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Discovery of endangered annual killifish *Austrolebias cheradophilus* (Aplocheiloidei: Rivulidae) in Brazil, with comments on habitat, population structure and conservation status

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Austrolebias genus comprises about 40 small annual killifishes endemic to South America and their highest diversity occurs in southern Brazil and Uruguay, especially in drainages of Patos-Mirim system. *Austrolebias* species are severely threatened with extinction because their life cycle and restricted ranges. Low dispersal ability and the extensive loss and fragmentation of freshwater wetlands contribute to this threat. Accurate information on the geographic distribution and ecology of the species, vital to plan conservation and management strategies, are scarce. In order to provide basic knowledge for annual fish conservation this paper reports the presence of *Austrolebias cheradophilus* and present data about its population structure (CPUA, size, sex ratio, length-weight relationships and condition factor) and conservation status in southern Brazil. The estimated CPUA of populations was 0.86 fish/m^2. Standard length (SL) of males ranged between 32.14 and 49.17 mm and for females between 25.11 and 41.6 mm. There were no differences in SL between the sexes (*t*-test = - 1.678; *P* = 0.105), and Chi-squared test demonstrated marginal differences in proportions of sexes (2.25:1; $\chi 2 = 3.846$; *P* = 0.07). Allometric coefficient of the *LWR* was slightly hyperallometric (*b* = 3.08) and *K* of the specimens ranged from 1.84 to 2.42 (mean ± S.E. = 2.12 ± 0.04). Populations have low density and their biotopes are under critical threat, mainly due to suppression by agriculture, pastures for livestock and increase housing. The species is considered "Critically Endangered" in Brazil, furthermore is strongly recommended its inclusion on lists of endangered fauna and ensures the protection of their remaining habitats.

O gênero *Austrolebias* compreende cerca de 40 espécies de peixes anuais endêmicos da América do Sul, cuja maior diversidade está concentrada no sul do Brasil e Uruguai, especialmente nas drenagens do sistema hidrográfico Patos-Mirim. A combinação de seu ciclo de vida peculiar com suas restritas distribuições, baixa capacidade de dispersão e ampla fragmentação das áreas úmidas fazem com que suas espécies sejam consideradas ameaçadas de extinção. Entretanto, informações sobre sua distribuição e ecologia são escassas. Visando fornecer informações básicas para a conservação, este estudo registra a presença de *Austrolebias cheradophilus* no sul do Brasil e apresenta dados sobre sua estrutura populacional (CPUA, tamanho, razão sexual, relação peso-comprimento e fator de condição) e estado de conservação. O CPUA total das populações foi de 0,86 peixe/m^2. Não foram encontradas diferenças no tamanho padrão entre os sexos (*t*-test = - 1,678; *P* = 0,105) e o teste do Qui-quadrado apresentou um valor marginalmente significativo para a proporção dos sexos (2,25:1; $\chi 2 = 3.846$; *P* = 0.07). O coeficiente de "*b*" calculado com base na relação peso-comprimento indica um padrão de crescimento levemente alométrico positivo (*b* = 3,08). O fator de condição dos indivíduos variou entre 1,84 e 2,42 (média ± S.E. = 2,12 ± 0,04). As populações identificadas têm baixa densidade e estão sob ameaça crítica, principalmente devido à supressão do habitat pela agricultura, pecuária e aumento da urbanização. A espécie foi considerada "Criticamente Ameaçada", portanto recomenda-se sua inclusão em listas de fauna ameaçada e a urgente proteção dos seus habitats.

Key words: Annual fishes, Restricted range, South America, Temporary ponds, Threatened fauna.

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Introduction

Austrolebias Costa, a specious genus of the family Rivulidae, comprises about 40 small annual killifishes (Costa, 2008; Ferrer *et al*., 2008; Loureiro & García, 2008; Loureiro *et al*., 2011) endemic to South America and distributed in southern Brazil, southern Bolivia, Paraguay, Uruguay, and north-eastern Argentina (Costa, 2010). The literature indicates that annual fishes, such as *Austrolebias* species, inhabit temporary wetlands formed during rainy periods and die when the ponds dry out (Costa, 2003, 2006). The embryos survive through resistant diapauses eggs during the dry periods, and hatch after the beginning of the rainy period (Wourms, 1972). When there are regions with no defined rainfall season the presence of water in temporary ponds is mediated by the balance between precipitation, temperature and evaporation (Lanés, 2011).

