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Morphological comparison of wild, farmed and hybrid specimens of two South American silversides, *Odontesthes bonariensis* and *Odontesthes hatcheri*

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Abstract

In this study, body shape of hybrid and presumptive introgressed South American silversides was studied. Body shape of *O. bonariensis* and *O. hatcheri* from wild populations and farmed stocks was compared to provide basic information on the effects of fish farming on morphometric parameters. Subsequently, wild presumptive introgressed individuals and artificially hybridized farmed individuals were morphologically analysed to assess the effects of hybridization on the same parameters. Most farmed purebred individuals were shorter and higher than their wild counterparts, which is probably due to the favourable growth conditions compared to the wild habitat. However, the results evidenced that purebred individuals were more slender than both hybrid (farmed) fish and introgressed (wild) fish. Further studies on the growth performance of hybrid *Odontesthes* will be required in order to assess whether the combination of hybridization and sterilization could produce, under farming conditions, growth performances which satisfy the requirements of aquaculture.

Keywords: external morphology, farming, hybrids, pejerrey

Introduction

The pejerrey *Odontesthes bonariensis* (Valenciennes 1835) and the Patagonian pejerrey *Odontesthes hatcheri* (Eigenmann 1909) are South American atherinopsid species of commercial and recreational importance (Somoza, Miranda, Berasain, Colautti, Remes & Strüssmann 2008; Inazawa, Hattori, Oura, Yokota & Strüssmann 2011). Species boundaries between *O. hatcheri* and *O. bonariensis* are revealed by the degree (7%) of genetic variation in the cytochrome b gene (cytb) (Conte-Grand 2012), other mitochondrial regions (12S rRNA, Cytochrome Oxidase I, and control region, Heras & Roldán 2011), and current taxonomy. Originally, both species had a disjoint distribution (Dyer 2006; Aigo, Cussac, Peris, Ortubay, Gómez, López, Gross, Barriga & Battini 2008) with *O. hatcheri* in the southwest (Andean Region) and *O. bonariensis* in the northeast (Pampean and Great Rivers Provinces, López, Menni, Donato & Miquelarena 2008). However, encouraged mainly by the potential of pejerrey aquaculture and sport fishing (Somoza *et al.* 2008), anthropogenic translocations of *O. bonariensis* into *O. hatcheri* original distribution areas were carried out during the 20th century, and have thus resulted in the formation of sympatric populations in several lakes and reservoirs of the Andean Region (Crichigno, Conte-Grand, Battini & Cussac 2013).

The low growth rates and early sexual maturation of *O. bonariensis* has limited the establishment of atheriniculture on a commercial scale (Strüssmann, Ng, Oshiro & Takashima 1993; Somoza *et al.* 2008; Inazawa *et al.* 2011). The same constraints also apply to *O. hatcheri* (Hualde, Torres, Moreno, Ferrada, Demicheli, Molinari & Luquet 2011; Conte-Grand 2012). Thus, the search for possible heterotic effects in hybrid and introgressed individuals (Rahman, Arshad, Marimuthu, Ara & Amin 2013) becomes relevant. However, the effects of hybridization on morphological characters are not clear. For example, although a number of hybridization studies in fish have been reported (Rahman *et al.* 2013), only small morphological variations have been found in a few cases of hybridization (Corse, Neve, Sinama, Pech, Costedoat, Chappaz & Gilles 2012).

The occurrence of hybridization between these two atherinopsids has been evidenced in fish held in captivity. *O. hatcheri* and *O. bonariensis* individuals kept together under spawning-inducing conditions were able to hybridize (Strüssmann *et al.* 1993) and generate fertile reciprocal hybrids (Inazawa *et al.* 2011). When two populations are largely allopatric but hybridize in a given area, a hybrid zone can be formed (Turner 1999). Multivariate analysis of morphology (Crespin & Berrebi 1999) and geometric morphometrics (Valentin, Sévigny & Chanut 2002; Tobler & Carson 2010) in pejerrey have revealed the presence of individuals with intermediate morphology in some lakes and reservoirs (Crichigno 2012; Crichigno *et al.* 2013), particularly in Lake Pellegrini (Conte-Grand 2012; Crichigno *et al.* 2013).

In this study, (i) wild and farmed *O. bonariensis* and *O. hatcheri* specimens, farmed hybrid specimens, and presumptive introgressed specimens from wild environments were morphologically and morphometrically compared to provide basic information on characters that are affected when fish are raised in captivity. Following this, (ii) hybrid and presumptive introgressed specimens were compared with purebreds to evaluate the effect of hybridization on morphological characters.

Materials and methods

Farmed individuals of *O. bonariensis* were obtained from Yoshida Experimental Station (Field Research Center, TUMSAT, Japan, water temperature 17 ± 1°C, SL = 330 to 419 mm, *N* = 15) and from Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH) rearing facilities (Argentina, water temperature 19 ± 0.5°C, SL = 202 to 294 mm, *N* = 15; Chalde, Fernández, Cussac & Somoza 2011). Both stocks were derived from Kanagawa stock (Inland Water Experimental Station, Kanagawa Prefecture, Japan).

Farmed *O. hatcheri* individuals (SL = 362 to 456 mm, *N* = 12) were derived from Ehi-M13 strain (Hattori, Oura, Sakamoto, Yokota, Watanabe & Strüssmann 2010) and were reared in the aquatic facilities of Shinagawa campus at TUMSAT (water temperature 17 ± 1°C). Hybrid fish (SL = 290 to 358 mm, *N* = 15) were obtained using one *O. hatcheri* female and one *O. bonariensis* male, from the Japanese stocks (TUMSAT, water temperature 17 ± 1°C).

