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Influence of pejerrey *Odontesthes bonariensis* (Valenciennes, 1835) broodstock age on gamete quality, reproductive performance and plasma sex steroid levels during the spawning season

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Abstract

This study evaluated the effect of pejerrey *Odontesthes bonariensis* (Valenciennes, 1835) broodstock age on gamete and spawning quality and its relationship with sex steroid plasma levels. Sperm was analysed using a computer-assisted sperm analysis (CASA) developed for pejerrey. Semen samples were taken from all fish from mid age groups (5- and 7-year fish), but it was not possible to obtain stripped semen samples from all fish of younger (3-year) or older (10-year) groups. The highest relative sperm weight and sperm concentration were recorded in 5- and 7-year groups respectively, while viability was not different between age groups. It was not possible to identify an effect of age on sperm quality by CASA. Non-viable spawned eggs were obtained in the 3-year group and, the relative fecundity and the relative number of fertilized eggs decreased with age in other groups. Fertilization and hatching rates showed the highest values in the 10-year group. However, the estimated number of hatched larvae was similar in 5-, 7- and 10-year fish. A clear effect of age on 11 ketotestosterone (11-KT) and estradiol (E_2) levels was identified, with the highest values in the 5- and 7-year groups. These results might be related to the onset of puberty in the 3-year group and ageing in the 10-year group. Considering that the estimated number of larvae obtained was similar among age groups, the results of this study suggest that broodstock maintenance cost could be reduced by using mid-age fish rather than older fish in pejerrey hatcheries.

Keywords: ageing, computer-assisted sperm analysis, gamete quality, sex steroids, puberty

Introduction

Efficient broodstock management and production of high-quality gametes in hatcheries are essential to produce fry to support the development of aquaculture production for any fish species. The supply of larvae and fry depends both on the quality of oocytes and the quality of sperm produced by broodstock (Bromage 1995).

The quality of fish gametes, both in the wild and in captivity, may be highly variable, even more in fish that are multiple spawners and have a long breeding season (Brooks, Tyler & Sumpter 1997). Some of these differences may be due to changes in female nutrition, physiology and environmental cues related to fish reproduction (Mylonas, Papadaki, Pavlidis & Divanach 2004; Papadaki, Papadopoulou, Siggelaki & Mylonas 2008; Bobe & Labbé 2010; Miranda, Chalde, Elisio & Strüssmann 2013). Another important factor that affects egg and sperm features is broodstock age (Brooks *et al.* 1997; Kamler 2005; Getinet 2008; Kanuga, Benner, Doble, Wilson-Leedy, Robison & Ingermann 2011; Jerez, Rodríguez, Cejas, Martín, Bolaños & Lorenzo 2012). Egg chemical composition changes with age of female broodstock.

For example, lipids and proteins amounts increase in middle-age females, and decrease in older ones (Kamler 2005). In some species, egg diameter and fecundity rate increase with broodstock age (Coward & Bromage 2000; Marteinsdottir & Begg 2002; Getinet 2008; Quintero, Hutson, Chaimongkol, Davis, Dunham & Abebe 2009), and larger eggs results in longer larvae with higher growth and survival rates (Miller, Herra & Leggett 1995; Chambers & Waiwood 1996; Trippel 1998). However, the highest fecundity and fertilization rates were obtained in young gilthead seabream (*Sparus aurata* Linnaeus, 1758) females (Jerez *et al.* 2012).

In the case of males, relatively few studies have been carried out to understand the effect of ageing on sperm quality, perhaps because the oocyte quality has been considered as the main component that directs the breeding success (Rurangwa, Kime, Ollevier & Nash 2004). Sperm concentration, velocity and motility are some of the semen characteristics that affect fertilization rate (Rurangwa *et al.* 2004; Bobe & Labbé 2010; Cabrita, Sarasquete, Martínez-Páramo, Robles, Beirão, Pérez-Cerezales & Herráez 2010; Fauvel, Suquet & Cosson 2010). In this sense, Casselman and Montgomerie (2004) observed that sperm swimming velocity increased with male age in the bluegill (*Lepomis macrochirus* Rafinesque, 1819), while old guppy males (*Poecilia reticulata* Peters, 1859) produced larger reserves of strippable sperm with low velocity (Gasparini, Marino, Boschetto & Pilastro 2010). Otherwise, in zebrafish (*Danio rerio* Hamilton-Buchanan, 1822) it was observed that male ageing is not associated with sperm quality, but alter reproductive behaviour decreasing the breeding success (Kanuga *et al.* 2011).

Pejerrey, *Odontenthes bonariensis* (Valenciennes, 1835), is one of the most appreciated native fish in Argentina, mainly for sport fishing and flesh quality (Somoza, Miranda, Berasain, Colautti, Remes Lenicov & Strüssmann 2008). Pejerrey, is a multiple spawning with a major breeding period from August until November in the wild (Elisio, Chalde & Miranda 2014). Nevertheless, under controlled temperature (ranging from 16 to 20°C) it is possible to obtain fertilized eggs, practically, along all the year (Miranda, Berasain, Velasco, Shirojo & Somoza 2006). Although, it should be noted, that the increase in the daylight length also promotes pejerrey reproduction (Miranda, Strüssmann & Somoza 2009). Despite the importance of this fish species, pejerrey aquaculture is employed only to repopulation of wild stocks (Somoza *et al.* 2008; Campanella, Gárriz, Colautti, Somoza & Miranda 2013). Two of the major difficulties for its cultivation are the low production of eggs per spawning and the lack of spawning synchrony among females (Somoza *et al.* 2008). For these reasons, a large number of parental stocks are required to meet the egg demand, and a hatchery operator has to maximize seed output by exploiting broodstock reproductive potential. Moreover, it is not known if pejerrey, in the maximum of its life (assumed 10 years) do or do not reproduce (Ringuelet 1943).

