of piscivorous and potential fish predators (*Crenicichla* spp., *Cichlasoma* spp., *Hoplias* spp., *Rhamdia* spp., *Synbranchus* spp.) of AF was registered.

Landscape variables

The sampling units were georeferenced and organized in the geographic information system (GIS). We created a digital terrain model using ArcGis 10.1 software and for each site, we generated bioclimatic and topographic variables - here named landscape variables, obtained from interpolations through WorldClim (Hijmans et al. 2005) and TopoData (Valeriano and Rossetti 2011) databases, respectively. We selected altitude (meters above

sea level), slope ($^{\circ}$), monthly and annual precipitation (mm), mean temperature ($^{\circ}$ C) and distance from the nearest river (m). These variables were selected because they are ecologically meaningful to explain AF occurrence and assemblage composition. We also obtained the hydrographic region identity (sensu SEMA 2004) using digital cartographic database provided by Weber and Hasenack (2007), and classified the land use and soil type of each site according to Hasenack and Cordeiro (2006) and Streck et al. (2008) digital cartographic databases, respectively.

Data analyses

Linear models

The effect of environmental predictors at local (pool size, depth, aquatic vegetation cover, habitat diversity and predatory non-annual fish presence) and landscape scale (altitude, slope, monthly and annual precipitation, mean temperature and distance from the nearest river)

on AF were tested using Generalized Linear Mixed Models (GLMM), with separate intercepts for each Hydrographic Region (random effect).

Before applying statistical models a data exploration was undertaken as recommended by Zuur et al. (2009, 2010). Continuous covariates were standardised (mean 0, s.d. = 1) to enhance numerical optimisation of the model. Collinearity between explanatory variables was tested by Variance Inflation factors and variables with GVIF >3 were eliminated. This prompted exclusion of habitat diversity and vegetation cover from some models. AF presence (*Austrolebias* and *Cynopoecilus* species combined) was modelled using binomial distribution (presence/absence) with log-link function, with a full set of predictor variables initially considered (local and landscape scale). The effect of habitat on AF abundance was studied on a subset of pools with fish presence ($n = 34$) using local habitat variables only. Landscape character is not predicted to directly affect fish abundance at a fine scale (as contrasted to fish presence). Poisson distribution was initially used, but negative binomial models were used for the final analysis due to major overdispersion of residuals (theta parameter >20). The assumptions of the models were inspected by comparing residuals against fitted values. All linear models analyses were performed using R statistical environment version 3.2.1 (R Development Core Team 2015) in lme4 package (Bates et al. 2015). We quantified model goodness-of-fit (R^2_{GLMM}) for the fixed part of a model (pseudo R^2_{GLMM} marginal) and for the complete model including its random effects (pseudo R^2_{GLMM} conditional; Nakagawa and Schielzeth 2013). These parameters were extracted using the “MuMIn” package (Barton 2015).

Multivariate analysis

Mantel tests were used to analyse the correlation of (i) AF dissimilarity with geographical distance, (ii) AF dissimilarity with environmental variables, and (iii) environmental variables with geographical distance. Three distance matrices were constructed: A) AF matrix based on species occurrence (dependent variable); B) environmental variables matrix (predictor variable); and C) geographic distance matrix based on latitude and longitude coordinates (predictor variable). The AF matrix was constructed using Jaccard distance. The environmental matrix was constructed using Gower distance and geographic matrix using Euclidean distance. Simple Mantel

tests were calculated using Pearson correlation. We performed these tests for all hydrographic regions combined, and separately for within each hydrographic region. The significance of correlations was tested by 999 permutations.

We used a variation partitioning routine (varpart; Peres-Neto et al. 2006) to assess the variation in AF assemblage composition and structure in relation to explanatory matrices. Thus, one response matrix (species composition of sites) and four explanatory matrices (X1 - local variables; X2 - landscape variables; X3 - hydrographic region; X4 - spatial factors) were built. We applied Hellinger transformation on species composition matrix (presence-absence data). We generated the spatial variables from the geographical coordinates from each site using Moran’s Eigenvector Maps (MEM; Borcard et al. 2011). This method compares geographical co-ordinates pair-to-pair using Euclidean distance, and capture spatial trends at different scale ranges. To identify the main contribution of matrix variables we used redundancy analysis (RDA function), followed by an analysis of variance (ANOVA with 999 permutations), as suggested by Oksanen et al. (2015). To assess the relative explanation of each explanatory matrices we perform partial redundancy analysis (pRDA).