Wild individuals were captured by means of angling, seine, or gillnets in three lakes; Pueyrredón (the type locality of *O. hatcheri,* SL = 120 to 390 mm, *N* = 15), San Lorenzo (a lake in the original distribution of *O. bonariensis,* SL = 202 to 240 mm, *N* = 15), and Pelegrini (the only lake with morphological intermediate individuals, Dyer 2006; Crichigno *et al.* 2013; SL = 250 to 314 mm, *N* = 8, Table 1), and identified using Dyer's (2006) dichotomous key. Some individuals were validated by means of Geometric Morphometrics (Crichigno *et al.* 2013) and mitochondrial DNA analyses (Cytb, Conte-Grand 2012).

The specific status of wild individuals was confirmed with Cytochrome Oxidase I (COI) data of *O. hatcheri* individuals (*N* = 19, SL = 92 to 233 mm) from Pueyrredón lake and public data (in www.boldsystems.org) of *O. bonariensis* individuals (*N* = 11, SL not available) from sites close to San Lorenzo lake (Salada de Monasterio, Adela, Alsina, Chascomús, and Pigüé lakes), within the original distribution area of *O. bonariensis*.

Specimens corresponding to Lake Pueyrredón, the type locality of *O. hatcheri* (Ringuelet, Arámburu & Alonso de Arámburu 1967), were deposited in *Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata,* Argentina (Voucher Museum ID: MLP 9858 to MLP 9877). The same Sample ID was used to include these individuals in The Barcode of Life Data System (Ratnasingham & Hebert 2013).

DNA of Pueyrredón lake individuals was extracted and polymerase chain reaction (PCR) and sequencing of the COI gene were performed according to standard DNA barcoding protocols (Ivanova, deWaard & Hebert 2006), using primer cocktails developed for fish (Ward, Zemlak, Innes, Last & Hebert 2005; Ivanova, Zemlak, Innes, Last & Hebert 2007). Extraction and amplification were carried

Table 1 Species and capture sites. Species presence was assessed using the Dyer (2006) key, Geometric Morphometric Analysis (Crichigno 2012; Crichigno *et al.* 2013) and/or mitochondrial DNA analyses (Conte-Grand 2012). Presumptive introgressed individuals could not be assigned to either species, exhibiting dorsal scale size (number of rows) typical of *O. hatcheri,* but dorsal fin position as expected for *O. bonariensis.* Some individuals were considered as F2 and back-crossed individuals in microsatellite DNA analyses (G. Ortí Pers. Com.)

Species	Capture site	Latitude Longitude	Mean summer air temperature (°C)	Distribution	Other silverside presence
O. bonariensis	Lake San Lorenzo	36°05′S 58°01′W	20 to 22	Original	No
Presumptive introgressed individuals	Lake Pellegrini	38°41′S 67°59′W	22	Original of *O. hatcheri*	Both
O. hatcheri	Lake Pueyrredón	47°23′S 71°55′W	12 to 14	Type locality (Ringuelet *et al.* 1967)	No

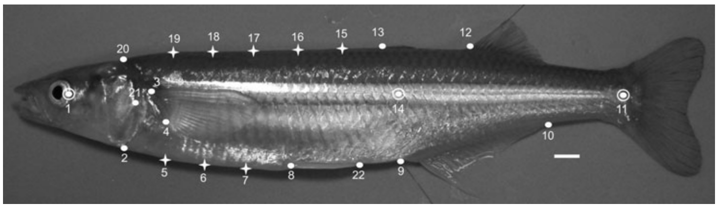

Figure 1 Landmarks (circles) and sliders (stars) digitalized on the left side of a pejerrey specimen. Landmarks 1, 11 and 14 (double circles) were used for unbending. 1: posterior border of the eye; 2: subopercular, at the lower body line profile; 3: upper anterior tip of pectoral fin base; 4: lower posterior tip of pectoral fin base; 5, 6 and 7: lower sliders (between 2 and 8); 8: anterior tip of pelvic fin base; 9: anus; 10: posterior tip of anal fin; 11: lateral line on the caudal fin base (unbending); 12: anterior tip of second dorsal fin base; 13: anterior tip of first dorsal fin base; 14: lateral line at the anus; 15, 16, 17, 18 and 19: upper sliders (between 13 and 20); 20: posterior tip of neurocranium 21: dorsal posterior process of subopercle; 22: posterior tip of pelvic fin (shown on the body). Bar = 1 cm.

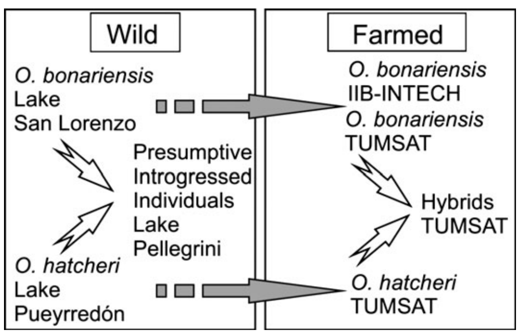

Figure 2 Schematic representation showing the comparative morphology analyses performed between groups. Grey arrows represent the comparison of wild with farmed fish, whereas open arrows represent comparison of pure species with presumably introgressed individuals, and of pure species with hybrid individuals.

out in the International Barcode of Life Argentinean reference Barcode Laboratory of CONICET at the Museo Argentino de Ciencias Naturales, Buenos Aires, Argentina. Sequencing was performed in the Canadian Centre for DNA Barcoding (CCDB) in Ontario, Canada. DNA sequences were aligned using the Muscle tool (Edgar 2004) and further double-checked visually. Taxon identification trees were performed using the Kimura two parameter (K2P) distance model (Kimura 1980). Analyses were conducted using the Maximum Composite Likelihood model (Tamura, Peterson, Peterson, Stecher, Nei & Kumar 2011). The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 630 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.* 2011).