In this context and in pursuit of better broodstock management, the aim of this study was to evaluate how the age of pejerrey broodstock can affects gametes and spawning quality parameters, using a specific CASA system to analyse the sperm. Furthermore, the study was designed to test the hypothesis that broodstock ageing is associated with changes in reproductive function, altering sex steroids production. For this reason, the parameters analysed were related with 11-ketotestosterone (11-KT) and estradiol (E_2) plasmatic levels in males and females respectively.

Materials and methods

Broodstocks and rearing conditions

Pejerrey groups of five males and five females of 3-; 5-; 7- and 10-years (see Table 1) were selected from the broodstocks of the *Instituto de Investigaciones Biotecnológicas – Instituto Tecnológico de Chascomús* aquaculture facilities (*Chascomús, Buenos Aires, Argentina*) on July of 2011. Sample period was extended from August to December, encompassing the principal reproductive activity of pejerrey. Fish were kept in 3000 L circular indoor tanks with flow-through underground water with 15 g L^{-1} salinity, oxygen concentration of 7.94 ± 0.85 mg L^{-1} (mean ± SD) and natural photoperiod (ranging from 10.5 up to 14.5 hours of light). Temperature was recorded every hour using waterproof electronic data loggers (Thermochron® iButton, Sunnyvale, CA, USA). Tank water temperature did not vary considerably during the experimental period, increasing from 16.4 ± 0.9°C in August to 18.6 ± 0.7°C in December (mean ± SD).

Table 1 Total length and weight of pejerrey broodstocks at the beginning of the study

	Females		Males	
Age (years old)	Weight (g)	Total length (cm)	Weight (g)	Total length (cm)
3	196.2 ± 13.9	28.8 ± 0.5	176.0 ± 15.2	28.0 ± 0.5
5	295.0 ± 20.5	31.9 ± 0.6	243.6 ± 17.0	30.0 ± 0.5
7	428.6 ± 25.4	37.6 ± 0.7	375.8 ± 42.6	37.7 ± 1.2
10	654.2 ± 50.2	43.3 ± 1.3	584.6 ± 38.4	41.7 ± 1.2

Means ± SEM (n = 5).

Fish were hand-fed to satiation three times a day with a commercial 3 mm pellet (Protein: 42.9%; Lipids: 1.5%; Carbohydrates: 43.8% Phosphorus: 2.9%; Shullet, Bs. As., Argentina).

Sperm quality measurements

Sperm samples were taken in mid-August, -September, -October, -November and -December. On each sampling date, the total sperm was stripped from each male (n = 5) by abdominal massage, collected with a syringe, drawn into a capped preweighed tube, weighed and kept on ice until used. The total sperm obtained from each male was relativized per fish weight (relative sperm weight). Since, it was already demonstrated that pejerrey spermatozoa are motile in hypoosmotic to slightly hyperosmotic media in relation to the seminal fluid (Renard, Strüssmann, Ling & Takashima 1994), all samples were diluted in tap water. Sperm concentration was determined counting spermatozoa in a 1000-fold dilution with tap water, using a Neubauer chamber under microscope (10X magnification). Sperm viability was assessed in another subsample of 1 μL of sperm diluted with 500 μL of Mounib modified solution (127 mM $NaHCO_3$, 159 mM sucrose, 0.025 g mL^{-1} reduced glutathione; pH: 8; osmolality 400 mOsm kg^{-1}; Lichtenstein, Elisio & Miranda 2010) and stained with eosin (10%) during 5 min as was described by Lahnsteiner, Berger, Weismann and Patzner (1996). About 100 spermatozoa were counted under a light microscope (10X magnification). The percentage of eosin-impermeable (non-staining) of total spermatozoa was estimated and considered as viable.

A CASA system was developed for the first time in pejerrey following Wilson-Leedy and Ingermann (2007) and Sanches, Bombardelli, Marcs, Neumann, Rebechi, de Toledo and Romagosa (2010) guidelines. The parameters analysed were: Motility (%), VCL (curvilinear velocity), VSL (straight line velocity) and VAP (velocity path average); using the configuration shown in Fig. 1. A subsample of 1 μL of semen was activated at room temperature (20°C) with 2 mL of tap water to record the videos. After gentler stirring, 10 μL of this dilution was quickly placed in a Neubauer chamber and covered with a coverslip (24 mm × 24 mm), previously pre-coated with 1% polyvinyl alcohol solution and dried at 60°C to avoid the adhesion of spermatozoa (Kime, Ebrahimi, Nysten, Roelants, Rurangwa, Moore & Ollevier 1996). Videos of each sperm sample were captured using a Basler 602fc camera (Ahrensburg, Germany) attached to a trinocular Olympus (Tokyo, Japan) CX 41 microscope at 10X magnification. The videos were recorded during 35 s at a rate of 100 frames s^{-1} in format '.avi' using the software AMCAP (Basler Vision Technologies, Ahrensburg, Germany). The videos were edited with the software VIRTUALDUB-1.9.0 (virtualdub.org) and exported as a sequence of images in format '.jpg'. The images corresponding to 1 s of video were edited in the software IMAGEJ (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) and compiled using the application CASA (University of California and Howard Hughes Medical Institute, USA). The sequences analysed were seconds 9 and 29 after activation.