In order to summarize the number of correlated variables and sort the sample units along environmental gradients, we used principal component analysis (PCA) to the set of environmental variables. We perform two PCA ordinations, one with all environmental variables and another with local habitat variables only. PCA axes with eigenvalue greater than 1 were accepted as a significant representation criterion of variance (McCune and Grace 2002). Correlations between variables and principal components were considered significant when presented values greater than or equal to 0.75 (Hair et al. 2005). All environmental variables were transformed to z-score standardization before PCA analysis.

Compositional changes in AF assembly among sites were ordinated using non-metric multidimensional scaling (NMDS). The analysis was performed with presence-absence data using Binomial dissimilarity index (Anderson and Millar 2004) and two axes. NMDS is generally used to graphically represent dissimilarity in species composition in multiple dimensions. However, NMDS is unconstrained by environmental variables and sites ordination is driven only by species composition. In order to identify the significant environmental predictors (at local and landscape scale) for AF composition, the

principal components generated by PCA and the qualitative variables (region, vegetation cover and predators) were correlated to NMDS axes a posteriori (McCune and Grace 2002) using the *envfit* function (Oksanen et al. 2009). Only species occurring in more than one site were included. We also used another NMDS to ordinate the multivariate pattern in AF abundances among sites and correlated principal components only with local habitat variables to NMDS axes using *envfit*. A subset of populations with at least three fish recorded and Binomial distance matrix were used.

Permutation Multivariate Analysis of Variance (PERMANOVA) is a useful tool for analysis and partitioning of sums of squares in a multivariate data set using distance matrix (metric or semi metric) and permutations to test hypothesis (Anderson 2001). One-way PERMANOVA with post-hoc pairwise comparison and Bonferroni correction was used to compare differences in all environmental variables among hydrographic regions studied. We also used PERMANOVA to compare differences in environmental variables among sites with presence and absence of AF (in this case without pairwise comparison). In both approaches the differences in environmental variables was assessed considering the full data set ($n = 82$) using Euclidian distance. Finally, we compared the variation in AF species composition among hydrographic regions, considering a subset of pools with AF presence ($n = 34$) and a presence-absence matrix using Jaccard distance. These tests were performed using 9999 permutations.

Distance comparisons in PERMANOVA can confound differences in multivariate dispersion with difference among centroids. Permutational analysis of multivariate dispersion (PERMDISP) is useful in determining whether the groups indicated by PERMANOVA are influenced by differences in the dispersions of groups (Anderson 2006). This procedure is a multivariate analogue to Levene's test for homogeneity of variance in ANOVA, and is informative about differences in beta-diversity (species turnover) among groups. We used PERMDISP with Tukey post-hoc pairwise comparison to determine whether the dispersions in assemblage composition of each hydrographic region around their group centroid were significantly different from one another. The analysis was performed with presence-absence data using Jaccard measure and 999 permutations.

All multivariate analysis, except PERMANOVAs, which were ran in PAST statistic software (Hammer

et al. 2001), were performed in R 3.2.1 environment (R Development Core Team 2015) using *vegan* (Oksanen et al. 2015) and *adespatial* (Dray et al. 2017) packages. The significant level considered was $P \leq 0.05$.

Results

Overview

We have recorded 23 AF species (18 *Austrolebias* and five *Cynopoecilus*) distributed across 34 (41%) pools (Online Resource 1). Most AF records occurred in Littoral hydrographic region (56%). Uruguay hydrographic region hosted 26% and Guaiba hydrographic region represented 18% of records (Fig. 1). All *Austrolebias* phylogenetic clades were recorded in our study area, except for subgenus *Acrolebias*, which is restricted to highlands of Atlantic Forest biome. Clades belonging to the subgenera *Austrolebias* (*Austrolebias adloffi* and *Austrolebias bellottii* species complexes) and *Argolebias* (*Austrolebias alexandrii* species group) reached the highest species richness. Particular species were endemic to small regions and most species have been recorded from a single site (12 species, 35.2% of pools with AF occurrence). *Cynopoecilus melanotaenia* occurred at five sites. *Austrolebias periodicus*, *Austrolebias minuano* and *Cynopoecilus fulgens* occurred at four sites (Online Resource 1). A total of three and four species co-occurred at three and two sites, respectively (Online Resource 1). Syntopic populations of AF occurred in eight sites (9.7%), always in the Littoral hydrographic region, involving species from different phylogenetic lineages. Among these, five sites presented co-occurrence of two species; two sites with three species, and one site with four species (Online Resource 1). The most frequent co-occurrence (50%) was across one species belonging *Austrolebias adloffi* complex and another species of *Cynopoecilus* genus. Within *Austrolebias*, co-occurrence was registered always with species from different phylogenetic clades. Different *Cynopoecilus* species never occurred in syntopy.