In all cases the individuals were anaesthetized with either benzocaine solution (1:10000) or phenoxyethanol (1: 2000) for the capture of images from the left side of the body (Fig. 1) for Geometric Morphometric analyses. Length data were recorded for all individuals and weight data only for lakes Pueyrredón and Pellegrini, farmed

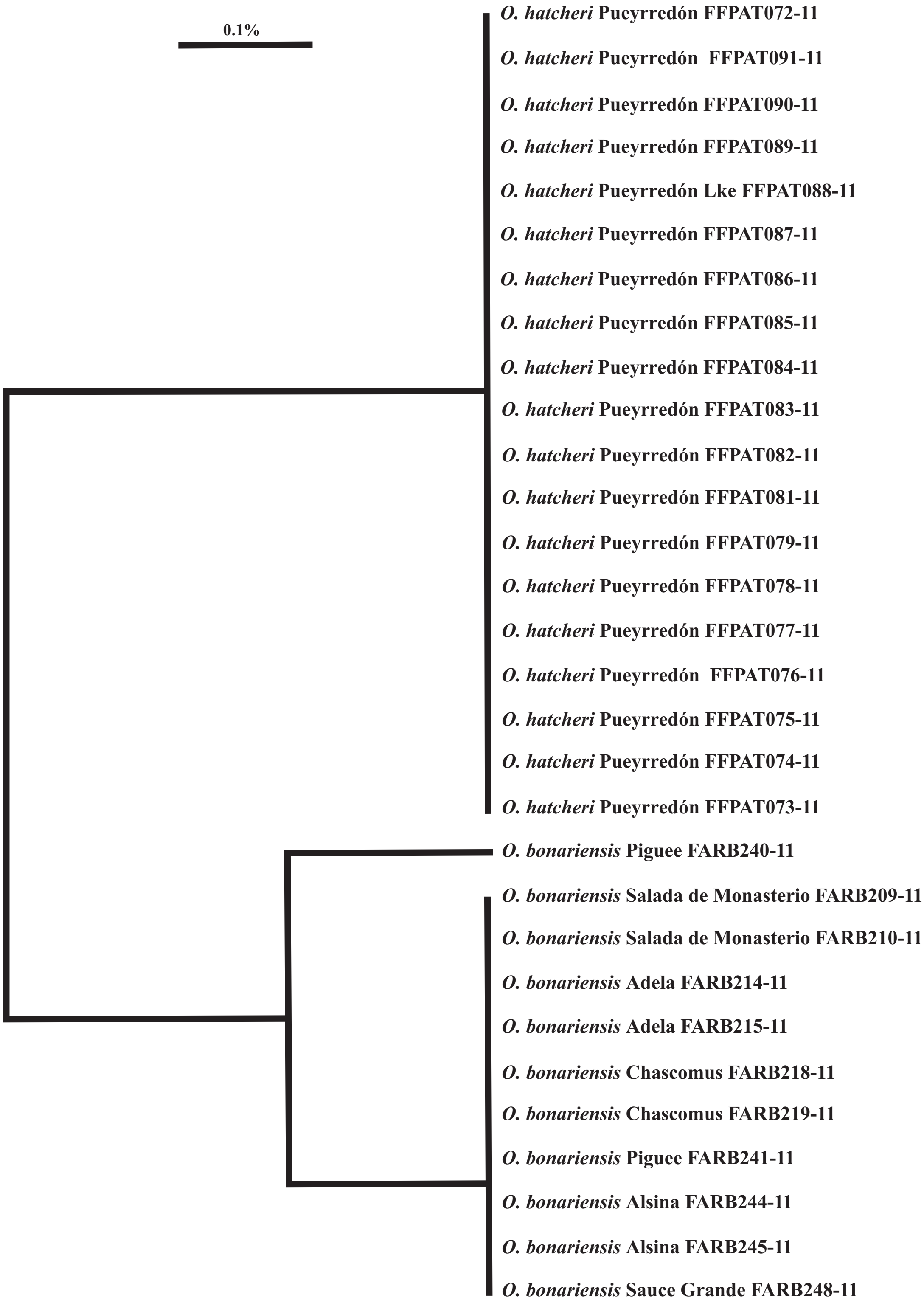

Figure 3 Maximum Composite Likelihood model tree based on Cytochrome Oxidase I (COI) data and Kimura two parameter (K2P) distance. Species, record identifications, and capture sites (Pueyrredón, Salada de Monasterio, Adela, Alsina, Chascomús and Pigüé lakes) are indicated.