Morphological (Costa, 2006) and molecular analysis (García, 2006) support the monophyly of the genus and its highest diversity occurs in southern Brazil and Uruguay, especially in drainages of Patos-Mirim hydrographic system (Costa, 2010; Loureiro *et al*., 2008). The genus was recently phylogenetically redefined, including the species previously referred to *Megalebias* (Costa, 2006), and divided into sub-genera (Costa, 2008). According to Costa (2006) the *Austrolebias elongatus* species-group, comprises five valid species [*Austrolebias cheradophilus* (Vaz-Ferreira, Sierra de Soriano & Scaglia-de-Paulete), *Austrolebias elongatus* (Steindachner), *Austrolebias monstrosus* (Huber), *Austrolebias prognathus* (Amato) and *Austrolebias wolterstorffi* (Ahl)], reaching a large size (about 70-120 mm SL), which makes some of them the biggest species of the family Rivulidae (Costa, 1998, 2002). After the division of *Austrolebias* into sub-genera, the *A. elongatus* species-group was allocated in the subgenus *Megalebias* (Costa, 2008).

These large species are generally top predators of temporary wetland systems, and are rarer than other smaller species of *Austrolebias* which are often abundant in their habitats (Costa, 2009). This pattern is probably a reflection of increased energy demand, as a function of a larger body size (Laufer *et al*., 2009; Arim *et al*., 2010).

The *Austrolebias* species are severely threatened with extinction because their peculiar life cycle and restricted ranges. Furthermore, their low dispersal ability and the extensive loss and fragmentation of freshwater wetlands and temporary ponds contribute to this threat (Reis *et al*., 2003; Rosa & Lima, 2008; Volcan *et al*., 2009, 2010a; Teixeira de Mello *et al*., 2011). However for the majority of *Austrolebias* species (and Rivulidae fishes in general), accurate and timely data on the distribution and ecological information vital to plan conservation and management strategies is absent (Lanés & Maltchik, 2010; Lanés, 2011). This degree of threat and demand of information about annual fishes resulted in the creation of the "Plano de Ação Nacional para a Conservação dos Peixes Rivulídeos Ameaçados de Extinção - PAN Rivulídeos" in Brazil (ICMBio, 2013). The main goal of this plan is establish mechanisms for ensure the protection of the species and avoid the loss of their habitats.

Austrolebias cheradophilus occurs in isolated coastal drainages (Costa, 2008) and the Mirim Lagoon basin in Uruguay, where is considered endemic and a priority species for conservation in this country (Teixeira de Mello *et al*., 2011). Recent studies have reported in southern Brazil the occurrence of species of *Austrolebias* formerly considered restricted to Uruguay. As an example *Austrolebias arachan* Loureiro, Azpelicueta & Garcia (Lanés *et al*., 2013), *Austrolebias melanoorus* Amato (Volcan *et al*., 2011a) and *Austrolebias vazferreirai* (Berkenkamp, Etzel, Reichert & Salvia) (Cheffe *et al*., 2010) were lately recorded in southern Brazil. Besides, several papers report the widening geographical distribution inside Brazil (Lanés & Maltchik, 2010; Volcan *et al*., 2010b; Volcan *et al*., 2010c; Volcan *et al*., 2011b), showing the incomplete knowledge about the real distribution of these threatened species.

The current paper reports the extension distribution and presence of *A. cheradophilus* in Brazil and provides ecological data about its population structure (results on the sex ratio, length-weight relationships and condition factor), population's threats and environment. Conservation status assessment, based on IUCN criteria is also presented.

