

The morphology–diet relationship and its role in the coexistence of two species of annual fishes

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Abstract – Annual fishes, which inhabit temporary pools with extremely limited habitat complexity and niche availability, display remarkable sexual dimorphism, rapid growth, and enormous investment into reproduction, all traits associated with high energy requirement. This study tests three hypotheses for two syntopic annual fishes (*Austrolebias minuano* and *Cynopoecilus fulgens*) found in six wetlands of southern Brazil: (i) considerable morphological differences result in low dietary overlap, (ii) sexual dimorphism in both species leads to intraspecific diet segregation, and (iii) dietary richness increases during ontogenetic development, and is narrower in *C. fulgens* than *A. minuano* due to morphological limitations imposed by reduced size. The diet of 82 *A. minuano* and 211 *C. fulgens* individuals was analyzed over two annual cycles. The morphology was characterized by 26 measurements covering the entire body of both species. There was no evidence of morphological specialization related to food competition and the diet of *A. minuano* and *C. fulgens* showed high overlap. High food availability, high predator abundance, and high connectivity of adjacent wetlands are likely the main mechanisms allowing coexistence of both species. Within species, sexual dimorphism did not result in a decrease in dietary overlap, which reinforces the idea that morphological differences between the sexes did not evolve as a mechanism to decrease food competition. Large *A. minuano* did not have a more diverse diet than the smaller *C. fulgens*; however, increase in body size allowed both species to ingest larger prey. Morphological variability in both species was mainly related to ontogenetic development and reproduction.

Key words: *Austrolebias minuano*; *Cynopoecilus fulgens*; niche overlap; sexual dimorphism; ontogeny

Introduction

A major topic of current research in evolutionary ecology is the study of mechanisms that allow sympatric occurrence of species from the same guild (Chunco et al. 2012). Interspecific competition has been addressed in several biological communities as it is a powerful force that leads to niche differentiation among sympatric species (Elliott & Mariscal 2001; Sánchez-Cordero et al. 2008). In a classic experiment, Gause (1934) demonstrated that for exploitative competition to occur, a resource has to be limited. In this circumstance, niche differentiation is frequently manifested through morphological variation resulting

from resource partitioning. At the same time, competition for food can exert powerful intraspecific selection, which can lead to morphological variations among individuals. This can lead to bimodal distribution within a population and, ultimately, may even contribute to sympatric speciation (Meyer 1990). Another possibility, however, is that phenotypic differences are manifested between the sexes, leading to a decrease in intraspecific competition in some populations (Rueffler et al. 2006; Cooper et al. 2011).

Ontogenetic growth affects morphological structures associated with feeding; and despite specific studies which have focused on these themes (Werner & Gillian 1984; Hjelm et al. 2001), the majority of

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ecomorphological studies have not addressed the role of ontogenetic growth and morphological change as regards intraspecific diet partitioning and dietary changes over an individual's development (Cassemiro et al. 2008). Growth of morphological structures (mainly mouth gape) during ontogeny allows for changes in diet, with larger fish being able to consume larger prey. Adult fishes generally take larger prey to maximize their energy intake (Wainwright 1995); though studies using high energy requirement generalists have shown that, even when larger prey are included in the diet of larger individuals, smaller prey still remain an important part of the diet (Woodward & Hildrew 2002; Laufer et al. 2009; Arim et al. 2010).

The family Rivulidae (Cyprinodontiformes) encompasses a species-rich group of Neotropical freshwater fishes (35 genera and 363 species; Eschmeyer & Fong 2012), distributed from the southern region of North America to southern Argentina (Costa 1998). Rivulid species display small body size (few species larger than 50 mm), low dispersal ability, and half of all species have annual life-cycles (Costa 2008, 2010). The annual fishes reach sexual maturity in just a few weeks and reproduce continuously throughout the wet season (Arenzon et al. 1999, 2001). Such rapid growth and high reproduction investment are both associated with elevated energy requirements (Polačik et al. 2011). Recent studies have shown that the generalist feeding habits displayed by annual fishes are an important strategy for coping with the harsh and unpredictable conditions prevalent in temporary habitats (Laufer et al. 2009; Polačik & Reichard 2010; Gonçalves et al. 2011).

The annual fishes live in small intermittent wetlands, environments of powerful natural selection (Hoffmann & Parson 1997). Harsh abiotic conditions strongly limit the number of species that can inhabit such ecosystems (Jocque et al. 2010), and presence of annual fishes is strictly related to embryo adaptations (developmental diapause in a specialized egg envelope) that enable survival during the dry season (Wourms 1972). Nevertheless, the coexistence of two or more annual fish species in small temporary pools is not rare and Costa (1998) observed up to seven Neotropical annual fish species living in the same pool, though the degree of niche partitioning was unknown. Laufer et al. (2009) suggested that coexistence of four Uruguayan annual fish species was due to interspecific differences in developmental time. On the other hand, Costa (2009) found morphological specializations related to diversification of feeding habits among species of *Austrolebias*, a common annual fish found in temporary wetlands. Interestingly, recent studies have demonstrated that the diet of some annual fish species varies between males and

females (Laufer et al. 2009; Keppeler et al. 2013). The males of these species were larger (Laufer et al. 2009) and displayed morphological differences (e.g., fin size) that could predispose the sexes to differences in diet (Keppeler et al. 2013).

The annual fishes of southern Brazil are represented mainly by species from 2 genera: *Cynopoecilus* Regan (Cynopoecilini tribe) and *Austrolebias* Costa (Cynolebiasini tribe) (Costa 2002). *Austrolebias minuano* Costa and Cheffe, 2001 and *Cynopoecilus fulgens* Costa 2002 are two small, sexually dimorphic species that co-occur in temporary wetlands across the entire range of *C. fulgens*. The body size of both species increases throughout the annual cycle, whereas their abundance tend to decline (March–November). *Austrolebias minuano* are typically larger (40 mm vs. 30 mm), with a deeper body and less abundant (1:4.16) than *C. fulgens* [Lanés et al. (in press)]. Keppeler et al. (2013) has observed that the taxonomic richness of ingested prey is positively related to body size in *C. fulgens* across the annual cycle, despite a decrease in the richness of food availability.

In this study, we test three hypotheses: (i) morphological differences between *A. minuano* and *C. fulgens* result in low dietary overlap, (ii) sexual dimorphism in both species leads to intraspecific diet segregation between the sexes, and (iii) prey taxonomic richness increases during ontogenetic development, and is narrower in *C. fulgens* than *A. minuano* due to morphological limitations imposed by reduced size.

Methods

Study area

The study was undertaken in the Lagoa do Peixe National Park (LPNP), a Ramsar Site situated in the middle of the Coastal Plain of Rio Grande do Sul State, southern Brazil (50°77'–51°15'W, 31°02'–31°48'S). The park contains 34,400 ha of estuarine, freshwater and terrestrial habitats in a region with flat topography and low altitude (<20 m.a.s.l.), a moist subtropical climate and a mean annual temperature of 17.5 °C (annual average ranging from 13 °C in winter to 24 °C in summer). Mean annual rainfall varies between 1200 and 1500 mm per year (Tagliani 1995).