Presence of potential fish predators on AF individuals was recorded in 23% of sampled pools (Online Resource 1). The most frequent fish predators found were *Hoplias* aff. *malabaricus* (nine cases) and *Cichlasoma dimerus* (seven cases); in two occasions they were found together. The fish predators *Crenicichla*

Table 1 Results of Generalized Linear Mixed Models (GLMM) calculated for environmental predictors of annual fish occurrence (binomial distribution with a random effect “Hydrographic

Region” at landscape and local habitat scales. Significant relationships are highlighted in bold

	Estimate	S.E.	z value	P
<i>Landscape Scale</i> (Intercept)	2.76	2.37	1.16	0.245
Monthly precipitation (mm)	-0.73	0.45	-1.61	0.106
Temperature (°C)	-0.18	0.35	-0.53	0.599
Altitude (m)	-1.08	0.44	-2.46	0.013
Slope (°)	-0.24	0.43	-0.558	0.576
<i>Local Habitat Scale</i> Area (m ²)	0.041	0.37	0.11	0.913
Depth (cm)	-1.24	0.57	-2.19	0.028
Emergent	0.03	0.40	0.08	0.936
Submerged	-0.54	0.32	-1.67	0.094
Floating	-0.27	0.37	-0.72	0.467
Habitat diversity	0.38	0.84	0.46	0.648
Predators	-2.85	0.99	-2.87	0.004
Vegetation cover	-1.99	1.05	-1.90	0.056

lepidota, *Rhamdia* aff. *Quelen* and *Synbranchus* aff. *Marmoratus* were captured from a single site.

AF habitats belonged to nine distinct soil types. Among these, most frequent soil types related with AF presence were represented by Planossoils (35.3%), Argissoils (23.5%), Plintossoils (11.7%), Gleissoils and Neossoils, both with 8.88% (Online Resource 2). The soils most often related to AF presence showed sedimentary characteristics and were derived from alluvial, lacustrine and arenitic materials, typically of Quaternary origin

Table 2 Results of Generalized Linear Mixed Model (negative binomial distribution with a random effect “Hydrographic Region”) calculated for environmental predictors of annual fish abundance at local habitat scale. Significant relationships are highlighted in bold

Variables	Estimate	S.E.	z	P-value
(Intercept)	4.67	1.27	3.67	0.0002
Area	0.24	0.17	1.37	0.1718
Depth	-0.40	0.16	-2.47	0.0134
Distance nearest river	-0.04	0.20	-0.21	0.8336
Emergent	-0.26	0.16	-1.56	0.1184
Submerged	-0.16	0.17	-0.92	0.3589
Floating	0.10	0.13	0.77	0.4415
Habitat diversity	-0.37	0.30	-1.23	0.2175
Predator	-0.38	0.78	-0.49	0.6254
Vegetation cover	0.12	0.54	0.23	0.8193

and are generally imperfectly drained. Agriculture and cattle raising were the most common land use and comprised 76.4% of temporary pools with AF records (Online Resource 3).

Predictors of annual fish presence and abundance

Presence of AF was negatively associated with altitude at the landscape scale and negatively associated with maximum pool depth and presence of predatory fish at the local scale (GLMM with binomial distribution and logit link, Table 1, pseudoR²_{GLMM} = 0.661). There was a negative association between AF abundance and water depth (GLMM with negative binomial distribution and log link; Table 2).