Material and Methods

Study area

The study area is located in the Coastal Plain of Rio Grande do Sul, the southernmost state of Brazil, and is inserted in the Pampa Biome (IBGE, 2004). Grasslands predominate on flat terrain in this region (IBGE, 2004). The hydrography is represented by the Patos-Mirim Lagoon system, Laguna dos Patos Ecoregion (Abell *et al*., 2008) which has a drainage area of 13.600 km², distributed in southern Brazil and eastern Uruguay (Malabarba, 2008). Surveys were directed to the lowlands of Jaguarão River one of the main tributaries of the Mirim Lagoon. The survey area falls within the subtropical climate area, with an average temperature of 13°C in the coldest month and above 22ºC in the warmest month. The hottest months are January and February and the coldest ones are June and July. The average annual rainfall (1200-1500 mm) is uniform in all seasons, but varies greatly in different years (Nobre *et al*., 1986). 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, 1997). In the studied region the land is used for agriculture and stockbreeding, mainly temporary crops such as rice and breeding of veal and sheep (IBGE, 2008).

Samplings

The data for this study were obtained specifically in samplings carried out during September 2009 and July 2011, corresponding to austral winter. During this period, the temporary ponds of the study area generally presents surface water. Although this study may seem casual, other numerous field surveys are being conducted, since 2005 (with exploratory and sporadic visits lasting one to five days), resulting in samples in more than 50 sampling units, distributed in main drainages of Mirim Lagoon, mostly in Jaguarão River basin, and resulting in the discovery of several other annual fish species and populations (Lanés *et al*., pers. obs.). Even these surveys resulted in the discovery of *Austrolebias univentripinnis* Costa & Cheffe and provided important material for describing *Austrolebias juanlangi* Costa, Cheffe, Salvia & Litz and *Austrolebias nachtigalli* Costa & Cheffe. For more details see Costa (2006).

At the sampling sites, the co-ordinates, area (m^2) and elevation (meters above sea level) were estimated using GPS, and the physical and chemical parameters of water (pH, dissolved oxygen (mg L^{-1}), conductivity (µS cm^{-1}) and temperature (°C)) were assessed with portable devices (Hanna HI 9828).

Fish samples were taken with a dip-net (D-shaped hand net, 60 cm x 30 cm, 2 mm mesh size), and then were euthanized with a lethal dose of clove oil, fixed *in situ* with 10% formalin, and later transferred into 70% ethanol. Sampling was performed by sweeping the net parallel to the pond bottom, exploring a variety of habitats of the wetlands. Fifty sweeps (corresponding each to approx. 0.6 m^2 of sampled area) were carried out per sampling site. The chosen sampler mesh-size was fine enough to capture all sizes of *Austrolebias* fishes present at a sampling site. Specimens were identified and sexed based on Costa (2006). The material was collected under IBAMA/ICMBio authorization (process number 18334-1 and 18334-2) and vouched in ichthyologic collection of Universidade Federal do Rio de Janeiro (UFRJ 6748 - nine specimens preserved in alcohol; UFRJ 6749 - five specimens cleared and counterstained).

Data analysis

Abundance was assessed by the total number of captured fishes and expressed as catch per unit area (CPUA: number of individuals/m^2). A Student's *t*-test was performed to explore differences between the mean standard body length (SL) of males and females. The possible bias of the sex ratio of the studied population from the expected 1:1 rate were determined by Chi-square test (with at 95% significance level) with randomization by Monte Carlo method.

The specimens were measured in standard length (SL) to the nearest 0.1 mm and weighed (body weight including the gonads) to the nearest 0.001 g to assess the length-weight relationship (*LWR*). The *LWR*, $W = aL^b$, was transformed into its logarithmic expression: $\log W = \log (a) + b * \log (SL)$, where *a*, is the intercept and *b* is the slope. The relationships between standard length and weight were determined by least square regression. The 95% confidence limits for *b* ($CL_{95\%}$) were calculated to determine if the hypothetical isometry value ($b = 3$) fell between these limits (Froese, 2006). Condition factor (*K*) coefficients of *A. cheradophilus* were calculated as the ratio between body weight and standard length, through the following equation: $K = (W / SL^b)*100$, where the (*b*) represents the slope of the length-weight relationship.