Sampling

While 14 wetlands were originally sampled in the LPNP, our study was completed using data from six wetlands where *A. minuano* and *C. fulgens* co-occurred (Fig. 1). The wetlands ranged in size from 0.5 to 1.0 ha and depth was always less than

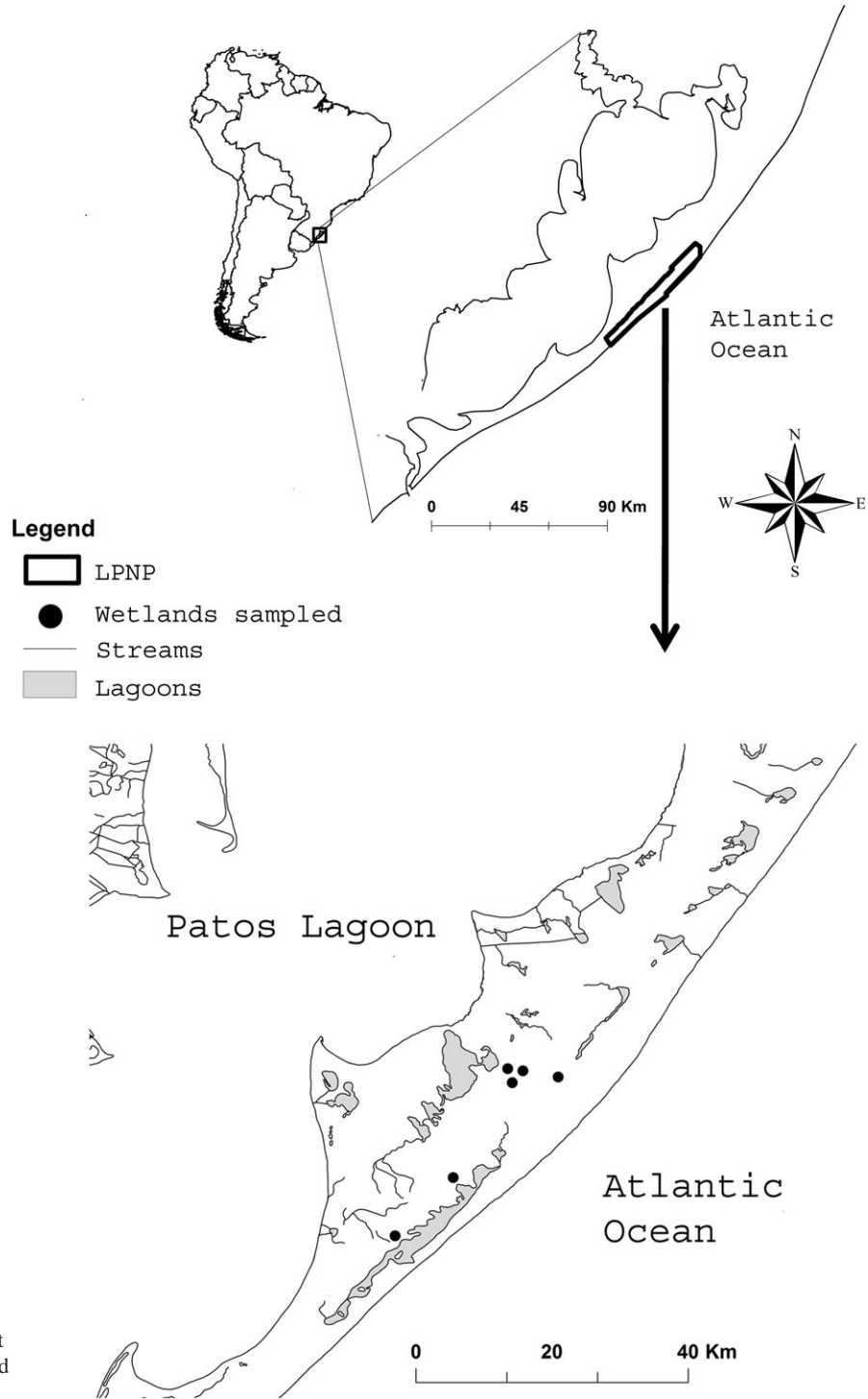


Fig. 1. Location of the Lagoa do Peixe National Park and the studied intermittent wetlands where *Austrolebias minuano* and *Cynopoecilus fulgens* co-occur.

40 cm. The surrounding landscape was characterized by a grassland matrix and aquatic vegetation was abundant, forming multispecies stands with no defined habitat. All wetlands dried out between December and February (summer season).

Seven sampling surveys were undertaken over 2 years, 4 in 2008 (May, June, August and October) and 3 in 2009 (March, August and October). Fishes were collected using a D-shaped hand net (60 cm

width, 1 mm-mesh), whereupon they were anesthetized with a lethal dose of benzocaine and fixed immediately in a 10% formalin solution. The sampling effort varied from 25 (areas smaller than 1 ha) to 50 sweepings (areas larger than 1 ha). This variation in the sampling effort was necessary to ensure a representative measure of the total area of each wetland (Gotelli & Colwell 2001). After 4 days, fish were transferred into 70% ethanol solution. All

specimens collected were identified and sexed according to Costa (2002, 2006) using color pattern and fin size. A total of 1871 individuals were collected over 2 years, comprising 216 *A. minuano* and 1655 *C. fulgens*. The difference in the number of collected individuals between species reflects the rarity of *A. minuano*. Note that the increase of sampling effort on *A. minuano* was not possible due to the endangered status of the species. The term ‘immature’ was used to define small individuals with no clear features to aid sex identification. Finally, all individuals were measured for standard length (mm) and weighed (g).

Analysis of gastrointestinal contents

Overall, 82 *A. minuano* and 211 *C. fulgens* were dissected for diet analysis, selection of individuals being based on stratified subsampling of all sampled wetlands, species and genders (see Supporting information for more details). Ingested prey was identified to the lowest taxonomic level possible. In most cases, aquatic macroinvertebrates (arthropods, worms,

leeches and molluscs) were identified to family level (Merritt & Cummins 1996; Fernández & Dominguez 2001) and microcrustaceans were identified to class or order levels. The contribution of each food item to fish diet was estimated using the numerical method proposed by Hyslop (1980).

Morphological analysis

Sixty-four *A. minuano* and 61 *C. fulgens* were used for morphological analysis. As this study was focused on trophic relationships, the functional morphological features selected were mainly centered on the head region (Table 1), though measurements related to body shape (diagnostic characters for inter-specific differences) and fin size (diagnostic for inter-sexual differences) were also included (Table 1) as these attributes can influence important aspects of feeding strategy (e.g., maneuverability, propulsion).

Images of the lateral view of body were generated using a video digitizer. Morphometric data were acquired as homologous landmark (geometric) coordinates with the TPSDig. v.1.18 software (Rohlf 1999). Aspects of head and body shape were quantified from 21 interlandmarks distances derived from a set of 31 homologous morphological landmarks, analyses obtained with the software LMDis v. 1.0 (Reis 1996). Dorsal view measurements and mouth gape size were taken using a digital caliper (0.01 mm).