Oocytes and eggs quality measurements

Oocytes samples were taken at mid-August, -September, -October, -November and -December. On each sampling date, oocytes were obtained by ovarian biopsies using a 2 mm internal diameter Silastic® catheter (Dow Corning, Midland, MI, USA) introduced into the genital pore. A subsample of oocyte was photographed under stereomicroscope (Nikon SMZ800, Tokyo, Japan) with a digital camera (Evolution BF 12-bit colour, cool phase) attached to it. The diameter of 200 oocytes was measured to the nearest 0.1 mm on these images using the software Image-Pro plus 4.5.

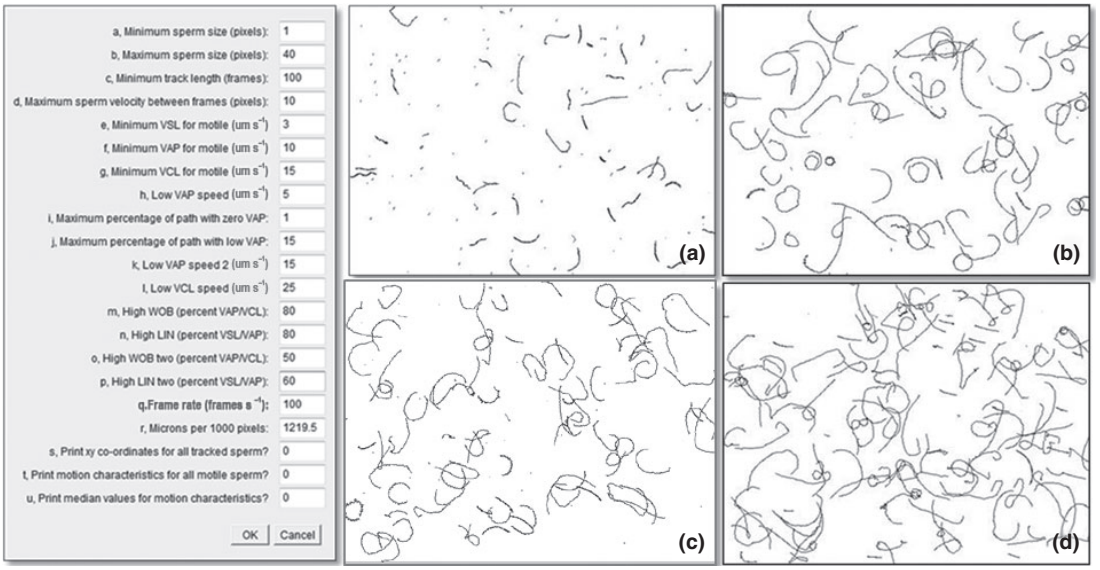

Figure 1 Images of the spermatozoa tracks of 3 (a), 5 (b), 7 (c) and 10 (d) year pejerrey generated by the application of CASA, using IMAGEJ software. The dialogue box on the left was generated by the application of CASA inside the IMAGEJ software with the configurations used in the analyses of pejerrey sperm motility. The images correspond to the spermatozoa tracks recorded during 1 s at 9 s post activation.

'Solver tool' application of Microsoft EXCEL was used to decompose polimodal distributions of oocyte diameter into their unimodal components to obtain the leading clutch diameter. Absolute fecundity (number of total eggs per female) and relative fecundity (number of total eggs per kg of female) were estimated volumetrically considering 250 eggs mL^{-1}. A subsample of at least 100 eggs of each spawn was photographed under stereomicroscope and analysed to calculate the fertilization rate. These data were used to determine the total number of fertilized eggs and the number of fertilized eggs per kg of female. The diameter of 50 fertilized eggs of each spawn was measured on the same photographs. In addition, from each spawn, three random samples of 10 fertilized eggs were weighed and used to measure the amount of protein by the method described by Lowry, Rosebrough, Farr and Randall (1951). Briefly, the eggs were ground in a 1.5 mL tube on ice with 1 mL of Tris-HCl pH 8, vortexed and incubated 10 min at 4°C. After a spin down, the total volume was transferred into another 1.5 mL clean tube and the original tube with 0.5 mL of Tris-HCl pH 8 was washed. Finally, both volumes were mixed and centrifuged at 4°C for 10 min at 2500 *g* and the supernatant was taken and stored on ice until the measurement was done. The results were expressed as mg of protein per egg and percentage of protein (Total eggs proteins eggs $weight^{-1}$).

Larvae quality measurements

Three replicates of 70 fertilized eggs from each spawn were incubated at 22.7 ± 0.4°C (mean ± SD) in a flow-through water (4–5 g L^{-1}

Table 2 Two-way ANOVA results showing main and interactive effects of age and month on sperm quality in *Odontesthes bonariensis*

Source	d.f.	MS	*F*-ratio	*P*-value
Relative sperm weight				
Age	3	20,770	3.67	0.017*
Month	3	15,617	2.76	0.049*
Interaction	9	9351	1.65	0.120
Error	64			
Sperm concentration				
Age	3	8.05E19	3.65	0.017*
Month	4	1.60E20	7.26	0.000*
Interaction	12	1.77E19	0.80	0.645
Error	63	2.20E19		
Sperm viability				
Age	3	185.37	1.86	0.148
Month	3	258.03	2.59	0.063
Interaction	9	177.01	1.77	0.096
Error	52	99.78		

*Significant effect ($P < 0.05$).

salinity) incubating jar (see Tsuzuki, Aikawa, Strüssmann & Takashima 2000). All hatched larvae of each replicate were counted to estimate the hatching rate and seven of these larvae were randomly sampled and photographed under a stereomicroscope to measure the total body length and the yolk-sac area to the nearest 0.1 mm using Image-Pro plus 4.5 software. The body weight was measured at the nearest 0.1 mg in an analytical scale. The remaining larvae were reared without food at the same temperature of incubation in a 50 mL beaker with flow-through water. The point of no return (PNR) was considered at the moment when at least 50% of larvae died.