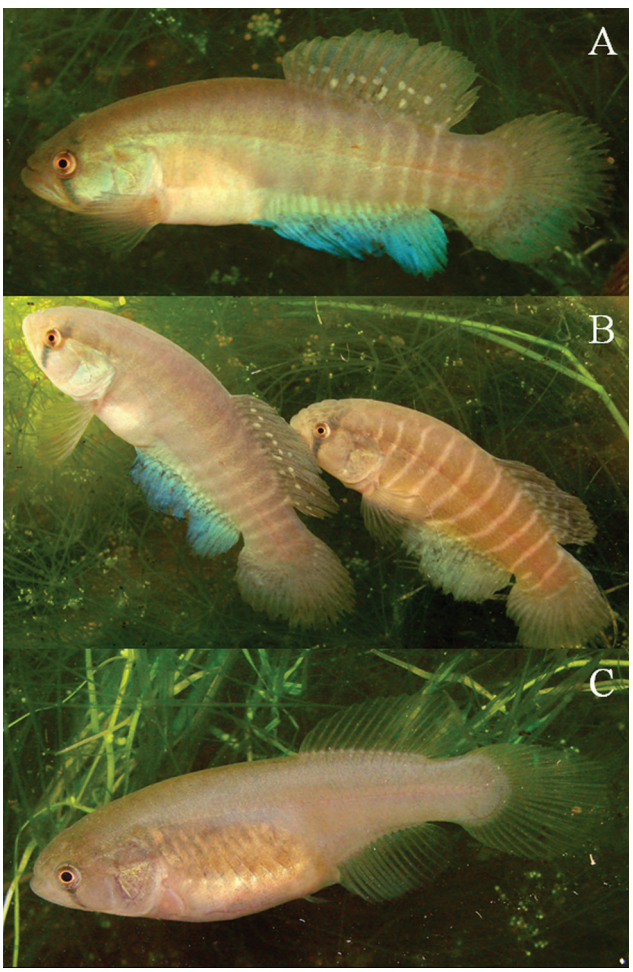

Fig. 1. *Austrolebias cheradophilus* sampled in Jaguarão River, Patos-Mirim Lagoon system, southern Brazil: (A) Male, 49.2 mm SL, (B) variation in color pattern of males (41.3 mm and 49.2 mm SL) and (C) female, 41.2 mm SL. Photos by Matheus Volcan.