Data analysis

Dietary overlap and dietary richness

Schoener's index (Schoener 1970) was calculated to evaluate interspecific (*A. minuano* vs *C. fulgens*) and intraspecific (between sexes) dietary overlap. Schoener's overlap coefficient (*C*) is determined using the formula:

$$C = 1 - 0.5 \left(\sum |p_{xi} - p_{yi}| \right)$$

where p_{xi} is the proportion of the *i*th category used by species *x*, and p_{yi} is the proportion of the *i*th category used by species *y*.

Index values range from 0 (no overlap) to 1 (complete overlap), and values above 0.6 are considered biologically significant (Wallace 1981). Comparison of dietary overlap between *A. minuano* and *C. fulgens* was undertaken using 82 individuals of each species, while 44 *A. minuano* (22 male and 22 female) and 132 *C. fulgens* (66 male and 66 female) were used for analysis at an intraspecific level. The dietary overlap in inter- and intraspecific comparisons was calculated among sampling surveys and among wetlands. Each comparison was made with the same

Table 1. List of traits measured for *Austrolebias minuano* and *Cynopoecilus fulgens*.

Abbreviation	Description of measured trait
M1	Dentary length
M2	Snout length
M3	Eye diameter
M4	Over-orbital height
M5	Under-orbital height
M6	Head height (eye level)
M7	Cheek length (tip of the snout to pre-operculum)
M8	Head length
M9	Head height (pre-operculum level)
M10	Total length
M11	Prepelvic length
M12	Predorsal length
M13	Body height
M14	Dorsal fin base (distance between bases of the first and the last ray)
M15	Dorsal fin length (the greatest distance between front and back end of the fin)
M16	Caudal peduncle length
M17	Caudal fin length (the greatest distance from the front end to the back end of the fin)
M18	Height of caudal peduncle
M19	Anal fin base (distance between bases of the first and the last ray)
M20	Anal fin length (the greatest distance between front and back end of the fin)
M21	Width between eyes
M22	Vertical mouth gape (distance between the lips with the mouth open without stretch the muscles)
M23	Mouth orientation (tangent of the angle formed by the upper and lower lips with the mouth fully open and the horizontal axis)
M24	Horizontal mouth gape (distance between the opposite corners of the mouth)
M25	Weight
M26	Standard length

number of individuals in each group, based on the number of individuals in the smaller group using random deletion. Only comparisons with at least three individuals in each group were considered.

Given the arbitrary delimitation of Schoener's index values (Feinsinger et al. 1981), the overlap coefficient obtained was compared with an appropriate null model. Null models were constructed from 1000 data randomizations using the reorganization algorithm RA4, as proposed by Winemiller & Pianka (1990). All simulations were carried out using the EcoSim program (Gotelli & Entsminger 2004). We also performed a permutation multivariate analysis of variance (PERMANOVA), based on Bray-Curtis dissimilarity, using wetland, sampling surveys, species and gender (and their interactions) as factors to test the effect of each variable on dietary data.

Quantile regressions were carried out between individual body size (independent variable) and ingested prey richness (the number of different taxonomic groups ingested by a single fish; dependent variable) for both species to identify possible limitations in prey item ingestion for small individuals. The use of quantile regression is more appropriate than standard regression as we are testing the hypothesis that body length is a limiting factor for prey richness. Body length, therefore, may set the upper limit for prey richness rather than affecting the mean values, the relationship being triangular in shape (Gotelli & Ellison 2004). Analysis of five quantiles (10th, 25th, 50th, 75th, and 90th) was carried out using the *quantreg* package within the R v 2.9.0 statistical program (R Core Team 2013).

Variation in prey richness (dependent variable) between species and between sampling months, as well as their interactions (independent variables), were analyzed using two-way ANOVA with Monte Carlo permutation (*lmPerm* package, R; Wheeler 2010).

Morphology

Both interspecific and intraspecific morphological variable matrices were reduced through Principal component analysis (PCA; hereafter named PCA-A). This procedure was used to analyze the pattern of morphological changes along species' development. Over-orbital height was not quantified in *C. fulgens* as the eye position was inserted at the upper limit of the head.

Relationship between morphology and diet

Apart from body weight and standard length, no other morphological variable was measured in individuals used for diet analysis. In order to study associations between individual morphology and diet, therefore, morphological characteristics of individuals used in diet analysis were estimated using linear regressions

(with individual standard length as the independent variable) obtained from the morphological analysis dataset (see above – Morphological analysis). Linear regressions were performed separately for each species and for each intraspecific group (i.e. immature, male and female). Morphological variables that were not precisely predicted by standard length ($r^2 < 0.7$) were discarded from the analysis. Following estimation, the variables were standardized according to Elliott et al. (1995) to remove size component from the shape measurements. Elliot et al.'s method standardizes morphometric character measurements utilizing the following formula:

$$M_s = M_0(L_s/L_0)^b$$

where M_s is standardized measurement, M_0 is measured character length, L_s is overall mean standard length for all fish, L_0 is standard length of specimen, and b is an estimate of the slope of the regression of $\log M_0$ on $\log L_0$. This method was carried out in PAST software (Hammer et al. 2001). The standardized data were reduced over two axes using PCA (hereafter named PCA-B). All head morphological variables were included in the analysis, as were variables responsible for the main differences between the interspecific and intraspecific groups obtained by PCA-A. Note that the addition of detailed morphometric measurements from all individuals used for diet analysis would add little insight as we were interested in variation among taxonomic and demographic groups rather than among individuals within each group.

To assess whether morphological traits were related to specific prey (morphological specialization), dietary composition was used to plot individuals utilizing nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity. Only taxa that comprised at least 0.1% of total abundance were included in the analysis to avoid the influence of rare prey items on the overall results. The first two axes of PCA-B (morphological variables without size effect) and the standard length (as a measured of fish size) were fitted to the ordination using the *vegan* package *envfit* function (Oksanen et al. 2009) in the R environment (R Development Core Team 2003). The *envfit* analysis fits environmental vectors of continuous variables and centroids of levels of class variables onto an ordination. The arrow shows the direction of the (increasing) gradient, and the length of the arrow is proportional to the correlation between the variable and the ordination. This procedure was necessary as our aims tested both ontogenetic and species-specific effects. The significance of the relationship ($P < 0.05$) was assessed after 4999 permutations.

Table 2. Mean percentage (m) and frequency of occurrence (f) of prey items ingested by *Cynopoecilus fulgens* and *Austrolebias minuano*. Values in parentheses represent standard errors (SE). Food items which comprised <0.01% of abundance were not included in this list.