Table 2 Discriminant Analysis across all (7) groups showing six significant DFs, classifying correctly 100% of cases, and explaining 94.7% of variation

Discriminant function (N = 95)	Variance explained (%)	Wilks' Lambda	Significance	Canonical correlation
1	52.1	0.003	0.001	0.933
2	17.1	0.023	0.001	0.830
3	14.2	0.075	0.001	0.804
4	9.5	0.214	0.001	0.741
5	4.3	0.474	0.001	0.597
6	2.8	0.737	0.009	0.513

O. bonariensis and *O. hatcheri*, and hybrids (TUMSAT).

Body shape was quantified by digitizing the Cartesian coordinates of 22 homologous landmarks on the left side of the body (Fig. 1) using Geometric Morphometric Analysis (GMA, Bookstein 1991; Adams, Rohlf & Slice 2004). Digitized images were first scaled to a common size and oriented using the generalized Procrustes superimposition approach (Bookstein 1991; Adams *et al.* 2004). Three homologous landmarks were used to unbend curved whole body lateral views (1, 14 and 11 slider). The consensus body shape for each population was estimated and quantified as partial warp scores using tpsRelw (http://life.bio.sunysb.edu/morph/index.html). Morphology was compared between all groups. Uniform and non-uniform components of partial warps were used in the Discriminant Analysis (DA). Mahalanobis distances for each individual were compared between groups using ANOVA. Comparisons of deformation grids between groups (Fig. 2) were performed, a new tpsRelw analysis being run for each comparison. TPSRegr 1.28 (Rohlf 2003) and polynomial regressions between discriminant functions among groups and standard length ere assayed to assess size effects. The grade of the polynomial regression was assessed on the basis of r^2 and the significance of the polynomial coefficients.

Condition factor was examined in six of seven groups analysed (fish from Lake San Lorenzo were not weighed). Condition Factor (CF) was calculated as CF = 100. Body mass (g). Standard length $(\text{cm})^{-3}$. Size effect was eliminated using unstandardized residuals of the regression of CF vs. Standard length. These residuals were subjected to parametric (one way ANOVA and Tukey) or non-parametric (Kruskal–Wallis, KW and Dunn) tests, according to equal variance verification (Levene test). Tests and plots were carried out with SIGMAPLOT (Systat Software, Inc., San Jose, CA, USA) and SPSS (Armonk, NY, USA).

Results

Confirmation of the specific status of O. hatcheri and O. bonariensis

Kimura two parameter (K2P) distance model based on COI gene generated a tree (Fig. 3) with *O. hatcheri* and *O. bonariensis* clustered separately (between group distance = 0.031).

Morphological comparison between all groups

The GMA for all individuals explained 53.52% of variation with the first two RWs (N = 95, RW1 = 37.75; RW2 = 15.77). Deformation grids show, in RW1, shorter higher to longer flatter individuals, with the first dorsal fin position varying from onto the anus to anterior to anus. In RW2, individuals were longer flatter to shorter higher. Regarding the size effect, TPSRegr test for common slopes was not significant (Wilks′ Lambda = 0.013, P = 0.75).

DA separated all seven groups, showing six significant DFs, which in turn classified 100% of cases correctly and explained 94.7% of variation (Table 2, Fig. 4). The large overlap between groups and the small sample sizes were in agreement with the lack of significant differences between groups for Mahalanobis distances extracted from step way DA (N = 95, H = 8.845, $P > 0.182$). The significant relationships of DF1, DF2, DF3 and DF6 with SL were considered (Table 2, Fig. 5). Thus, it can be seen that the *O. bonariensis* individuals from IIB-INTECH, *O. bonariensis* individuals from lake San Lorenzo, presumptive introgressed individuals from lake Pellegrini, and hybrid individuals from TUMSAT differentiated from the others.

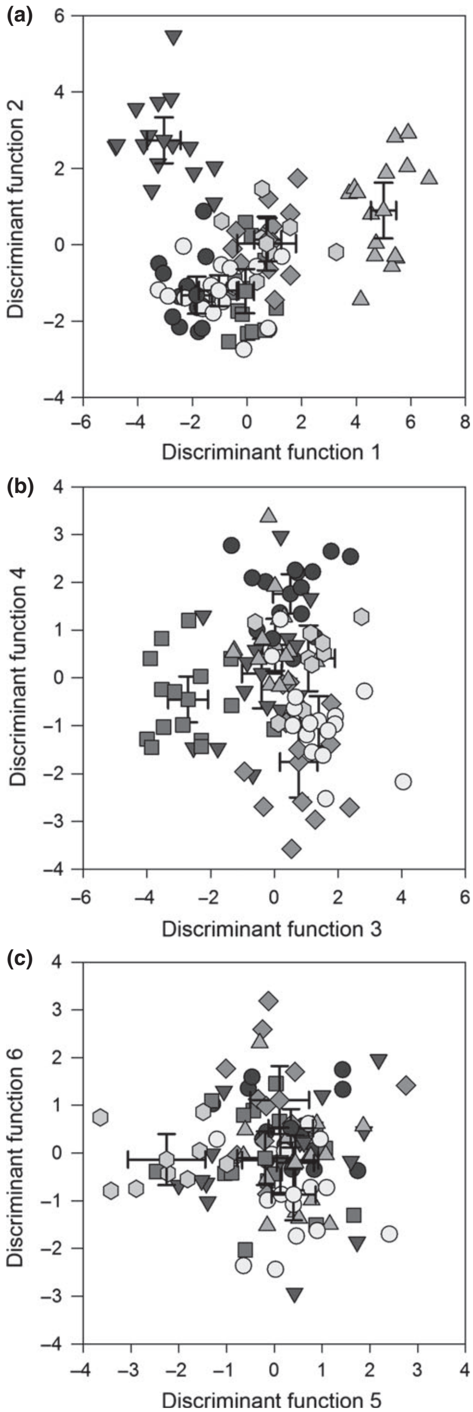

Figure 4 Discriminant analysis of all individuals. (a) DF1 vs. DF2, (b) DF3 vs. DF4, (c) DF5 vs. DF6. *O. bonariensis* individuals farmed at TUMSAT (●); *O. bonariensis* individuals farmed at INTECH (▼); *O. bonariensis* individuals from San Lorenzo Lake (■); *O. hatcheri* individuals farmed at TUMSAT (◆); *O. hatcheri* individuals from Pueyrredón Lake (▲); presumptive introgressed individuals from Pellegrini Lake (⬢); hybrid individuals farmed at TUMSAT (●). Means and 95% confidence intervals are indicated.