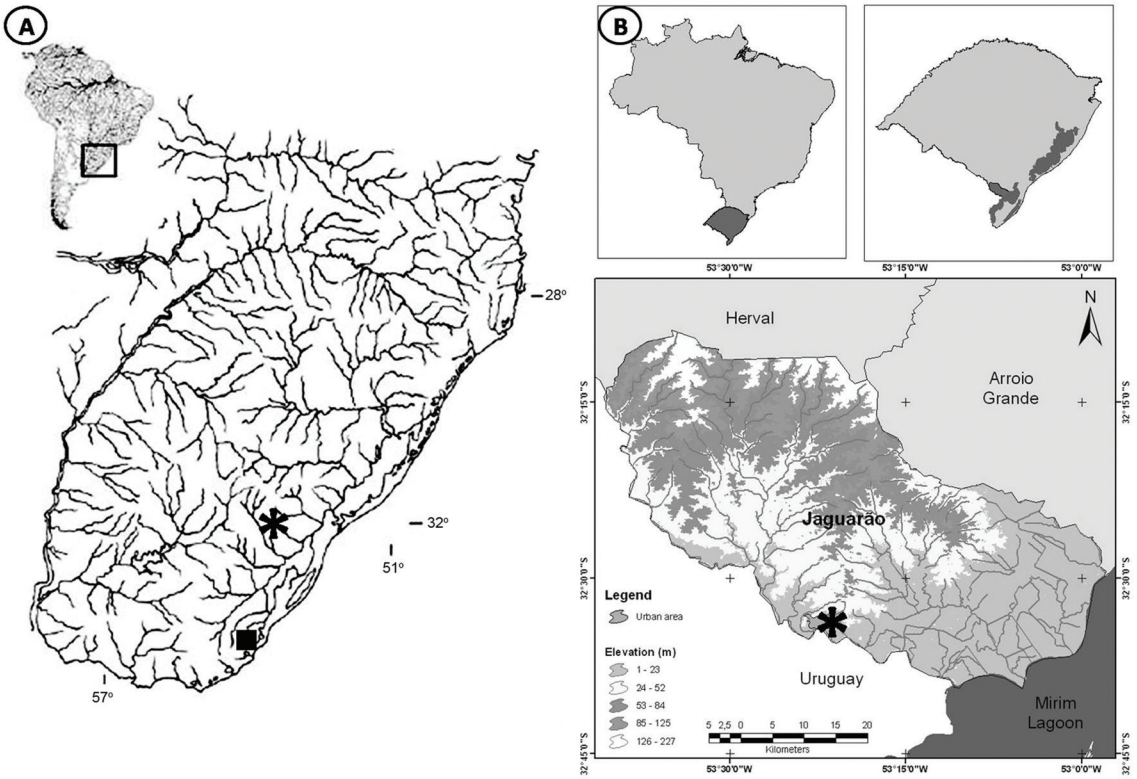

Fig. 2. (A) Occurrence of *Austrolebias cheradophilus* in Jaguarão River basin, Patos-Mirim Lagoon system, southern Brazil (asterisc), and previous record of the species closest to Brazil (Black square). (B) Map detailing the occurrence of the species in lower Jaguarão River, Patos-Mirim Lagoon system, southern Brazil.

Conservation assessment

Conservation status assessment was based on field surveys. To establish the vulnerability category the IUCN criteria were applied (IUCN, 2011), using criterion B2 (Geographic range in the form of either B2 - area of occupancy). Currently this approach is applied in the revaluation of the red lists of threatened fauna of Rio Grande do Sul State (Fontana *et al.*, 2003), Brazil (Rosa & Lima, 2008), and "Plano de Ação Nacional para a Conservação dos Rivulídeos - PAN Rivulideos" (ICMBio, 2013). Areas of occupancy (AOO) and distances were calculated using satellite images available in Google Earth (earth.google.com). Wetland boundaries were determined 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).

Results

Two populations of *A. cheradophilus* (Fig. 1) were discovered in southern Brazil, Jaguarão town, associated with a small tributary stream located in lowlands of Jaguarão River drainage, Patos-Mirim Lagoon hydrographic system (Fig. 2). This study provides the first record of the species in Brazil, extending its distribution 150 km northwards (Fig. 2) and constitutes the first citation of *Megalebias* subgenus in Jaguarão River basin.

Both populations (Site 1 and Site 2) were found in similar environments, represented by small (< 1 km^2) and shallow (< 30 cm) temporary palustrine ponds (represented by small ground depressions) located in grassland areas of private farms surrounding Jaguarão town (Fig. 3). The two newly-discovered localities inhabited by *A. cheradophilus* are hydrologically isolated and separated by *ca.* 750 meters. Soil preparation activities for exotic pasture culture were observed on the surrounding areas after the species discovery. There are a local road, small groves of eucalyptus, houses and pastures for cows and horses between the two identified locations. The sites are not covered by protected areas. Wetland vegetation was dense and abundant, composed mostly of emergent macrophytes; mainly *Eryngium* sp. and floating rooted (*Echinodorus* spp., *Leersia hexandra*, *Luziola peruviana*, *Ludwigia peploides*, *L. grandflora*, *Marsilea* spp., *Myriophyllum aquaticum*, *Nymphoides indica*, *Regnellidium diphyllum*, *Polygonum hydropiperoides*) and submerged plants (*Scirpus submersus*, *Urtricularia gibb*), forming

multispecific stands. The only other fish species found in co-occurrence with *A. cheradophilus* were the non-annual fishes *Cheirodon interruptus* (Jenyns), *Callichthys callichthys* (Linnaeus) and *Phalloceros caudimaculatus* (Hensel). Details of environmental and water characterization of each sampling site are shown in Table 1.