Prey category	<i>C. fulgens</i>				<i>A. minuano</i>			
	Male		Female		Male		Female	
	m (%)	f (%)	m (%)	f (%)	m (%)	f (%)	m (%)	f (%)
Aquatic insects (holometabolous)								
Larvae								
Chironomidae	10.97 (18.15)	65.91 (5.65)	7.82 (2.15)	63.24 (5.57)	6.29 (4.99)	66.67 (9.32)	6.64 (6.44)	59.46 (19.68)
Chaoboridae	0.00	0.00	0.00	0.00	0.00	0.00	0.22 (0.14)	2.70 (1.85)
Coleoptera	2.83 (0.77)	28.41 (4.87)	0.95 (0.42)	22.06 (6.29)	0.58 (1.85)	12.50 (12.25)	0.67 (0.57)	21.62 (4.7)
Hydroptilidae	0.37 (0.31)	5.68 (2.09)	0.09 (0.11)	2.94 (3.12)	0.13 (0.14)	4.17 (2.56)	0.22 (0.14)	5.41 (2.29)
Leptoceridae	0.00	0.00	0.00	0.00	0.00	0.00	0.78 (0.49)	2.70 (1.85)
Trichoptera morph1	0.55 (0.40)	10.23 (5.38)	0.66 (0.49)	8.82 (2.79)	0.19 (0.44)	8.33 (5.82)	0.06 (0.03)	2.70 (1.85)
Pupae								
Culicidae	0.03 (0.05)	1.14 (1.23)	0.00	0.00	0.00	0.00	0.11 (0.15)	2.70 (5.55)
Other Diptera morph1	0.22 (0.15)	7.95 (3.25)	0.09 (0.05)	2.94 (1.97)	0.19 (0.10)	8.33 (11.11)	0.33 (0.45)	5.41 (11.11)
Pupa morph1	0.00	0.00	0.14 (0.06)	1.47 (0.86)	0.00	0.00	0.00	0.00
Aquatic insects (hemimetabolous)								
Nymph								
Baetidae	0.65 (0.21)	18.18 (5.45)	0.38 (0.31)	8.82 (5.53)	0.06 (0.07)	4.17 (2.56)	0.56 (0.53)	10.81 (4.58)
Caenidae	0.22 (0.27)	4.55 (2.13)	0.05 (0.16)	1.47 (2.27)	0.00	0.00	0.11 (0.16)	2.70 (2.56)
Ephemeroptera morph1	0.37 (0.43)	12.50 (7.72)	0.00	0.00	0.19 (0.07)	8.33 (4.81)	0.50 (0.23)	16.22 (6.57)
Corixidae	0.06 (0.11)	2.27 (2.46)	0.05 (0.16)	1.47 (2.27)	0.06 (0.07)	4.17 (2.56)	0.17 (0.04)	8.11 (3.4)
Belostomatidae	0.15 (0.12)	4.55 (4.33)	0.09 (0.04)	1.47 (0.86)	0.00	0.00	0.06 (0.08)	2.70 (2.56)
Hemiptera morph1	0.00	0.00	0.00	0.00	0.13 (0.14)	8.33 (5.12)	0.17 (0.13)	5.41 (4.89)
Microcrustaceans								
Cladocera	48.21 (15.01)	81.82 (11.18)	60.98 (4.95)	89.71 (4.55)	61.39 (12.85)	91.67 (5.82)	53.68 (15.46)	94.59 (4.81)
Copepoda	21.26 (1.52)	67.05 (19.42)	15.74 (1.00)	75.00 (11.83)	13.50 (5.11)	79.17 (8.89)	18.92 (5.09)	75.68 (4.1)
Conchostraca	0.00	0.00	0.00	0.00	0.13 (0.14)	4.17 (2.56)	0.06 (0.03)	2.70 (1.85)
Ostracoda	7.09 (2.61)	47.73 (14.79)	3.56 (1.09)	30.88 (10.11)	11.75 (4.46)	54.17 (14.32)	7.65 (1.99)	64.86 (6.41)
Other Aquatic Arthropods								
Hydracarina	1.45 (0.45)	21.59 (6.28)	2.56 (0.80)	33.82 (10.14)	0.26 (0.29)	12.50 (7.69)	0.45 (0.19)	13.51 (5.35)
Dogielinotidae	3.60 (1.05)	45.45 (1.70)	5.36 (4.06)	44.12 (17.94)	3.63 (2.05)	58.33 (18.02)	6.47 (5.13)	43.24 (6.12)
Other Aquatic Invertebrates								
Nematoda	0.12 (0.16)	4.55 (3.44)	0.28 (0.13)	8.82 (2.75)	0.26 (0.41)	16.67 (9.62)	0.73 (0.33)	13.51 (9.62)
Hirudinea	0.00	0.00	0.05 (0.05)	1.47 (1.56)	0.00	0.00	0.00	0.00
Oligochaeta	0.12 (0.22)	3.41 (3.7)	0.05 (0.02)	1.47 (0.86)	0.00	0.00	0.11 (0.16)	2.70 (2.56)
Planorbidae	1.14 (0.38)	14.77 (5.83)	0.52 (0.15)	8.82 (2.73)	0.97 (0.75)	29.17 (19.54)	0.78 (0.25)	27.03 (15.7)
Mollusca morph1	0.34 (0.31)	7.95 (4.32)	0.43 (0.31)	8.82 (3.2)	0.06 (0.23)	4.17 (6.66)	0.33 (0.15)	10.81 (4.58)

Results

Dietary overlap and dietary richness

A total of 38 types of prey were found in the gastrointestinal tract of the studied species (Table 2). The diets of both species were represented mainly by microcrustaceans (Cladocera, Copepoda, Ostracoda), and immature aquatic insects (Chironomidae). Schoener's Index was higher than 0.6 in all subsamples (collection dates and wetlands), indicating high dietary overlap between *A. minuano* and *C. fulgens*. Dietary overlap was high between sexes of both species, the only C coefficients lower than 0.6 being that for *A. minuano* in June 2008 (C = 0.58) and for *C. fulgens* in wetland 5 (C = 0.52). For all collection surveys and wetlands,

the nonsignificance of null model levels ($P > 0.05$) indicated dietary overlap was higher between species and between sexes than would be expected under resource partitioning (for more details see 'Supporting information').

The PERMANOVA test revealed differentiation between the diet of *A. minuano* and *C. fulgens* ($F_{1,176} = 3.07$, $P = 0.009$). This differentiation does not appear to change between wetlands ($F_{5,176} = 1.33$, $P > 0.05$), but varies over sampling surveys ($F_{5,176} = 2.47$, $P = 0.002$). There were no differences between the sexes of both species ($F_{1,176} = 0.39$, $P > 0.05$), and this pattern hold across wetlands ($F_{3,176} = 0.77$, $P > 0.05$) and over sampling surveys ($F_{4,176} = 0.95$, $P > 0.05$). The interactions between wetland, sampling surveys and species was not significant ($F_{16,176} = 0.97$, $P > 0.05$), and this