Shape changes: wild and farmed fish

Deformation grids of wild (Lake San Lorenzo) vs. farmed (IIB-INTECH) *O. bonariensis* (see Fig. 2 grey arrows) showed longer flatter (wild) versus shorter higher (farmed) individuals (Fig. 6a). Wild *O. bonariensis* (Lake San Lorenzo) compared with farmed *O. bonariensis* (TUMSAT) showed individuals with the margin of the neurocranium in the anterior (wild) or posterior position (farmed), shorter pectoral fin base (wild), and shorter pelvic fin (wild, Fig. 6b). Wild (Lake Pueyrredón) vs. farmed (TUMSAT) *O. hatcheri* showed longer flatter (wild) or shorter higher individuals (farmed, Fig. 6c).

Effect of hybridization and introgression on body shape (white arrows in Fig. 2)

The results shown by deformation grids are summarized in Table 3 and Fig. 7. The comparison of CF showed a significantly higher value for the farmed *O. bonariensis* of IIB-INTECH (KW, $n = 81$, $H = 47.980$ $P < 0.001$, Dunn, $P < 0.05$, group 4 in Fig. 8). When this group was eliminated from the analysis, in consideration of a particular husbandry effect, other differences arose: the CF of farmed hybrids (TUMSAT) was higher than those of farmed *O. bonariensis* (TUMSAT), farmed *O. hatcheri* (TUMSAT), and wild *O. hatcheri* (Lake Pueyrredón) individuals (ANOVA, $n = 61$, $F = 10.280$, $P < 0.001$, Tukey, $P < 0.05$, Fig. 8). In addition, the CF of wild presumptive introgressed individuals (Lake Pellegrini) was higher than those of farmed *O. bonariensis* (TUMSAT) and farmed *O. hatcheri* (TUMSAT) (Tukey, $P < 0.05$, Fig. 8).

Discussion

The results obtained by GMA and DA evidenced the occurrence of two main morphologies among the specimens examined in this study: slender or thicker individuals, each with a different dorsal fin position, a character used for taxonomic discrimination at species level in atherinopsids (Dyer 2006). Remarkably, both wild presumptive introgressed individuals and farmed hybrids were