A total of 26 specimens of *A. cheradophilus* (8 males and 18 females) were sampled throughout the study. The specimens were found only in shallow areas (mean ± S.E. = 10 ± 1.30 cm) of the ponds. The total CPUA of populations was 0.86 fish/m^2. There was a variation of CPUA values among sampling sites, being higher in the site 1 (1.2 *vs*. 0.6 fish/m^2 in site 2) (Table 2). The SL of males ranged between 32.14 and 49.17 mm (mean ± S.E. = 39.27 ± 2.41 mm) and of females between 25.11 and 41.6 mm (mean ± S.E. = 35.06 ± 1.29 mm). Even though the larger caught fish were males, there were no significant differences in SL between the sexes (*t*-test = - 1.678; $P = 0.105$). The Chi-squared test demonstrated marginal significant differences in proportions of sexes (2.25:1; $\chi 2=3.846$; $P = 0.07$). Information about variations in the number of sampled specimens, CPUA, and SL range, in each sampling site for each sex are given in Table 2.

Length-weight relationship of *A. cheradophilus* ($r^2 = 0.961$; $P = 0.0001$) in the form of a regression equation was estimated as log W = - 2.596 + 3.083 log SL and the allometric coefficient "*b*" of the LWR was slightly hyperallometric ($b = 3.08$; $CI_{95\%} = 2.348$ to 3.519). The condition factor of the specimens ranged from 1.84 to 2.42 (mean ± S.E. = 2.12 ± 0.04).

In accordance with application of IUCN criteria, *Austrolebias cheradophilus* was considered as "Critically Endangered" species in Brazil, being included in the criteria CR B2ab (ii, iii). The species presents reduced area of occupancy (AOO less than 10 km²), populations severely fragmented (a) and continued decline (b) in area of occupancy (ii) and quality of habitat (iii).

Table 1. Environmental and water characterization of sampling sites of *Austrolebias cheradophilus* populations found in Jaguarão River basin, Patos-Mirim Lagoon system, southern Brazil.

Variables	Site 1	Site 2
Geographic coordinates	32°33'S 53°21'W	32°33'S 53°20'W
Elevation (m)	25	23
Estimated area (m^2)	3776	7500
Depth (cm)	25	19
Dissolved oxygen (mg/L)	4.28	4.63
pH	6.85	8.23
Conductivity (mS/cm)	0.026	0.019
Temperature (°C)	10.87	12.13

Fig. 3. Temporary ponds (Site 1 above and Site 2 below) inhabited by *Austrolebias cheradophilus* in lowlands of Jaguarão River basin, southern Brazil.

Discussion

This paper reports a significant range extension in the known distribution of *A. cheradophilus*, especially considering the low vagility of the *Austrolebias* species (Loureiro *et al.*, 2011; Garcia *et al.*, 2012). Until recently *A. cheradophilus* was considered exclusive of isolated coastal drainages (Costa, 2003, 2006, 2008), despite the existence of an unpublished record in the Cebollati Stream, Mirim Lagoon basin, in Uruguay (Muséum national d'histoire naturelle, MNHN 1997-411) (Froese & Pauly, 2011), 150 km far from this record. Recently Teixeira de Mello *et al*. (2011) formalized the occurrence of the species in Lagoa Mirim basin. The present-day distribution of *A. elongatus* species-group was hypothesized as a result of dispersal event from Patos (ancestor) to the Paraguay hydrographic system through La Plata area (Costa, 2010), and the occurrence of species in the Patos-Mirim system is probably related to ancient connections between this system and coastal drainages of Uruguay.

Table 2. Sex ratio, number of sampled specimens (*n*), CPUA (fishes/m^2), and standard length (SL in mm; minimum - maximum) of *A. cheradophilus* between sampling sites in southern Brazil.

	Male	Female	*n*	CPUA	SL Male	SL Female	SL M+F
Site 1	6	11	17	1.2	32.14 to 49.17	25.11 to 41.24	25.11 to 49.17
Site 2	2	7	9	0.6	33.1 to 44.9	31.7 to 41.6	31.7 to 44.9
Total	8	18	26	0.86	32.14 to 49.17	25.11 to 41.6	25.11 to 49.17

Considering also the present study there are 22 species of the genus *Austrolebias* in Rio Grande do Sul State (Lanés, 2011; Lanés *et al*., 2013), which represent more than half of all diversity of the genus (Costa, 2006, 2008; Loureiro *et al*., 2011). It shows that this territory is an important center of endemism and diversity of this group. Six of them (*A. arachan*, *A. cheradophilus*, *A. juanlangi*, *A. melanoorus*, *A. nachtigalli*, and *A. univentripinnis*) has records in Brazilian drainages of Jaguarão River basin (Costa, 2006; Volcan *et al*., 2011a; Lanés *et al*., 2013). Moreover, the recent descriptions of *Austrolebias reicherti* Loureiro & García and of *Austrolebias quirogai* Loureiro, Duarte & Zarucki in Jaguarão River basin in Uruguay, indicate that probably they also occur in Brazil. Unfortunately, despite the large representation of species with conservation importance, there is no protected area in Jaguarão River drainages (Lanés *et al*., 2013).