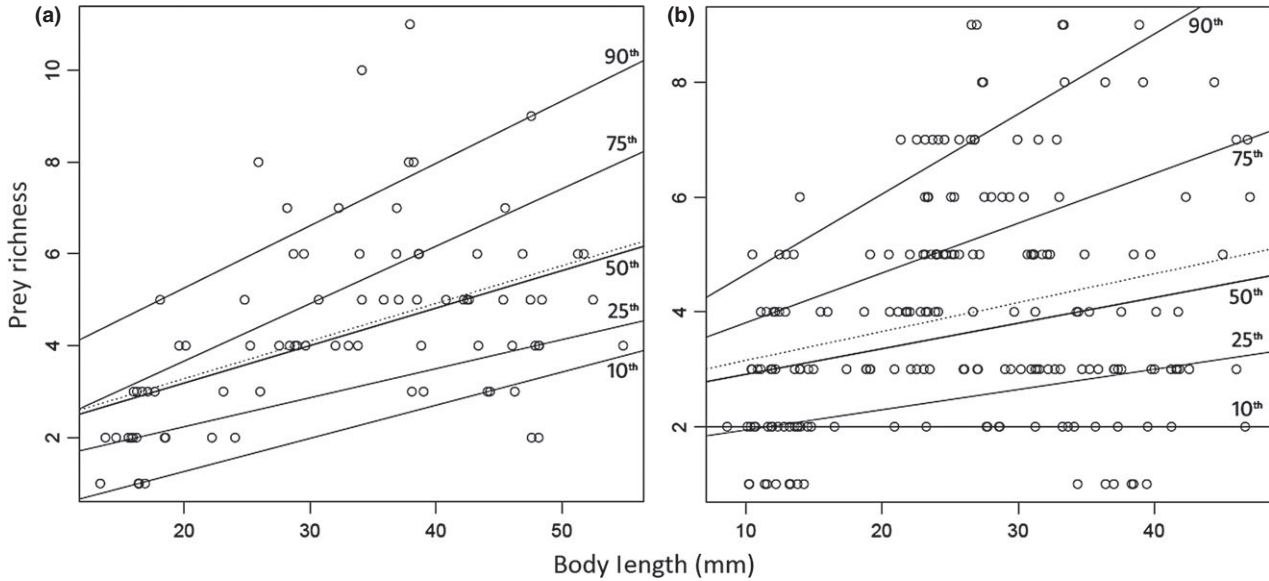


Fig. 2. Quantile Regression between body length and prey richness for (a) *Austrolebias minuano* and (b) *Cynopoecilus fulgens*.

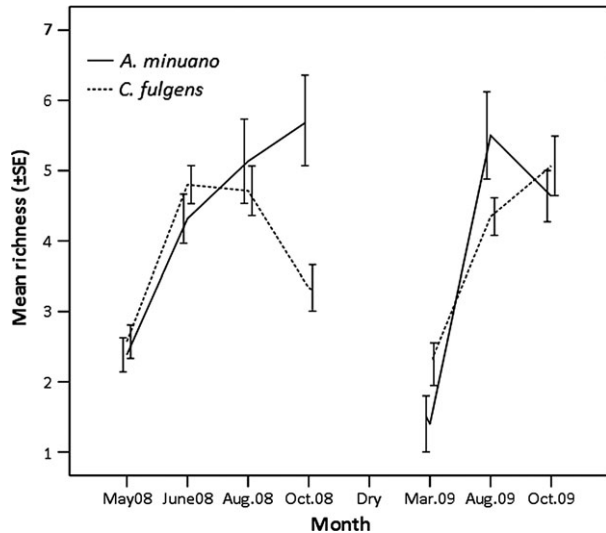


Fig. 3. Comparison of diet richness (mean \pm SE) for *Austrolebias minuano* and *Cynopoecilus fulgens* over both study years.

pattern did not change after sex was included into the analysis ($F_{1,176} = 0.77, P > 0.05$).

Both species showed increasing dietary taxonomic richness throughout their life cycles (Fig. 2). There was a significant positive relationship between dietary taxonomic richness and standard length for all quantiles, except for the 10th quantile in *C. fulgens* and the 90th quantile in *A. minuano*, which were marginally nonsignificant (for more details see ‘Supporting information’). The interaction of dietary richness between species and sampling months was significant ($F_{6,265} = 2.69, P = 0.015$), indicating that variation in dietary richness between species showed a dissimilar pattern over time. Both species

displayed a trend toward increased prey taxonomic richness over the year for both years of the study (Fig. 3), though *C. fulgens* dietary richness decreased at the end of the 2008 annual cycle (October).

Morphology

The first 2 axes explained 96.3% of total interspecific morphological variation in PCA-A (Fig. 4; for details of PCA’s scores see ‘Supporting information’). The first axis (93.6%) was related to an increase in size of all morphological structures over the life cycle of both species, and axis 2 (2.7%) was related to characteristics that distinguished both species. *Austrolebias minuano* had a deeper body, deeper head, and larger dorsal and anal fin bases, while *C. fulgens* had a more fusiform body shape, with higher values for predorsal and pre-anal length.

The first ordination axis for intraspecific PCA-A (*A. minuano*: 94.3%; *C. fulgens*: 97.6%) was related to growth of morphological structures over the fish’s life cycle, while axis 2 (*A. minuano*: 2.2%; *C. fulgens*: 1%) was related to morphological features that distinguished the sexes of both species. Whereas *A. minuano* males showed a relatively deep body and longer dorsal and anal fin bases, females had higher body weight, a larger caudal peduncle and longer predorsal and pre-anal length (Fig. 5a). In *C. fulgens*, males had longer dorsal and anal fins and a relatively deep body, while females had higher body weight and a more elongated body shape (Fig. 5b). For both species, differences between the sexes increased with fish growth.