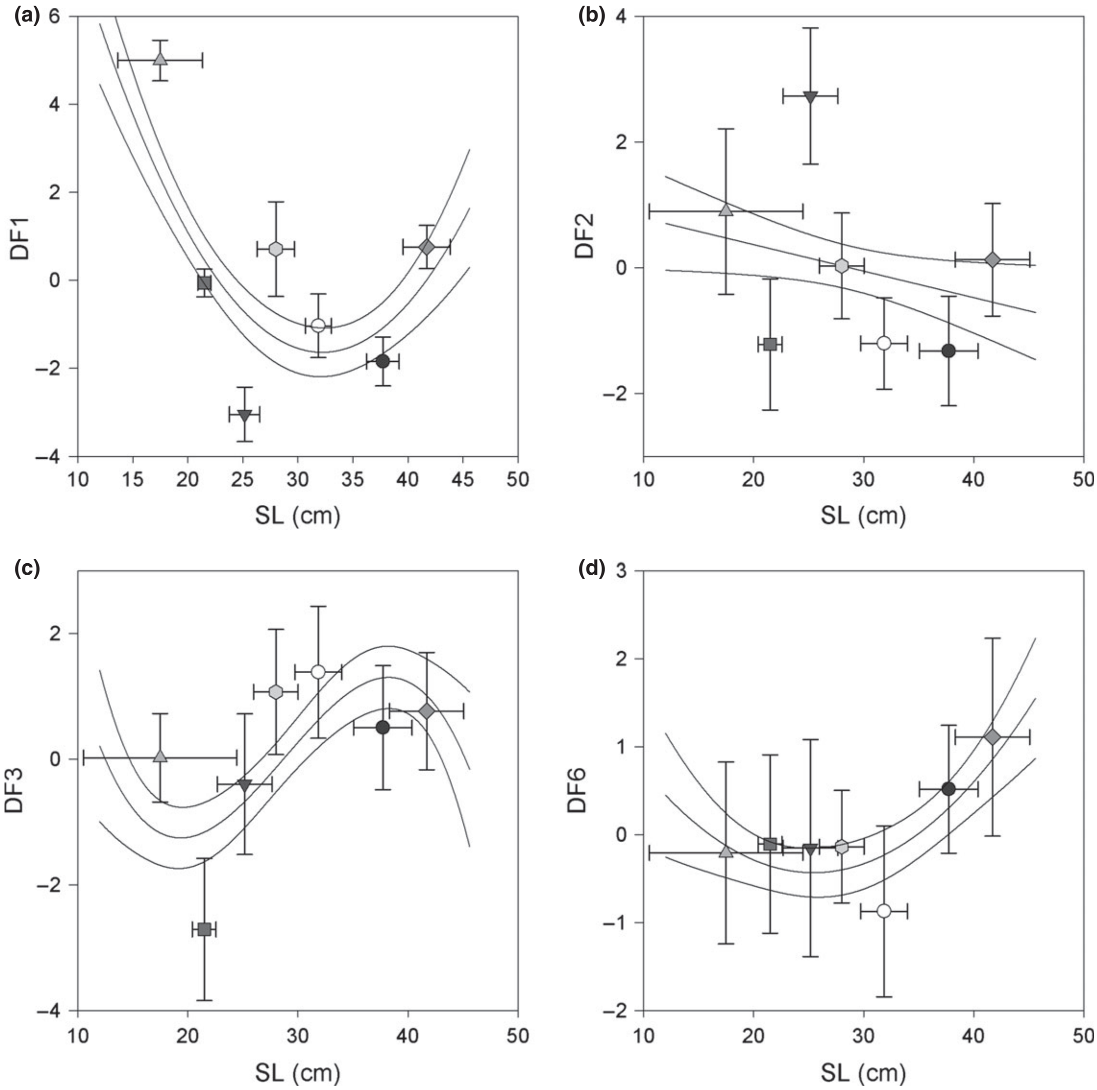

Figure 5 (a), (b), (c) and (d) Discriminant Functions (DFs) with significant correlation with SL. Fitted polynomial line, centroids, and 95% confidence intervals are indicated [*O. bonariensis* individuals farmed at TUMSAT (●); *O. bonariensis* individuals farmed at INTECH (▼); *O. bonariensis* individuals from San Lorenzo Lake (■); *O. hatcheri* individuals farmed at TUMSAT (◆); *O. hatcheri* individuals from Pueyrredón Lake (▲); presumptive introgressed individuals from Pellegrini Lake (●); hybrid individuals farmed at TUMSAT (●)].

shorter and higher, with higher CF, than both wild and farmed IIB-INTECH individuals of *O. bonariensis*. Moreover, at TUMSAT, the CF showed higher values for farmed hybrids when compared either with farmed *O. bonariensis* or farmed *O. hatcheri* individuals.

The rearing environment can affect not only the biochemical composition of the flesh (Johnston, Li, Vieira, Nickell, Dingwall, Alderson, Campbell & Bickerdike 2006) but also many morphological or morphometric characters. When compared to wild organisms, those reared in captivity usually present a thicker or taller trunk and a less elongated head, due to nutritional or feeding differences (Laird & Needham 1990; Fleming, Jonsson & Gross1994; Arechavala-Lopez, Sanchez-Jerez, Bayle-Sempere, Sfakianakis & Somarakis 2011; Rogdakis, Koukou, Ramfos, Dimitriou & Katselis 2011). Particularly, Strüssmann and Takashima (1989) observed size and histological effects of incubation and rearing temperatures on first-feeding *O. bonariensis* larvae, and Chalde *et al.* (2011) documented the depen-