Annual fish assembly composition

For the full dataset (all hydrographic regions combined), there was no spatial autocorrelation in environmental characteristics (Mantel test, $r = 0.119$, $P = 0.105$), but significant autocorrelation in AF assemblage composition ($r = 0.356$, $P = 0.001$). The sites that were similar environmentally also possessed similar AF assembly composition ($r = 0.163$, $P = 0.001$). When we analysed each hydrographic region separately, Uruguay presents autocorrelation in environmental characteristics ($r = 0.527$, $P = 0.004$), Littoral presents results marginally significant ($r =$

Table 3 Principal Component Analysis (PCA) results with eigenvalues and percentage of variance explained by each axis and eigenvectors of each associated variable

a) Landscape and local variables related to annual fish composition (occurrence)													
Axis	Eigenvalue	Percentage	Slope	Prec Month	Temp	Alt	Area	Depth	Habdiv	Submer	Emerg	Floating	Dist
Axis 1	2.867	26.060	0.677	1.048	0.984	0.971	-0.223	-0.451	0.035	0.234	0.353	-0.426	-0.483
Axis 2	2.0424	18.570	0.160	0.084	-0.221	-0.034	-0.548	0.151	0.970	-0.316	0.896	0.885	-0.206
Axis 3	1.447	13.160	0.746	0.187	-0.047	-0.454	-0.005	-0.117	0.297	-0.222	0.136	-0.478	0.991
Axis 4	1.252	11.390	-0.184	0.095	0.063	0.113	-0.850	0.887	-0.149	0.383	-0.002	-0.266	0.278
Axis 5	1.039	9.446	0.132	0.237	-0.0629	0.356	-0.023	0.380	-0.0701	-0.966	-0.511	0.118	-0.007
b) Local variables related to annual fish composition (abundance)													
Axis	Eigenvalue	Percentage	Area	Depth	Habdiv	Submer	Emerg	Floating	Pred	Dist			
Axis 1	2.017	25.22	0.751	0.164	1.059	-0.404	0.943	0.900	-0.5193	-0.241			
Axis 2	1.398	17.49	-0.777	-0.276	0.164	0.172	0.295	-0.028	-0.975	0.965			
Axis 3	1.175	14.7	0.156	1.098	0.380	-0.272	-0.434	-0.122	0.207	0.762			
Axis 4	1.125	14.07	0.234	0.467	0.121	1.092	0.558	-0.598	-0.068	-0.150			

Significant relationships are highlighted in bold. Slope: standardize terrain slope (°); Prec month: monthly precipitation (mm); Temp: mean temperature (°C); Alt: altitude (meters above sea level); Area: pool size in m²; Depth: pool depth (cm); Habdiv: habitat diversity; Submer: submerged vegetation cover (%; ordinary variable); Emer: emergent Vegetation cover (in %; ordinary variable); Floating: floating vegetation cover; Pred: presence or absence of potential fish predator; Dist: Distance nearest river (m)

0.188; $P = 0.067$) and there was no spatial autocorrelation in Guaíba ($r = 0.576$, $P = 0.208$).

Variation partitioning analysis has shown that when combined (shared variation), all the exploratory matrices have an effect and accounted for 11.5% of total variation in assemblage composition. However only space and hydrographic region components presents pure effects and significance. Pure spatial predictors explained 10.5% of total variation ($P = 0.001$), while pure hydrographic region accounted for a small, but statistically significant fraction of 0.43% ($P = 0.001$) (Online Resource 4).

The environmental variables related to temporary pools were reduced by PCA (Table 3). AF assemblage dissimilarity (based on presence-absence data) among sites was represented by two axes of NMDS ordination (stress = 0.0321). According to *envfit* analysis, the PC1 vector that represented monthly precipitation, temperature and altitudinal increase ($r^2 = 0.610$, $P = 0.001$) (Table 4; Fig. 2) was the strongest driver of the differences in AF composition, followed by the factor representing hydrographic regions ($r^2 = 0.30$, $P = 0.003$) (Fig. 2). Temporary pools located in Uruguay and Guaíba regions coincided with higher monthly precipitation, temperature and altitude (Fig. 2; Online Resource 5; Online Resource 6). The presence of *Cynopoecilus nigrovittatus* and *Austrolebias periodicus* were more associated with Uruguay and Guaíba hydrographic regions,

respectively, as well as with an increase in monthly precipitation, temperature and altitude. In contrast, *Austrolebias wolterstorffi*, *Cynopoecilus melanotaenia* and *Austrolebias minuano* were more associated with Littoral hydrographic region. When AF abundance data was used, no environmental variable was related to variation in species composition among sites ($P > 0.05$; Online Resource 7).