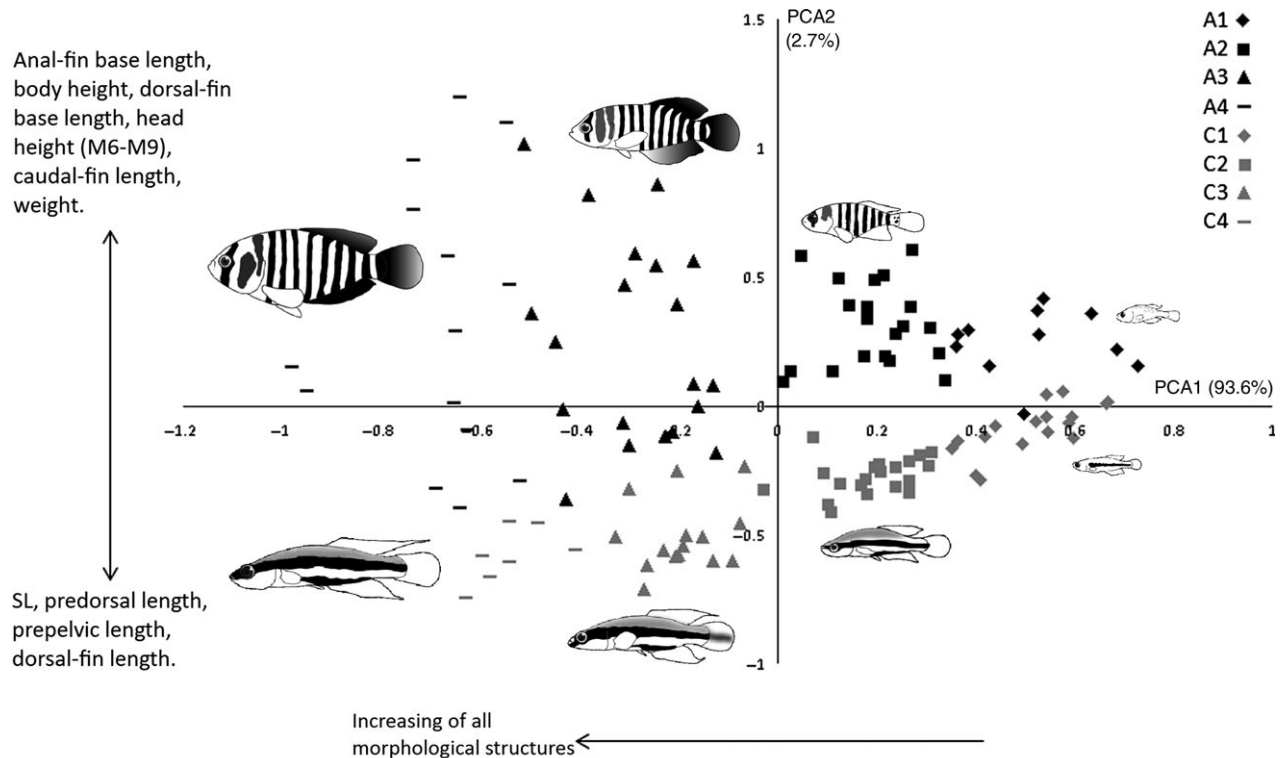


Fig. 4. PCA ordination (axis 1 – axis 2) of *Austrolebias minuano* and *Cynopoecilus fulgens*. Colors represent species and symbols represent size classes (classified just for graphic purposes). A = *A. minuano* and C = *C. fulgens*. 1 = 15–25 mm. 2 = 25–35 mm. 3 = 35–45 mm and 4 = 45–55 mm.

Relationship between morphology and diet

Despite all morphological variables having a significant relationship with standard length (univariate regressions, $P < 0.01$), six variables (M2, M3, M4, M5, M7, and M22) in the interspecific comparison, four variables in the intraspecific comparison of *A. minuano* (M2, M3, M4, and M5) and three variables in the intraspecific comparison of *C. fulgens* (M22, M2, and M5) were not included into PCAs-B due to their low coefficients of determination ($R^2 < 0.7$). All these nonincluded variables showed high individual variation and appear to have no relation to the differences among groups (sexual dimorphism, interspecific differences) as they had low contribution to the PCA-A axes. Equations generated by the linear prediction model and PCA-B's scores are listed in 'Supporting Information'.

There was a significant relationship between the first PCA-B axis (morphological variables without size effect) and diet for both inter- ($P = 0.05$, $r^2 = 0.02$, Fig. 6a) and intraspecific comparisons (*A. minuano*: $P < 0.001$, $r^2 = 0.37$, Fig. 6b; *C. fulgens*: $P = 0.04$, $r^2 = 0.04$, Fig. 6c), which led to a small variation in dietary composition. The second PCA-B axis was not significant for both inter- ($P > 0.05$) and intraspecific comparisons ($P > 0.05$).

The standard length (fish body size) showed to have influence over both interspecific ($P < 0.001$, $r^2 = 0.23$, Fig. 6a) and intraspecific comparisons (*A. minuano*: $P < 0.001$, $r^2 = 0.44$, Fig. 6b; *C. fulgens*: $P < 0.001$, $r^2 = 0.16$, Fig. 6c). Whereas some prey items, such as Cladocera and Copepoda, were consumed throughout the species' life cycle (with higher representation in smaller fish), other items, such as Amphipoda and Planorbidae, were consumed only by larger individuals (Fig. 6a,b,c).

Discussion

Two annual fish genera, *Austrolebias* and *Cynopoecilus*, sympatrically inhabit the geologically recent coastal region of southern Brazil and Uruguay. Over the Quaternary period, at least four successive transgressive–regressive sea level cycles have shaped the regional landscape (Villwock 1977); the latest transgression probably occurring during the Holocene, approximately 5500 years ago (Calliari 1998). Hence, the coexistence of *A. minuano* and *C. fulgens* may be a relatively recent evolutionary event, and the difference in body shape is most likely related to their ancestral origin. Indeed, each of the two genera has a characteristic body shape, each having evolved in two distinct environments (Nielsen 2010), *Austrolebias*