Blood collection and sex steroid plasma measurements

Blood samples were taken only at the beginning (mid-August), middle (mid-October) and at the end

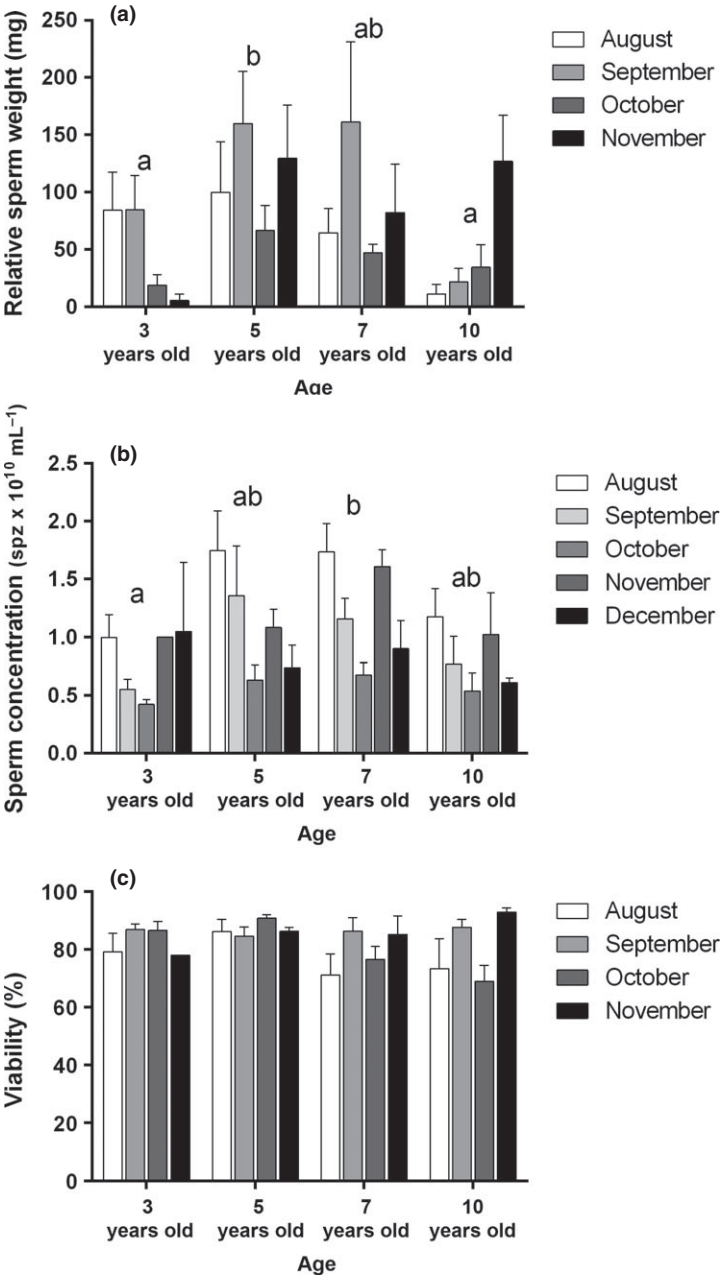

Figure 2 Changes in relative sperm weight (a), sperm concentration (b) and sperm viability (c) observed in pejerrey males of different ages during the breeding season. Results are expressed as means ± SEM. Different letters represent significant differences resulting from Tukey's *post hoc* tests ($P < 0.05$).

(mid-December) of the spawning season not to overstress the animals. After fish were anesthetized by immersion in a 100 ppm benzocaine, blood samples (300–500 μL) were taken from caudal peduncle and collected in 1.5 mL tubes by heparinized syringes. Samples were centrifuged at 4°C for 15 min at 3000 *g* and the plasma was stored at −80°C until used. Sex steroids, 11-KT in males and E_2 in females, were measured by an Enzyme-linked immunoabsorbent assay (EIA) using commercial kits (Cayman Chemicals, Ann Arbor, MI, USA for 11-KT and DRG Instruments GmBH, Frauenbergstr, Germany for E_2).

Steroids were extracted before measurements with diethyl ether and suspended in their initial volume with EIA buffer. A standard curve was run for each EIA plate.

Statistical analysis

Results are presented as means ± SEM unless otherwise mentioned. All data were statistically analysed using Statgraphics Centurion XVI version 16.2.04 (StatPoint Technologies, Warrenton, VA, USA). The data were checked for normal distribution with the Kolmogorov–Smirnoff test. The effects of age and month and their interaction on sperm quality, oocyte size and steroids levels were tested using two-way ANOVA. Data with significant differences were further compared among the means using *post hoc* Tukey's HSD test ($P < 0.05$). The effect of age on fecundity and eggs quality was tested using one-way ANOVA and *post hoc* Tukey's multiple comparison test ($P < 0.05$).

Results

Sperm quality

It was not possible to obtain semen from some of the fish from 3- and 10-year groups in some months. In December, only a small amount of semen (<0.1 mg) could be obtained from every fish, consequently, the only parameter measured in these samples was sperm concentration.