Although there were differences between multivariate dispersion (PERMDISP found differences in AF assemblage composition among hydrographic regions - $F_{2,3} = 5.899$, $P = 0.002$), PERMANOVA based on the presence-absence data showed highly significant variability in AF assemblage composition among hydrographic regions (*Pseudo*- $F_{2,33} = 3.12$, $P = 0.0001$). Pairwise comparisons with Bonferroni correction demonstrated significant multivariate difference among all three compared hydrographic regions ($P = 0.003$ to $P = 0.006$). There was significant difference in the values of environmental variables among hydrographic regions (*Pseudo*- $F_{2,81} = 4.38$, $P = 0.0001$), and among sites with presence and absence of AF (*Pseudo*- $F_{1,81} = 1.985$, $P < 0.05$). The distance to the centroid across hydrographic regions, was 0.54 in Guaíba, 0.56 in Uruguay and 0.65 in Littoral, and PERMDISP pairwise comparisons shown significant differences among hydrographic regions ($P = 0.002$ to $P = 0.007$), except between Guaíba and Uruguay ($P = 0.68$).

Table 4 Correlation between principal components, hydrographic region, vegetation cover and predators and the axis 1 and 2 of NMDS based on annual fish composition (with occurrence data). Significant correlations ($P < 0.05$) are highlighted in bold

Variables	NMDS 1	NMDS 2	r^2	P
PC1	-0.955	-0.296	0.610	0.001
PC2	0.629	-0.777	0.162	0.1179
PC3	-0.335	-0.942	0.014	0.8272
PC4	0.150	-0.989	0.108	0.2557
PC5	-0.615	-0.788	0.008	0.9181
<i>Hydrographic Region</i>			0.300	0.003
Guaíba	-0.592	0.022		
Littoral	0.472	0.060		
Uruguay	-0.521	-0.135		
Vegetation cover (vegcov)			0.012	0.7672
vegcov presence	0.123	0.212		
vegcov absence	-0.022	-0.039		
Predation (pred)			0.022	0.7213
Pred presence	-0.692	-0.048		
Pred absence	0.028	0.002		

Discussion

Environmental predictors

Our study provides a first evaluation on the influence of environmental variables on Neotropical AF assemblages under natural conditions. We demonstrated a significant effect of environmental variables at landscape and local habitat scale on the variation of occurrence and abundance of AF species. Generally, pools that supported AF species were shallow, situated in lowland areas and without predatory fishes. AF abundances were negatively affected by water depth.

Some studies reported that Neotropical AF species inhabiting shallow habitats, in lentic temporary pools filled with abundant aquatic vegetation and located in lowland areas (Nico and Taphorn 1984; Volcan et al. 2011a; Lanés et al. 2014a, b). AF records in Pampa biome are usually associated with low altitudes (Costa 2006) and few species were registered in relatively more elevated areas (i.e., 100 m. a.s.l) (Ferrer et al. 2008; Cheffe et al. 2010; Volcan et al. 2011b, 2014a, b). Wetlands at higher elevations presents greater isolation (less connectivity), and the founded pattern is probably related to a lack of historical dispersal, since evolutionarily, the common ancestors of both genera (*Austrolebias* and *Cynopoecilus*) originated in lowlands (Costa 2010; Ferrer et al. 2014). The AF presence was also negatively related to the altitude in African AF (Reichard et al. 2009; Reichard et al. 2017).

During extensive and intense precipitation periods temporary pools increase in size and can connect with permanent waters, resulting in greater predation

pressure from non-annual fish (Lanés et al. 2016). In addition, swimming ability of AF is low (Bartáková et al. 2015), making them particularly vulnerable to predation from large aquatic macroinvertebrates and fishes. Accordingly, we found a strong negative effect of potential fish predators on AF occurrence. Predation by non-annual fish was recognised as an important driver in eliminating adult AF in temporary pools (Vaz-Ferreira et al. 1966; Nico and Thomerson 1989; Winemiller and Jepsen 1998). The most frequent fish predator recorded was *Hoplias* aff. *malabaricus*. This species is cryptically coloured and active mostly at night, captures its prey by ambush and frequently occurs in habitats with abundant marginal vegetation (Corrêa et al. 2012). The negative effect of water depth in AF occurrence and density can also be a consequence of predation, since depth differences provide heterogeneous microhabitats, allowing the establishment of various organisms (Maltchik et al. 2010, 2014), including potential macroinvertebrate and fish predators.