Morphology–diet relationship of annual fishes

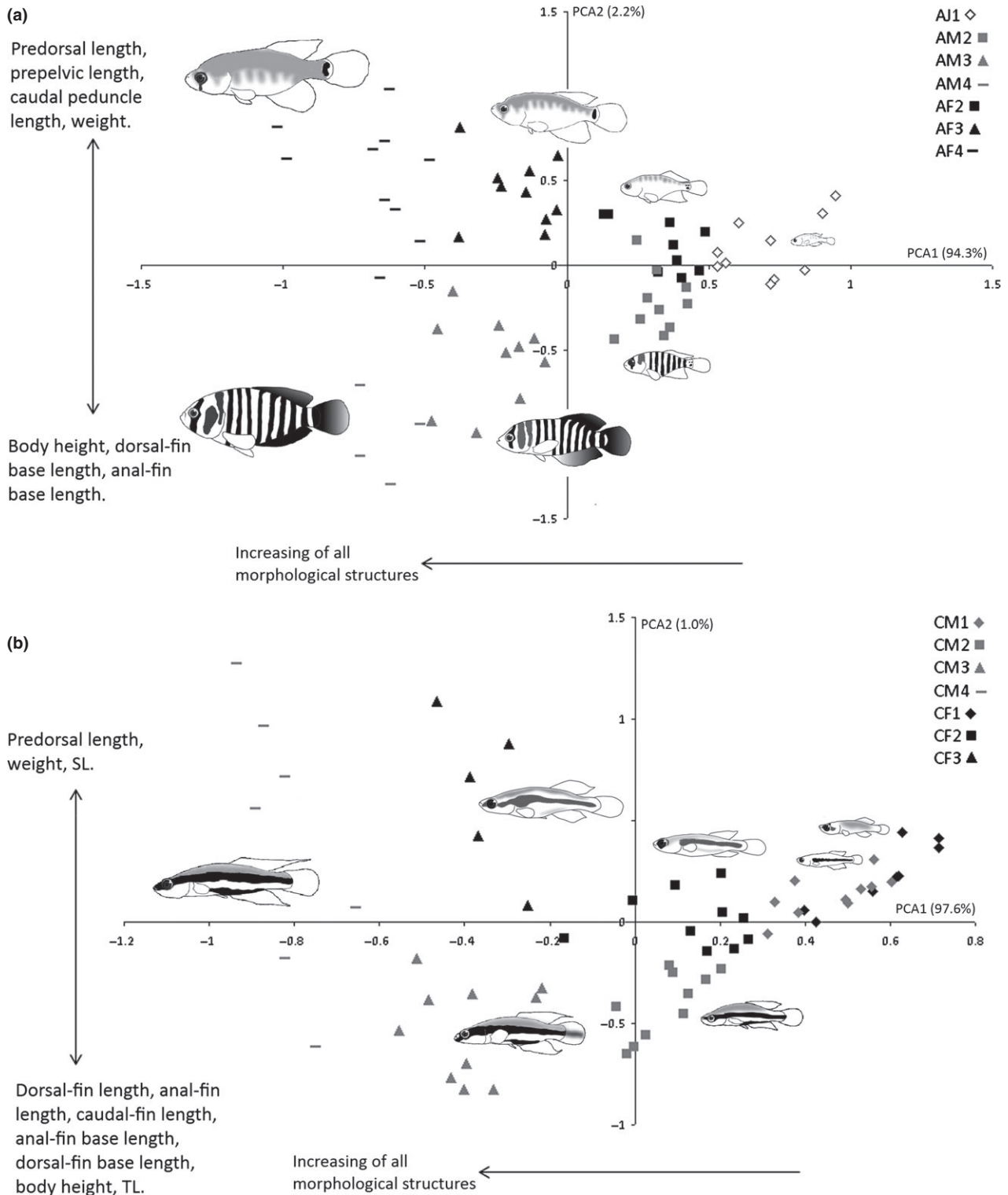


Fig. 5. PCA ordination (axis 1 – axis 2). Colors represent intraspecific groups (gray-males. black-female. white-immature) and symbols represent size classes (classified just for graphic purposes). (a) *Austrolebias minuano* (F = female. M = male and I = immature) and (b) *Cynopoecilus fulgens* (F = female. M = male.). 1 = 15–25 mm. 2 = 25–35 mm. 3 = 35–45 mm and 4 = 45–55 mm.

typically inhabiting intermittent open-field wetlands and *Cynopoecilus* having evolved in the Atlantic forest region (Nielsen 2010).

Variation in *C. fulgens* and *A. minuano* morphological traits were mainly related to species ontogeny and growth, and resulted in clear differences in

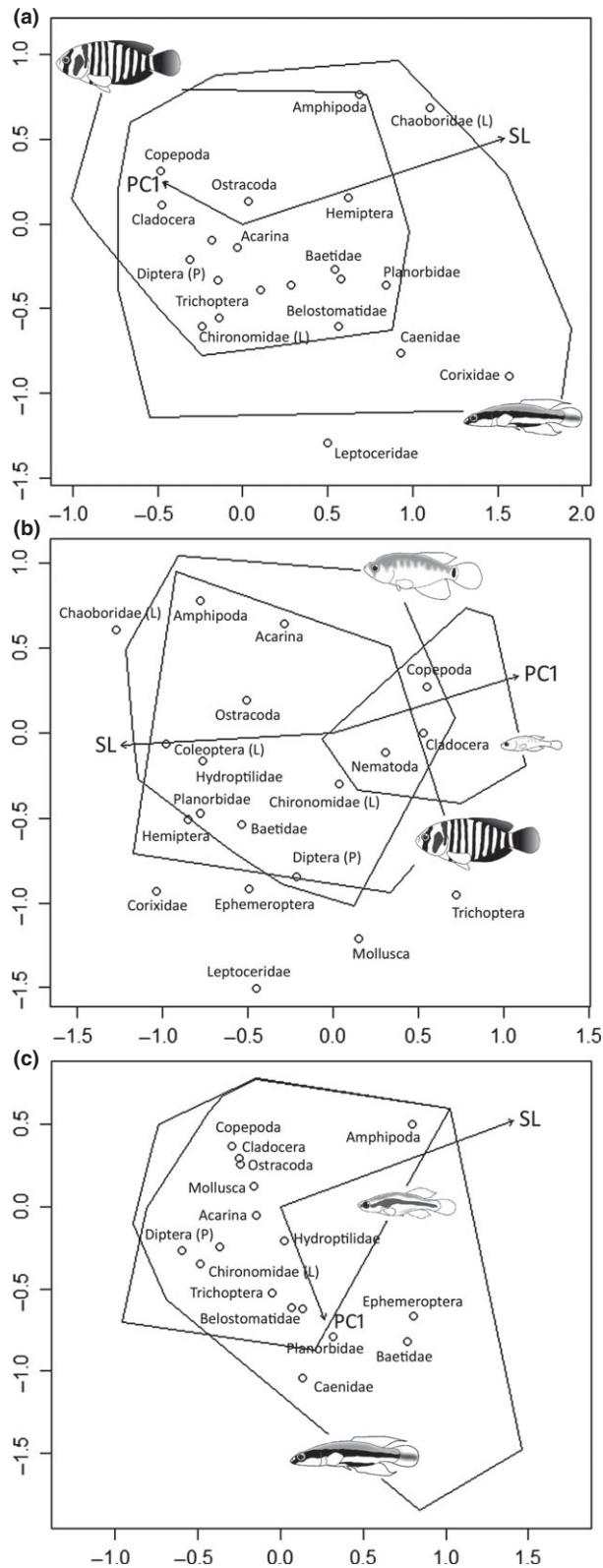


Fig. 6. Results from nonmetric multidimensional scaling analysis (NMDS) of *Austrolebias minuano* versus *Cynopoecilus fulgens* (a) and intraspecific groups of *A. minuano* (b) and *C. fulgens* (c). Fish were plotted on the basis of their diet. The arrows represent the first axe of PCA-B (transformed morphological traits to achieve size-independence) and standard length (SL).

diet. Smaller individuals of both species ingested lower prey diversity compared to larger adults. Hence, an increase in the size of key morphological structures resulted in an increase in prey item diversity. Several studies suggest that the increase in fish size enables ingestion of larger prey items due to an increase in handling efficiency and searching ability (Mittelbach 1981; Hairston & Hairston 1993; Brose et al. 2006). Ingestion of larger prey items by adults is essential to support increased energetic demands (Arim et al. 2010). Our results support the hypothesis that generalist organisms display negligible changes in dietary composition during ontogeny but a minor increase in niche width as they grow (Cassemiro et al. 2008).

Keppeler et al. (2013) suggested that the increase of ingested prey richness over the annual cycle is a reflection of an increase of annual fish body size. The small size of individuals in the beginning of the annual cycle would lead to a lower richness of ingested prey than in the middle/end part of the annual cycle when fish are larger. This difference would occur due to morphological limitations (e.g., mouth gape) of prey item ingestion in smaller individuals. As both fish density [Lanés et al. (in press)] and prey availability (Keppeler et al. 2013) are lower at the end of the annual cycle in southern Brazil wetlands, our study increases evidence of the key role of morphological structures in the seasonal increase of ingested prey richness.

We found some differences in prey ingested between species, and the dynamic of these differences appear to change seasonally. However, the magnitude of these differences was small, as the dietary overlap was high over the two study years. In this sense, the relationship between specific morphological traits and different types of prey ingested was not strong enough to decrease interspecific competition for food. The few studies that have analyzed coexistence of annual fishes have produced mixed results. Whereas Polačik & Reichard (2010) found dietary overlap in three sympatric African annual fish species (where sympatry was considerably older than in our study area; Dorn et al. 2011), Laufer et al. (2009) demonstrated differences in dietary richness and dietary composition among four annual fish species in Uruguay. Costa (2009) found strong support for trophic radiation among 15 *Austrolebias* species, as indicated by both morphological specialization and dietary composition. In contrast, Nico & Thomerson (1989) found significant dietary overlap among six species of syntopic annual fishes in the Orinoco Basin (Venezuela). However, significant variation in adult body size, body form, and spatial distribution within the pools was demonstrated, and the latter was suggested as the main factor explaining coexistence

(Nico & Thomerson 1989). A study of dietary overlap across a gradient of annual fish species richness within a restricted biogeographical area could provide illuminating insight.