Significant effects of age and month for relative sperm weight and sperm concentration were observed while the interaction between factors was not significant ($P > 0.05$). Fixed factors did not show any significant effects on sperm viability

Table 3 Two-way ANOVA results showing main and interactive effects of age and month on sperm parameters obtained in *Odontesthes bonariensis* using CASA over 1 s taken 9 s (A) and 29 s (B) after sperm activation

Source	d.f.	MS	*F*-ratio	*P*-value	d.f.	MS	*F*-ratio	*P*-value
(A)								
	Sperm motility				VCL			
Age	2	105.3	0.20	0.821	2	839.8	1.25	0.301
Month	3	2118.3	4.00	0.019*	3	13753.4	20.45	0.000*
Interaction	6	687.8	1.30	0.296	6	910.4	1.35	0.263
Error	24	529.7			32	672.5		
	VSL				VAP			
Age	2	518.8	1.36	0.271	2	1053.5	1.79	0.183
Month	3	1563.3	4.10	0.014*	3	11278.7	19.14	0.000*
Interaction	6	817.3	2.15	0.075	6	1001.8	1.70	0.151
Error	32	380.9			34	589.2		
(B)								
	Sperm motility				VCL			
Age	2	312.6	0.90	0.418	2	80.75	0.13	0.879
Month	3	13626.9	39.07	0.000*	3	1104.7	1.77	0.172
Interaction	6	476.3	1.37	0.258	6	1250.7	2.01	0.094
Error	32	348.7			32	622.6		
	VSL				VAP			
Age	2	168.9	1.29	0.289	2	174.6	0.32	0.727
Month	3	746.4	5.71	0.003*	3	905.6	1.67	0.192
Interaction	6	231.8	1.77	0.136	6	1083.6	2.00	0.095
Error	32	130.8			32	541.2		

*Indicates significant effect ($P < 0.05$).

VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.

(Table 2). The *post hoc* test for two-way ANOVA indicated that 5-year group was significantly higher than 3- and 10-year groups for relative sperm weight (Fig. 2a). Meanwhile, sperm concentration of 7-year group was significantly higher than the one of the 3-year group (Fig. 2b). Sperm viability was not statistically different between age groups (Fig. 2c).

The 3-year group was excluded from the CASA analysis, because there was not enough sperm to record the videos. The analysis of sperm parameters obtained using CASA showed no significant effects of age for sperm motility, VCL, VSL and VAP at second 9 and 29 after activation (Table 3) and Tukey's *post hoc* test indicated homogenous age groups (Fig. 3). On the other hand, the variable month showed significant effect for all parameters at second 9, while only sperm motility and VSL at second 29 was significantly affected by month. No significant interaction ($P > 0.05$) was observed between age and month for all CASA parameters (Table 3).

Oocyte growth, spawning activity and eggs quality

Two-way ANOVA showed significant effects of age but not of month for oocyte leading clutch size, while the interaction between factors was not significant (Table 4). The *post hoc* test for two-way ANOVA indicated that the oocyte leading clutch size of 5-year fish was significantly higher than 3- and 10-year fish (Fig. 4). A single spawn was found in females from the 3-year group during the reproductive season (2000 eggs) with a very low fertilization rate (2%). In the 5-, 7- and 10-year females it was possible to collect 12, 12 and 4

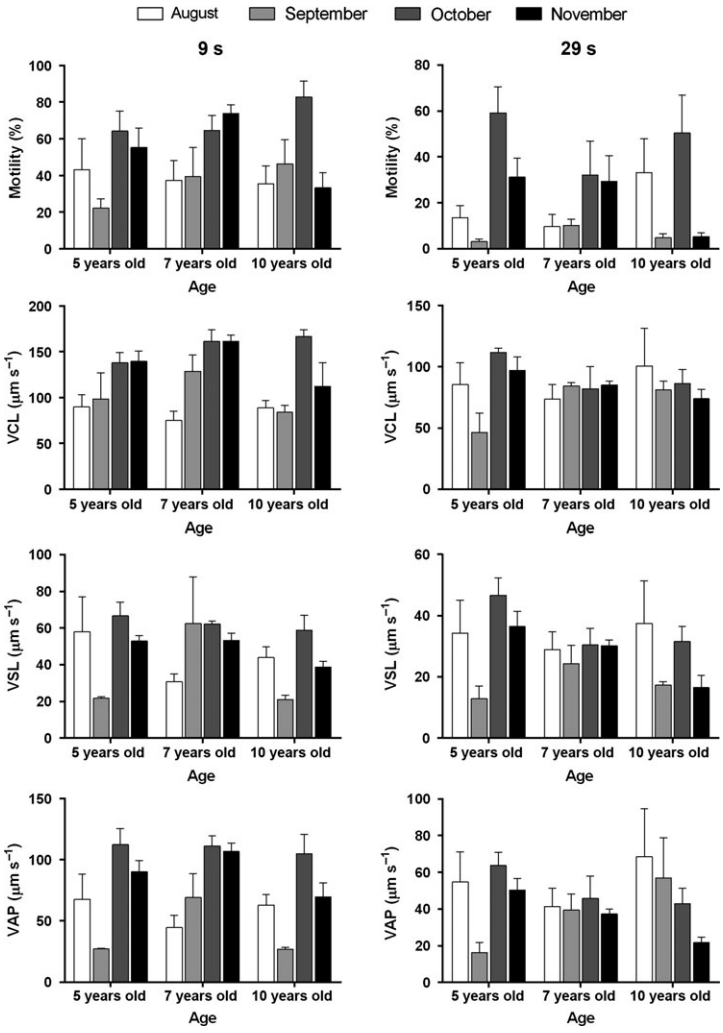

Figure 3 Sperm parameters obtained from pejerrey of different ages during the breeding season using CASA over 1 s taken at 9 s (left panel) and 29 s (right panel) after sperm activation. Results are expressed as means ± SEM. VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.