Annual fish assembly composition, distributional pattern and spatial factors

Our results showed that AF assemblages in *Pampas* are strongly spatially structured and many individual species form discrete units. Broad scale space and hydrographic region were the main filters shaping the assemblage composition. In communities that exhibit spatially structured pattern, sites located close to each other are generally compositionally more similar than distant sites (Soinin 2015).

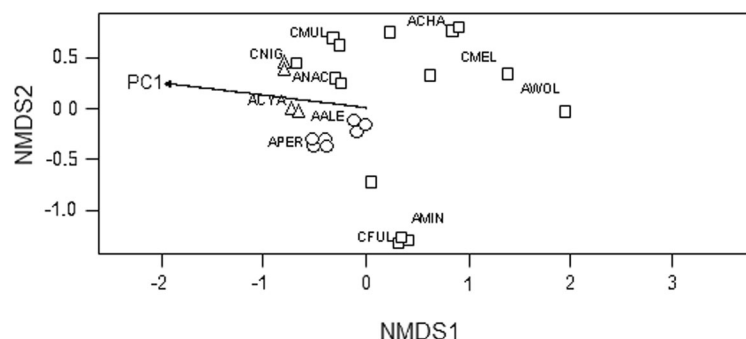


Fig. 2 Annual fish assemblage similarity (occurrence data) sampled across sites of southern Brazil grasslands, represented by two axes of Non-metric multidimensional scaling (NMDS) ordination, and fitted by hydrographic regions (Guaíba, Littoral and Uruguay) Acronyms used: PC1: Principal Component axis 1. Sampling units in Guaíba Δ , Littoral \square , and Uruguay \circ . Species: AALE:

Austrolebias alexandri; ACHA: *Austrolebias charrua*; ACYA: *Austrolebias cyaneus*; AMIN: *Austrolebias minuano*; ANAC: *Austrolebias nachtigalli*; APER: *Austrolebias periodicus*; AWOL: *Austrolebias wolterstorffi*; CFUL: *Cynopoecilus fulgens*; CMEL: *Cynopoecilus melanotaenia*; CMUL: *Cynopoecilus multipapillatus*; CNIG: *Cynopoecilus nigrovittatus*

The structured pattern of individual species occurrence and distribution found here reflect historical biogeographic factors shaping composition of Neotropical AF assemblages in subtropical grasslands and evidence the dispersal limitation. The typical mode of AF dispersal and colonization of new habitats remains unknown (Dorn et al. 2012; Bartáková et al. 2015). AF habitats are very fragmented in general and our result is not an artefact of supposedly incomplete knowledge of the extent of their distributions. Recently, Volcan et al. (2015) corroborated the same pattern during an extensive survey of AF from Pampa biome. The location of suitable habitats for AF is extremely patchy (Loureiro et al. 2015; Reichard 2015; Volcan et al. 2015) and in modern landscapes, temporary waters are even more widely spaced and rare (Schwartz and Jenkins 2000). Present-day distribution of Pampas's AF assemblages is hypothesised as a combined result of vicariance events, congruent with tectonic episodes and dispersal events, and caused by river captures (Ribeiro 2006; Costa 2010; Loureiro et al. 2011; García et al. 2012; Ferrer et al. 2014; Loureiro et al. 2015).

Each hydrographic region presented a unique species composition. Littoral showed more AF records, which covers a more extensive area, and presents higher richness and beta diversity. Despite not sharing species, we found that Guaíba and Uruguay (which are spatially closer) are more similar to each other. These regions coincided with higher monthly precipitation, temperature and altitude and presented similar number of AF records and richness. According to Abell et al. (2008) hydrographic regions capture broad patterns of fish species associated to ecological and evolutionary processes generated primarily by continental (mountain formation, speciation and glaciation) and regional scale filters (broad climatic and physiographic patterns, and regional catchments). AF species have been used to freshwater ecoregion delimitation (Abell et al. 2008). However, unlike most freshwater fishes, AF species are also highly influenced by surrounding terrestrial biome (Costa 2009). Distributional pattern usually associated with terrestrial animals has been recorded for African AF and, interestingly, large river channels formed main barriers to their gene flow (Bartáková et al. 2015). While *Austrolebias* comprises typically grasslands and genuine Pampas species (Costa 2006, 2010), Ferrer et al. (2014) suggested an ancestral relationship to Atlantic Forest in *Cynopoecilina* tribe, suggesting that occurrence in grassland type habitat constitute a recent

evolutionary adaptation during diversification of the genus *Cynopoecilus*. Similarly, Dorn et al. (2014) proposed a scenario where initial diversification of African AF coincided with aridification of East Africa and consequent grassland habitats establishment.