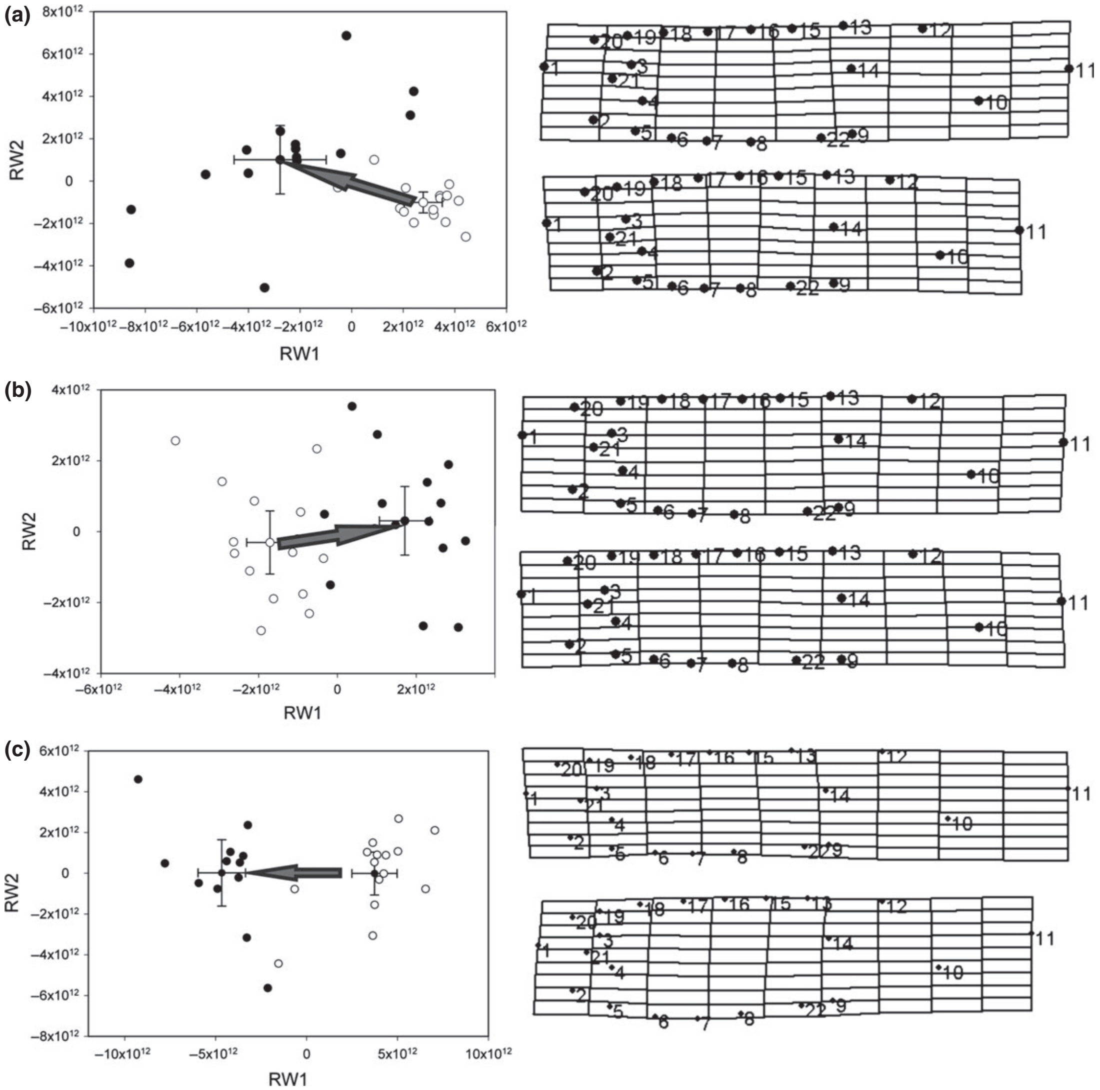

Figure 6 RW1 and RW2 in wild and farmed fish. Grey arrows run from wild (white circles and upper deformation grids) to farmed fish (black circles and lower grids). (a) Wild Lake San Lorenzo *O. bonariensis* to farmed IIB-INTECH *O. bonariensis*; (b) Wild Lake San Lorenzo *O. bonariensis* to farmed TUMSAT *O. bonariensis*; and (c) Wild Lake Pueyrredón *O. hatcheri* to farmed TUMSAT *O. hatcheri*. Means and 95% confidence intervals are indicated.

Table 3 Shape comparison between wild pure species and presumptive introgressed individuals and between farmed pure species and farmed hybrids

Shape	Compared with	Observation
Longer flatter wild *O. bonariensis* individuals (Lake San Lorenzo)	Shorter higher wild presumptive introgressed individuals (Lake Pellegrini).	Slender individuals had the anterior landmarks more retracted than fatter individuals (Fig. 5a).
Longer flatter wild *O. hatcheri* individuals (Lake Pueyrredón)	Shorter higher wild presumptive introgressed individuals (Lake Pellegrini).	Slender individuals had the anterior landmarks higher up than fatter individuals (Fig. 5b).
Slender *O. bonariensis* individuals from farmed stocks (TUMSAT)	Shorter higher farmed hybrids (TUMSAT).	Slender individuals had shorter pectoral fin base than fatter individuals (Fig. 5c).
Farmed *O. hatcheri* individuals (TUMSAT) with the first dorsal fin located more anterior and the pelvic fin more posterior	Farmed hybrids (TUMSAT).	(Fig. 5d).