The CPUA values obtained suggest that the species occur in low densities in the investigated localities. Other studies found 2.2 individuals per sampled area of 1 m^2 for a population of *A. arachan* (Lanés *et al*., 2013), and 1.97 for *Atlantirivulus riograndensis* (Costa & Lanés) (Lanés *et al*., 2012). Larger species of rivulids (such as *Megalebias* subgenus) are generally rarer than small species. Species of *Megalebias* subgenus were found at relatively low densities in areas where distinct rivulid species co-occurs (Laufer *et al*., 2009; Arim *et al*., 2010; Volcan *et al*., 2013).

Although males of *A. cheradophilus* were less abundant and larger than the females, there were no significant differences in the proportion of individuals and standard size between the sexes. This result may be assigned to the natural rarity of the species and to the small size of our samples, since the males of *Austrolebias* usually are larger and less abundant than females (Costa, 2003; Lanés *et al*., 2013). Laufer *et al*. (2009) found differences in these aspects in other species of *Austrolebias* with abundant population, but as in this study, also hasn't found difference in *A. cheradophilus*, attributing this result to its small sample size. The *P*-values of 0.10 for size differences and of 0.07 for sex ratio can therefore be considered as marginally significant and they suggest a trend of difference in these parameters between the sexes.

Allometric coefficients (*b*) of *LWR* estimated for the species fell within the expected range of 2.5-3.5 (Froese, 2006). The slightly hyperallometric growth found in this study suggests that analyzed specimens increases minimally more in weight (W) than predicted by the increase in length (SL). No previous references for *LWR* of *A. cheradophilus* were available making difficult the comparisons with other previous studies. *Austrolebias wolterstorffi*, other member of *Megalebias* subgenus showed positive allometric growth ($b = 3.04$) when kept under laboratory conditions (Fonseca *et al*., 2013), with values very similar to those found in this study and Volcan *et al*. (2013) found highly positive allometric growth for wild populations of *A. wolterstorffi* ($b = 3.26$). The same growth pattern found in this study was observed for other *Austrolebias* species, such as *Austrolebias viarius* (Vaz-Ferreira, Sierra-de-Soriano & Scaglia-de-Paulete) (Errea & Danulat, 2001), *Austrolebias nigrofasciatus* (Costa & Cheffe) (Volcan *et al*., 2013) and *A. arachan* (Lanés *et al*., 2013).

Austrolebias cheradophilus is considered "Priority Species for the Conservation in Uruguay" (Teixeira de Mello *et al*., 2011), because it's restricted distribution, low representation in fish collections and economic value (for aquarium). Although these new populations' records and the distribution expansion of the species to Brazil are important, is not sufficient to ensure its conservation. In accordance with application of IUCN criteria, the species is considered "Critically Endangerd" in Rio Grande do Sul State and Brazil. The Red List Criteria represents a first step in setting priorities for conservation action (IUCN, 2011).

Therefore, the inclusion of *A. cheradophilus* in future listings of threatened fauna is strongly recommended. However the most important and effective action for conservation species in Brazil is the protection of their remaining habitats, since the populations are highly restricted, have low density and their biotopes are under critical threat, mainly due to suppression by the land use for agriculture, livestock (replacement of natural grassland areas by pastures for grazing) and increase urbanization. The conservation of small and seasonal ponds is essential for the maintenance of annual fish species. Knowing the geographic distribution and environment of endangered species is critical as baseline for future studies, conservation strategies and environmental licensing. In this sense, our study provides a first insight about population parameters and habitat features related to the occurrence of *Austrolebias cheradophilus* in Brazil.

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