Table 4 Two-way ANOVA results showing main and interactive effects of age and month on oocyte leading clutch size

	Leading clutch size			
Source	**d.f.**	**MS**	***F*-ratio**	***P*-value**
Age	3	8.14E5	7.46	0.000*
Month	4	3.35E4	0.31	0.873
Interaction	12	8.99E5	0.82	0.627
Error	79	1.09E5		

*Indicates significant effect ($P < 0.05$).

viable spawns, respectively, during the whole analysed period (Fig. 5). The length of the spawning activity was longer in 5- and 7-year groups (108 and 106 days, respectively), extending from the last week of August through the second week of December. Meanwhile, the 10-year group presented a shortened spawning activity (66 days), extending from the first week of September through the first week of November (Fig. 5).

During the complete reproductive period, absolute and relative fecundity and the number of fertilized eggs decreased with age. However, the fertilization rate showed an opposite pattern, with the highest values (84.9 ± 6.2%) in the oldest group (Table 5). The diameter and weight of spawned eggs were not different between groups meanwhile the egg protein % values were high in 7-year group (Table 5).

Hatching rate increased with the age of fish, being higher and statistical significantly in the 10-year group than in the 5-year group (Table 6). However, the estimated number of hatched larvae (no. of fertilized eggs kg of females^{-1} × hatching rate) was similar in 5-, 7- and 10-year groups (~5000). For other parameters, no differences were found between age groups, with the exception of larvae length being significantly longer in the 10-year group (Table 6).

Sex steroid levels

Two-way ANOVA revealed significant effects of age for both 11-KT levels in males and E_2 levels in females, while month effect was observed only for E_2 levels. The interaction of fixed factors for both steroids levels was not significant (Table 7). The *post hoc* test for two-way ANOVA showed that E_2 levels in 5-year group were significantly higher than in 3 year, while for 11-KT levels in 5- and 7-year fish were significantly higher than in the 3- and 10-year fish (Fig. 6).

Discussion

The analysis of sperm parameters between age groups showed the best values for relative sperm weight and sperm concentration in mid-age animals.

The most likely reason for not being able to obtain stripped semen in the younger and older fish could be related to the onset of puberty in 3-year males and ageing in 10-year males. The absence of age effect by CASA analysis could be indicate the existence of some other factors affecting male reproduction than the sperm quality. According to this observation, in zebrafish it was reported that male ageing could be associated with altered reproductive behaviour and/or female response, but not with the sperm quality (Kanuga *et al.* 2011). On the other hand, a strong effect of spawning season time was recorded. In this sense, it was obtained very scarce releasable sperm in December, in agreement with the ending time of

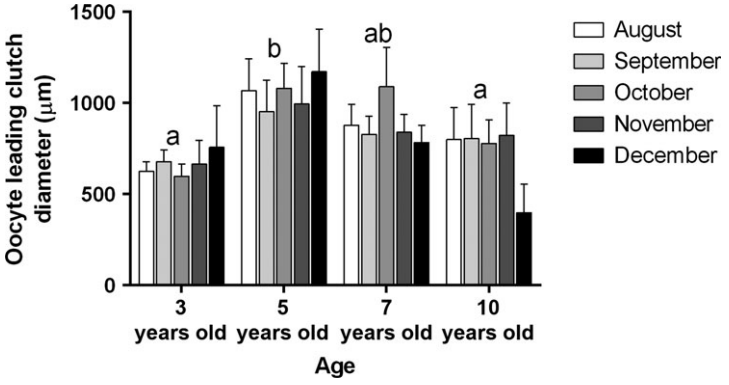

Figure 4 Oocyte leading clutch diameter obtained from pejerrey females of different ages during the breeding season. Results are expressed as means ± SEM. Different letters represent significant differences resulting from Tukey's *post hoc* tests ($P < 0.05$).

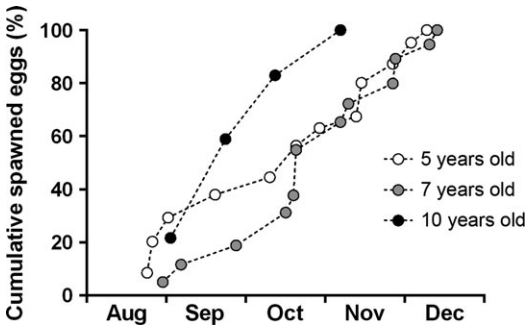

Figure 5 Cumulative percentages of spawned eggs from pejerrey females of different ages during the breeding season.

the reproductive activity, as it was reported by Miranda *et al.* (2006) in the same rearing conditions. Some studies have as well described a low sperm production at the beginning of the reproductive season (Büyükhatipoglu & Holtz 1984; Dreanno, Suquet, Fauvel, Le Coz, Dorange, Quemener & Billard 1999).