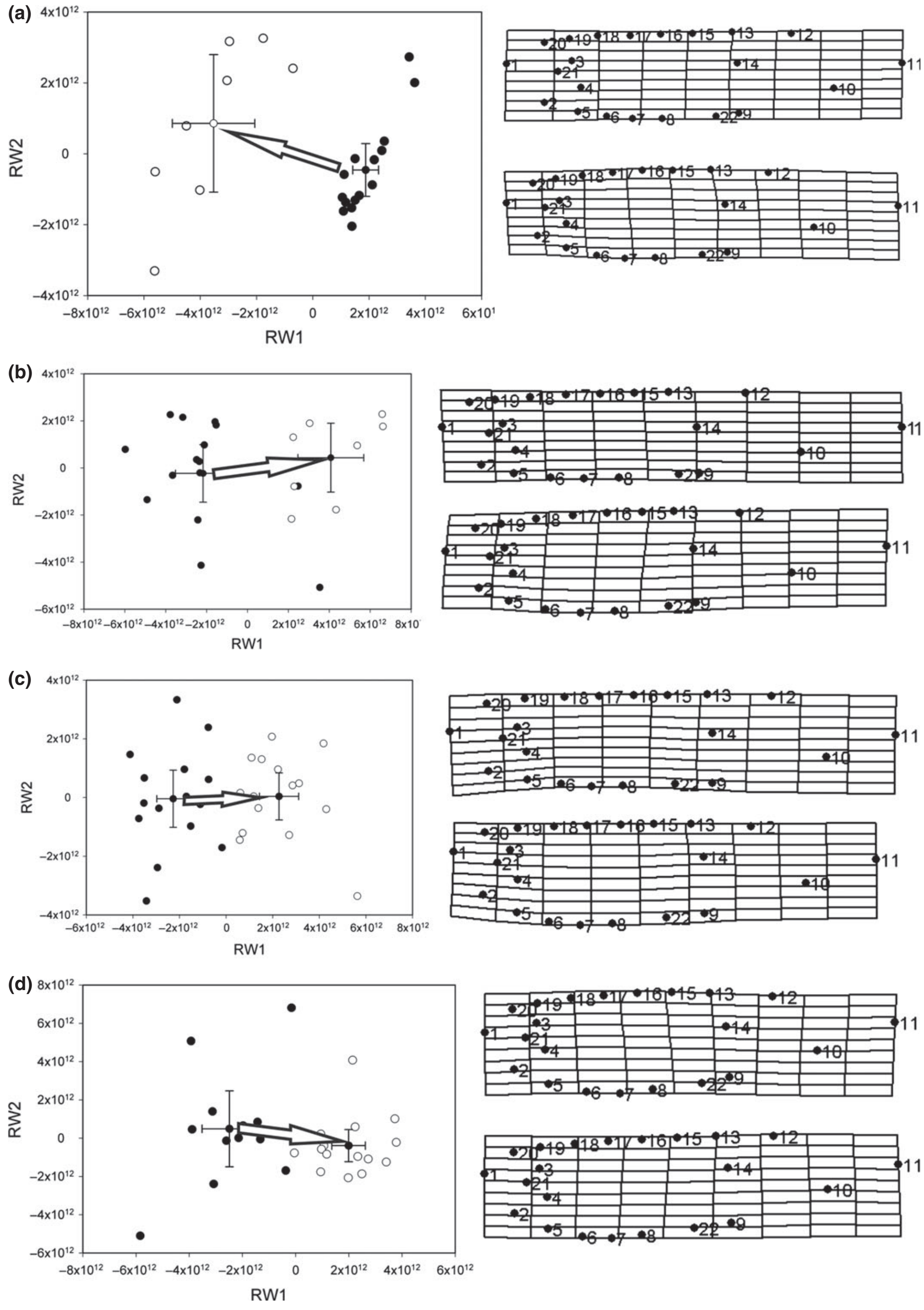

Figure 7 RW1 and RW2 for shape effects of hybridization. White arrows run from purebreds (black circles and upper deformation grids) to hybrid or introgressed fish (white circles and lower grids). (a) Wild individuals from Lake San Lorenzo (*O. bonariensis*) to wild individuals with intermediate characteristics (Lake Pellegrini), (b) wild individuals from Lake Pueyrredón (*O. hatcheri*) to wild individuals with intermediate characteristics from Lake Pellegrini, (c) farmed *O. bonariensis* (TUMSAT) to farmed hybrid fish (TUMSAT) and (d) farmed *O. hatcheri* (TUMSAT) to farmed hybrid fish (TUMSAT).

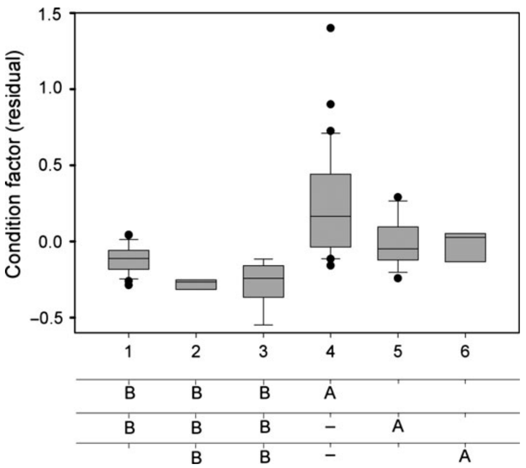

Figure 8 Box and whisker plots (median, quartiles and data outside 5 and 95 percentiles) for the comparison of residual CF (unstandardized residual from the regression of CF vs. SL) between 1: Wild *O. hatcheri* individuals from Lake Pueyrredón, 2: Farmed *O. hatcheri* individuals from TUMSAT, 3: Farmed *O. bonariensis* individuals from TUMSAT, 4: Farmed *O. bonariensis* individuals from IIB-INTECH, 5: Farmed hybrid individuals from TUMSAT, and 6: Wild individuals with intermediate characteristics from Lake Pellegrini. Different letters in the same line indicate a significant difference.

dence of *O. bonariensis* larval morphology on temperature. Crichigno, Battini and Cussac (2012) documented the dependence of newly hatched *O. hatcheri* free embryo morphology on temperature, and the induction of morphological changes by controlled feeding in juveniles, in agreement with Battini, Alonso and Cussac (1995) who pointed out the relationship between larval morphology and nutritional status in the same species. Crichigno *et al.* (2013) related morphological variation in *O. hatcheri* to environmental cues such as the availability of littoral shelter.

Biological constraints for pejerrey farming, such as low growth rates and early maturation, may require considerable effort to solve (Somoza *et al.* 2008). The use of hybrids has promoted interest (Strüssmann, Akaba, Ijima, Yamaguchi, Yoshizaki & Takashima 1997) and this is reinforced by our results, since the hybrids and presumptive introgressed individuals were more robust and presented higher CF values than purebreds. Under laboratory conditions, hybrids also showed absence of body deformations at high temperature during early life, which are common in purebred *O. hatcheri* (at 25°C or higher) and *O. bonariensis* larvae (at 29°C or higher), suggesting that hybrids have higher tolerance to high temperatures during development (Inazawa *et al.* 2011). In agreement with previous information (Heras & Roldán 2011; Conte-Grand 2012), COI results showed a clear genetic differentiation between *O. hatcheri* individuals of lake Pueyrredón and *O. bonariensis* individuals of Pampean lakes. On the basis of morphological and genetic data (cytb and microsatellite markers), Conte-Grand (2012), Crichigno *et al.* (2013), and recent results of E. Rueda (personal communication) agree on considering a hybrid zone in the original distribution area of *O. hatcheri*. In this context, further studies on the growth performance of hybrid *Odontesthes* will be required in order to assess whether the combination of hybridization and sterilization (Inazawa *et al.* 2011) could produce, under farming conditions, growth performances which satisfy the requirements of aquaculture.

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