Conservation

All AF species sampled, except *C. melanotaenia* are listed as vulnerable, endangered or critically endangered (FZBRS 2014). Endemic and threatened species constitute priority species for conservation, although in many cases they are common in their limited distribution area. There are relatively few conservation initiatives covering South American grasslands region, although assessments of species-extinction risks have been produced (Azpiroz et al. 2012; Saraiva and Souza 2012; Saraiva et al. 2014). The Brazilian government launched an action plan to ensure AF conservation (ICMBio 2013), but few concrete actions have been taken to prevent loss of these species and habitats.

Main land use practices observed in the studied sample units were livestock, agriculture, exotic afforestation and urbanization. These activities cause an intense fragmentation and habitat loss in South America subtropical grasslands (Vega et al. 2009; Azpiroz et al. 2012; Maltchik et al. 2014). Our results indicate that AF inhabit mainly shallow and relatively small-size pools, containing surface water only during a short period of the year, and located at flat and lowland grassland areas. These features make these habitats extremely fragile and vulnerable to human activities. Among our 82 sampled pools, at least 12 have been completely destroyed by the advancing agriculture in less than a year after sampling was undertaken (L. Moreira pers. comm. to LEKL).

Most AF records were found in temporary pools located on private farms used for cattle grazing. Temporary pools are important to provide food and drinking reservoirs to cattle, mainly during driest and hottest periods. Although domestic cattle trample, urinate and defecate into the pools this seems to have no significant deleterious effect on AF populations and environmental quality. Several studies have drawn attention to grasslands conservation (Overbeck et al. 2007; Bond and Parr 2010). In ecosystems where climate favours forest expansion, grasslands without

management by grazing or fire are generally subject to shrub encroachment and subsequently converted into forests (Overbeck et al. 2007). Subtropical grasslands of South America has been historically inhabited by extinct megafauna, and extensive cattle grazing is considered as a way to simulate the effects of disturbances that have occurred since millennia and retained its grassland areas (Pillar and Vélez 2010).

Contrary to livestock, agriculture promotes strong modification and destruction of natural grasslands, wetlands and temporary pools (Machado and Maltchik 2010; Rolon and Maltchik 2010). AF records in agricultural areas occurred only in small portions not directly affected by this activity. Despite harbouring several endemic and endangered species, only 0.5% of South Brazilian grasslands are under legal protection in conservation units (Overbeck et al. 2007) and studies suggested that grasslands, in particular, cannot be maintained in integral protection areas, because the lack of management provided by cattle grazing and fire (Oliveira and Pillar 2004). Habitat maintenance should be considered as principal strategy for AF conservation. However, conservation strategies directed to AF and their biotopes should not be limited to the simple establishment of protected areas. Taking the degree of threat and increasing habitat loss for agriculture and exotic afforestation into account, one alternative of AF conservation is to promote wildlife-friendly practices in livestock and agricultural lands. Similar initiatives were established by The Alliance for the Grasslands to promote certified beef production in natural areas of Río de la Plata grasslands and reconcile production with wild bird species conservation (Azpiroz et al. 2012).

Here we provide the first approach on the broad-scale patterns of AF assemblage structure and diversity in South Brazilian grasslands. The restricted geographic range, low dispersal ability and high habitat specificity highlights the importance of AF as target group for conservation and environmental licensing. In this sense, conservation actions should be considered at regional hydrographic scale level, and future studies could be directed to investigate the role of these predictors at specific species-groups and distribution areas. As biologically interesting and attractive charismatic animals, AF can be used as flagship species for conservation of temporary pools in South America subtropical grasslands.

Acknowledgements This research was supported mainly by funds from CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazil (52370695.2) and UNISINOS. The biological data were obtained as part of doctorate project of LEKL (CNPq process number 132343/2009-6). The Ph.D. course of LEKL was partially funded by PROSUP/CAPES scholarship (2011/2). Part of the manuscript was prepared during the period covered by the Doctorate Sandwich Abroad scholarship (SWE) of LEKL through Science without Borders program from CNPq (200165/2015-1). LM holds Research Productivity grant and MR Special Visiting Research (PVE) grant, both from CNPq. The AF collections complied with Brazilian current laws (IBAMA-02001.001148/2007-61).

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