The reduced body size of annual fishes is another issue that requires attention. Usually, the reduction of body size through evolution opens new possibilities of niche acquisition (Miller 1996). Thus, smaller fish could have access to food resources not available to larger fish. However, in this case, the small body of *A. minuano* and *C. fulgens*, and associated constraints related to the size and nature of prey that can be consumed, could have limited the development of pronounced resource differentiation. This morphological constraint, termed ‘gape limitation’, leads to a limitation in the acquisition of prey and could be a more important factor than energetic constraints for small animals (Arim et al. 2007). Reduced body size of annual fish may be a simple consequence of their life history, where rapid growth and fast maturation is critical (Blažek et al. 2013).

Mechanisms explaining the coexistence of annual fishes are complex. Intermittent wetlands are typically small ecosystems, with extreme fluctuations in physical and chemical conditions. Even within the same region, individual wetlands may vary in many factors, such as pool morphology, bottom composition, nutrient concentration, light availability, temperature, and pH (Williams 2006). In this case, both biotic and abiotic factors may have a strong influence on community structure (Serrano & Fahd 2005), and the role of each factor in each community probably varies. The co-existence of two or more annual fish species, therefore, could be attributable to historic events and ecosystem singularities rather than morphological specializations related to resource exploitation.

Macroinvertebrate diversity and abundance are high in the intermittent wetlands of southern Brazil (Stenert et al. 2008), and the study area represents an important feeding and resting site for many migratory bird species, important predators of annual fishes (Costa 1998, 2009). In this sense, the co-existence of *A. minuano* and *C. fulgens*, despite a high dietary overlap, may result from high overall food availability and its association with other population regulation factors (in this case predation). Local topography may represent a further strong factor allowing the coexistence of similar species (Huston 1994). While the pools themselves do not exhibit high structural complexity, the flat topography of the Rio Grande do Sul coastal plain results in high connectivity between water bodies, forming a complex matrix of habitats at the landscape scale. In this sense, the process of competitive exclusion could be considerably weaker than in isolated areas (Chesson 2000).

Austrolebias minuano is both larger and less abundant than *C. fulgens* [Lanés et al. (in press)]. Laufer et al. (2009) reported that body size differences among annual fish species may allow coexistence. As we observed high dietary overlap over the entire annual cycle, however, size difference is unlikely to be a factor minimizing food competition. The relative abundance of individuals from the two genera probably varies regionally as data from Uruguay demonstrates that *Austrolebias* fishes are more common when occurring in sympatry with *Cynopocilus* (Laufer et al. 2009; Arim et al. 2010), the opposite situation to that found in southern Brazil (Quintela et al. 2007; Gonçalves et al. 2011). At present, regional differences in relative abundance between *Austrolebias* and *Cynopocilus* are not understood.

Sexual dimorphism is a widely observed phenomenon among fishes (Ward et al. 2006), and intersexual differences in activity and habitat use may occur as a consequence (Sims et al. 2001). Three mechanisms may explain sexual dimorphism: sexual selection, fecundity selection, and ecological causation (Hedrick & Temeles 1989). In annual fishes, structural and behavioral differences between males and females are significant and mainly related to color, body size, and morphological structures (Belote & Costa 2004). Laufer et al. (2009), for example, identified male and female size difference as the main reason for diet variation in *C. melanotaenia*, *A. cheradophilus*, *A. viarius*, and *A. luteoflammulatus*. Gonçalves et al. (2011), however, found no difference in diet between males and females of *C. melanotaenia*. In our study, intersexual differences in morphological traits were not large enough to result in dietary differentiation in either species, reinforcing the view that morphological differences between sexes in these species did not evolve as a response to intraspecific competition for food.

The main morphological differences between males and females in *A. minuano* and *C. fulgens* were related to reproductive features. Males had a relatively deep body and larger anal and dorsal fins than females (features related to courtship displays), while females were characterized by a more elongate body and higher body weight (related to the presence of eggs in the body cavity). These differences were especially evident as individuals became mature. Male annual fishes display complex courtship behavior (Vaz-Ferreira & Sierra 1972; Belote & Costa 2004) related to female mate choice (García et al. 2008). Long fins and deep bodies in males, therefore, may act to amplify body-size perception by rival males in male–male competition for access to females. Sexual selection pressure is the likely reason, therefore, for morphological differentiation between the sexes in both species.

It should be noted that our study, as most other studies on annual fishes (Nico & Thomerson 1989; Laufer et al. 2009; Abilhoa et al. 2010, Contente & Stefanoni 2010, Poláčik & Reichard 2010; Gonçalves et al. 2011; Keppeler et al. 2013), is limited by relatively small sample size and unequal distribution of samples among groups. Consequently, some effects, such as diet segregation, could have been underestimated, although we believe it is unlikely a major concern in our analysis. A limited sample size in most studies on annual fish is an inevitable outcome of the trade-off between sampling effort, conservation, and ethical issues. Future studies should overcome this potential limitation, perhaps using nonlethal methods, to identify when and where particular coexistence mechanisms are most important.

Our study demonstrates that, despite clear morphological differences, the diet of *A. minuano* and *C. fulgens* shows a high degree of overlap, indicating that coexistence is not related to resource partitioning. To a certain extent, this conflicts with previous studies on resource partitioning in annual fish communities that detected both evolutionary and ecological drivers of dietary diversification (Costa 2009; Laufer et al. 2009). We suggest that the coexistence of sympatric annual fish species could be enabled by regional singularities (e.g., productivity, pond connectivity, predation levels), or chance events. From an intraspecific point of view, sexual dimorphism in both species was not associated with diet and all sex-specific differences could be attributed to reproduction. The main effect of morphology on diet was along the ontogenetic axis, with increase in size allowing these generalist fish to widen their prey spectrum. Any future studies on food webs and community dynamics in intermittent wetlands should take this finding into account.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The sampling effort in each wetland and abundance of both species of dissected fish per wetland along all sampling surveys.

Table S2. Results of Schoener's index (C) for *Austrolebias minuano* and *Cynopocilus fulgens* and for males and females of each species, for all sampling surveys.

Table S3. Results of Schoener's index (C) for *A. minuano* and *C. fulgens* and for males and females of each species, for all wetlands sampled.

Table S4. Quantile regression parameters for *A. minuano* (*A. m.*) and *C. fulgens* (*C. f.*) over the five quartiles analyzed.

Table S5. Contribution of morphological variables to axes created by PCA-A for interspecific and intraspecific comparisons.

Table S6. Contribution of morphological variables (estimated using a linear predictive model) to the relationship between diet and morphology for significant axes created by PCAs-B for interspecific and intraspecific comparisons.

Table S7. Linear predictive model generated from morphological measurements made on 64 individuals of *A. minuano* and 61 of *C. fulgens*.