Both age and size during puberty varies within species and strains, and are modulated by genetic and environmental factors (Duponchelle & Panfili 1998; Taranger, Haux, Stefansson, Björnsson, Walther & Hansen 1998; Olsen, Lilly, Heino, Morgan, Brattey & Dieckmann 2005; Matic-Skoko, Kraljevic, Dulcic & Jardas 2007; Taylor, Porter, Bromage & Migaud 2008). In this study, non-viable eggs were found in 3-year group during the whole experimental period, meaning that these females would be reaching puberty soon. These results differ in part to the ones reported by Strüssmann (1989) where a complete experimentally maturity was achieved 3 years after hatching, in the same species. Nevertheless, this difference may be due to the author testing the presence of vitellogenic oocytes in ovarian histological sections as criterion for maturity. Furthermore, it has been reported that age of the first and the last sexual maturity in pejerrey wild populations is 1 and 5 years old, respectively (Calvo & Morriconi 1972; Grosman, Sanzano, Agüeria,

Table 5 Fecundity and eggs quality obtained from different age broodstocks during the spawning season

Age (years old)	3	5 (*n* = 12)	7 (*n* = 12)	10 (*n* = 4)
Absolute fecundity (eggs per female)	ND	15,800	12,950	6450
Relative fecundity (eggs female kg^{-1})	ND	58,483	29,506	10,803
Fertilization rate (%)	ND	34.3 ± 5.4a	53.6 ± 6.5b	84.9 ± 6.2c
No. of fertilized eggs per female	ND	5633	6850	2284
No. of fertilized eggs female kg^{-1}	ND	20,888	15,664	8895
Egg diameter (μm)	ND	1623 ± 12	1631 ± 19	1641 ± 18
Egg weight (mg)	ND	2.34 ± 0.04	2.38 ± 0.07	2.51 ± 0.10
Egg protein (mg per egg)	ND	1.37 ± 0.07	1.51 ± 0.05	1.22 ± 0.14
Egg protein (%)	ND	58.1 ± 3.0ab	59.9 ± 2.1a	47.2 ± 3.5b

Values are expressed as means ± SEM. Different letters represent significant differences between age groups (Tukey's multiple comparison test, $P < 0.05$).
ND, no data; n, number of spawning.

Table 6 Characteristics of hatched larvae obtained from different age broodstocks during the spawning season

Age (years old)	3	5 (*n* = 8)	7 (*n* = 11)	10 (*n* = 4)
Hatching rate (%)	ND	25.3 ± 6.7a	33.9 ± 6.5ab	58.9 ± 15.2b
No. of estimated hatched larvae	ND	5222	5310	5239
Weight (mg)	ND	1.29 ± 0.04	1.38 ± 0.04	1.40 ± 0.05
Total length (mm)	ND	6.70 ± 0.12a	6.72 ± 0.09a	7.24 ± 0.17b
Yolk-sac area (mm^2)	ND	0.74 ± 0.05	0.86 ± 0.03	0.75 ± 0.12
PNR (days)	ND	6.0 ± 1.3	6.7 ± 0.7	7.3 ± 1.2

Values are expressed as means ± SEM. Different letters represent significant differences between age groups ($P \leq 0.05$).
No. of estimated hatched larvae = No. of fertilized eggs female kg^{-1} × Hatching rate (%) = number of spawning.
ND, no data.

	11-KT				E_2			
Source	**d.f.**	**MS**	***F*-ratio**	***P*-value**	**d.f.**	**MS**	***F*-ratio**	***P*-value**
Age	3	4.58E7	16.22	0.000*	3	9.49E5	3.84	0.015*
Month	2	1.48E6	0.52	0.595	2	4.20E6	16.98	0.000*
Interaction	6	1.32E6	0.47	0.828	6	6.39E4	0.26	0.953
Error	48	2.82E6			47	2.47E5		

*Indicates significant effect ($P < 0.05$).

Table 7 Two-way ANOVA results showing main and interactive effects of age and month on 11-KT levels in males and E_2 levels in females of *Odontesthes bonariensis*

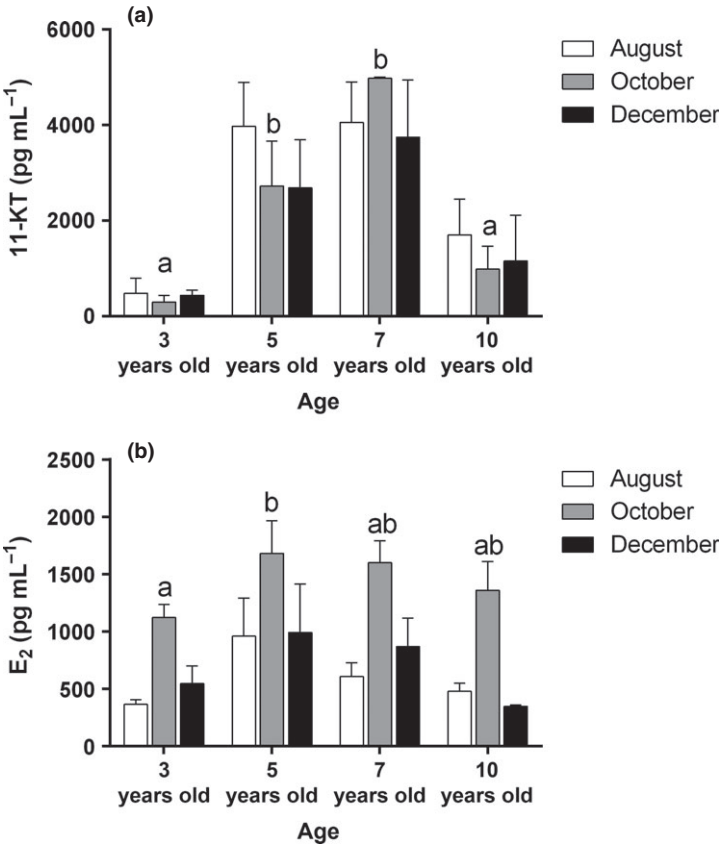

Figure 6 Plasma levels of 11-KT in males (a) and E_2 in pejerrey females (b) of different ages during the breeding season. Results are expressed as means ± SEM. Different letters represent significant differences resulting from Tukey's *post hoc* tests ($P < 0.05$).

González & Sergueña 2001). Fast growth rates have been reported from pejerrey of natural water bodies, where these fish had supposedly access to abundant live food (Somoza *et al.* 2008). This fact may explain the difference in puberty time between wild and captive fish, since it has been reported a positive correlation between rapid growth and early puberty in fish (Taranger, Carrillo, Schulz, Fontaine, Zanuy, Felip, Weltzien, Dufour, Karlsen, Norberg, Andersson & Hansen 2010). Moreover, it is important to highlight that there is not a specific food formulation for pejerrey available, and it is known that food quality and quantity may modulate the onset of puberty as it has been reported in Atlantic salmon (Thorpe, Talbot, Miles & Keay 1990). In addition, the apparent puberty delay observed in this study could also be due to a lack of environmental inputs in the reared system. Age at puberty can be controlled by constant photoperiod (Taranger *et al.* 1998; Taranger, Haux, Hansen, Stefansson, Björnsson, Walther & Kryvi 1999; Carrillo, Zanuy, Felip, Bayarri, Molés & Gómez 2009) meanwhile it is not well established if constant temperature also could delay the onset of puberty. In this sense, the onset of the first reproductive period in *Ictalurus punctatus* (Rafinesque, 1818) is a developmental event that requires three cycles of warm and cold

periods, and that photoperiod have little influence on the onset of puberty (Davis 2009).

Absolute and relative fecundity of pejerrey were affected by age, as it was reported in tilapia *Oreochromis niloticus* Linnaeus, 1758 (Getinet 2008), sea bream *Diplodus puntazzo* Cetti, 1777(Papadaki *et al.* 2008) and gilthead seabream *Sparus aurata* (Jerez *et al.* 2012). Moreover, pejerrey older females had less spawning events and shortened the spawning season compared with younger females. According to these results, Getinet (2008) found that the number of spawns per female was negatively correlated with maternal age in tilapia. On the other hand, the fertilization rate increased with age as it was demonstrated in Atlantic halibut, *Hippoglossus hippoglossus* Linnaeus, 1758 (Evans, Parrish, Brown & Davis 1996) and in red porgy, *Pagrus pagrus* Linnaeus, 1758 (Mylonas *et al.* 2004).

In many fish species, the egg size is considered as a determinant of egg quality due to the fact that larger eggs may contain more yolk reserves and hatched larvae may endure starvation for longer periods (Brooks *et al.* 1997; Kamler 2005). In the present study, no difference in egg size was found between age groups; nevertheless, the maximum egg protein % was observed in the 7-year old group. The values obtained for the pejerrey hatching rate followed the same pattern than the observed for fertilization rate, obtaining the best values in the oldest groups. However, no difference in this variable was reported in relation to fish maternal age for other species (Mylonas *et al.* 2004; Papadaki *et al.* 2008; Jerez *et al.* 2012).

Regarding androgens production and fish age, it has been suggested that 11-KT is a key factor for the regulation of the onset of puberty in teleost (Cavaco, Vilrokx, Trudeau, Schulz & Goos 1998; Rodríguez, Begtashi, Zanuy & Carrillo 2005). The results obtained showed highest 11-KT plasma levels in mid-age males which may be associated to the high values of relative sperm weight and sperm concentration. Nevertheless, it was not possible to identify an effect of age on sperm quality obtained by CASA.

A reduction in egg quality and survival to hatching was associated with low levels of E_2 in plasma in sea bass (Cerdà, Carrillo, Zanuy & Ramos 1994; Cerdà, Zanuy, Carrillo, Ramos & Serrano 1995; Navas, Mananos, Thrush, Ramos, Zanuy, Carrillo, Zohar & Bromage 1998). In this study, the lowest value of sex steroid plasma levels was found in the 3-year group, demonstrating the onset of puberty and that maturity was not fully achieved in those fish yet. High values of E_2 levels observed in the mid-age groups may be supporting an active vitellogenesis that explain the high leading clutch and consequently the high relative fecundity in these groups.

Considering the overall results obtained, it is possible to conclude that older pejerrey spawn less times with fewer eggs during the breeding season, shortening the reproductive period, perhaps as a strategy for ensuring high fertilization and hatchability. It has been experimentally verified that in captivity, pejerrey can live well beyond 10 years although, in natural environments, its average life span seems to be around 3–4 years (Strüssmann & Yasuda 2005). Therefore, taking into account that energy demands for reproduction increase with age (Hutchings & Myers 1994; Jonsson, Jonsson & Hansen 1997), it is expected that 10-year pejerrey begin to display some symptoms of senescence adopting reproductive strategies to diminish energetic costs. Further researches are necessary to validate these observations.

In summary, considering that the estimated number of larvae obtained was similar in the 5-, 7- and 10-year groups, this study suggests that it would be better to use mid-age fish to improve pejerrey hatcheries since it would allow a reduction in broodstock maintenance costs, without affecting spawning quality. In addition, due to the small difference observed in sperm quality between groups, the oocyte quality should be considered as the main component that direct breeding success.

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