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Sperm quality of Brazilian flounder *Paralichthys orbignyanus* throughout the reproductive season

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Abstract

The aim of this study was to evaluate the sperm quality of Brazilian flounder *Paralichthys orbignyanus* throughout its reproductive season. Sperm was collected at the beginning, middle and end of the breeding period. Spermatozoa density was maximum at the beginning ($12.7 \pm 0.92 \times 10^9$ cells mL^{-1}) and at the end ($11.8 \pm 0.39 \times 10^9$ cells mL^{-1}) of the breeding season ($P < 0.05$). Sperm production and the percentage of spermatozoa moving fast forward increased significantly towards the end of the breeding season ($P < 0.05$). The mean duration of progressive motility of spermatozoa was around 10 min. No difference was observed during the reproductive season in the percentage of motile cells, pH, osmolality and K^+, Cl^- and Mg^{2+} concentrations in seminal plasma. The concentration of Na^+ increased throughout the breeding season, reaching 174.62 ± 12.68 mmol L^{-1} at the end ($P < 0.05$). There was a decline in the concentration of Ca^{2+} (12.31 ± 3.08 mmol L^{-1}) in the middle of the breeding season, which coincided with the shortest motility duration of spermatozoa. The information reported in this study should help to improve management and optimize the development of protocols for short-term storage and cryopreservation of Brazilian flounder semen.

Keywords: reproduction, sperm quality, Spermatozoa, Pleuronectiformes, *Paralichthys orbignyanus*

Introduction

The Brazilian flounder *Paralichthys orbignyanus* (Valenciennes) inhabits estuarine and coastal waters from Rio de Janeiro (Brazil) to Mar del Plata (Argentina) (Figueiredo & Menezes 2000), and constitutes an important fishing resource in these areas. There is increasing interest in the culture of this species due to its high demand, elevated market price and meat quality. Studies related to physiology (Bianchini, Wasielesky Jr & Miranda 1996; Wasielesky Jr, Bianchini & Miranda 1998; Sampaio & Bianchini 2002), reproduction (Lanes, Okamoto, Cavalcanti, Collares, Campos, Deschamps, Robaldo, Marins & Sampaio 2008; Sampaio, Robaldo & Bianchini 2008; Radonic 2009), embryology (Cerqueira 2005), larviculture (Sampaio, Freitas, Okamoto, Louzada, Rodrigues & Robaldo 2007) and grow-out (Sampaio, Bianchini & Cerqueira 2001) support the development of *P. orbignyanus* culture.

Silveira, Cousin and Haimovici (1995) studied the gonadal development of *P. orbignyanus* and divided spermatogenesis into five stages and oogenesis into six stages. Moreover, they observed a long breeding season for this species, lasting up to 6 months, between mid-spring (October) and early fall (April). Besides this work, studies related to the reproduction of Brazilian flounder have focused on the development of techniques for obtaining natural and induced spawning in captivity (Cerqueira, Mioso, Macchiavello & Brugger 1997; Sampaio *et al.* 2008). Therefore, there

is little knowledge on Brazilian flounder sperm characteristics, even though Lanes, Okamoto *et al.* (2008) described a protocol for cryopreservation of semen.

Adequate knowledge of the physical and chemical characteristics of sperm is important to understand the responses of fish kept in captivity (Ciereszko, Glogowski & Dabrowski 2000). Likewise, it is the key step to optimize the fertilization protocols as well as to apply new biotechnological research such as short-term storage, cryopreservation and transgenic animal production (Chao & Liao 2001; Zohar & Mylonas 2001; Lanes, Sampaio & Marins 2008). Biomarkers of sperm quality documented so far include spermatocrit, sperm density, osmolarity, pH and chemical composition of seminal plasma, enzymatic activity, adenosine triphosphate (ATP) concentration, motility, morphology and ultrastructure and fertilizing capacity (Rurangwa, Kime, Ollevier & Nash 2004). Besides these parameters, the percentage of motile cells and duration of motility are considered to play an important role in sperm-fertilizing ability in fish (Alavi & Cosson 2005; Cosson, Groison, Suquet, Fauvel, Dreanno & Billard 2008).

Changes in the sperm characteristics of fish occur naturally during the breeding season. Parameters such as semen volume, spermatozoa concentration, percentage of motile cells, motility duration, morphology, pH and fertilizing capacity vary throughout the reproductive season, as described previously for ocean pout *Macrozoarces americanus* (Bloch & Scneider) (Wang & Crim 1997), red porgy *Pagrus pagrus* L. (Mylonas, Papadaki & Divanach 2003), Atlantic halibut *Hippoglossus hippoglossus* L. (Babiak, Ottesen, Rudolfsen & Johnsen 2006), Senegalese sole *Solea senegalensis* (Kaup) (Cabrita, Soares & Dinis 2006), spiny eel *Mastacembelus mastacembelus* (Bank & Solender) (Sahinöz, Aral & Dogu 2007) and Atlantic cod *Gadus morhua* L. (Rouxel, Suquet, Cosson, Severe, Quemener & Fauvel 2008). However, the changes observed in the sperm quality for different species do not follow the same pattern. Thus, alterations in sperm production during the reproductive period should be species-specific.

The aim of this study was to evaluate the sperm quality of Brazilian flounder *P. orbignyanus* throughout the reproductive season.

Materials and methods

Fish

Brazilian flounder broodstock were captured during its breeding season (from October-2006 to April-2007) at Cassino Beach (Southern Brazil, 32°12′S – 52°10′W) and taken to the Laboratory of Marine Fish Culture at the Federal University of Rio Grande (FURG). Males (306 ± 24 g, 30 ± 1 cm, mean ± SE) were kept in the laboratory no longer than 3 days before sperm was collected, and they were not fed during this period. Holding tanks (1000 L) were placed in a room with a controlled photoperiod (14 h light/10 h dark). Temperature and salinity were normal for the season and varied according to Fig. 1. Water was renewed at least 100% daily and feeding was withheld before sperm sampling.

Sperm sampling

Sperm samples were collected at the beginning (October, $n = 15$), middle (January, $n = 8$) and end (April,

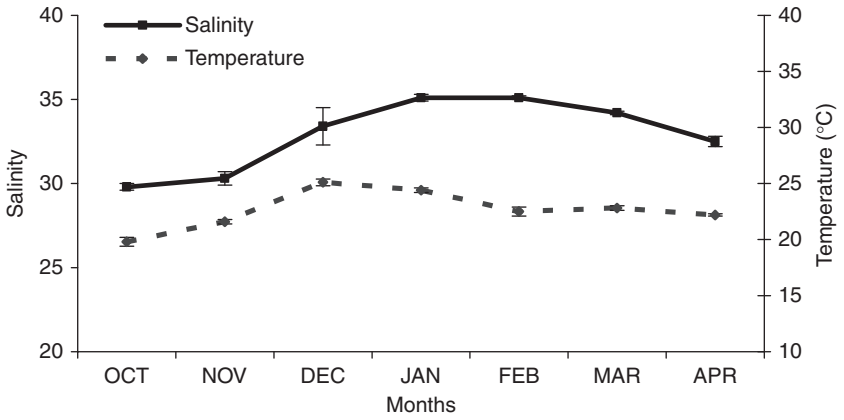

Figure 1 Mean values of water temperature and salinity during the reproductive season of Brazilian flounder *Paralichthys orbignyanus*. Flounders were caught in the beginning (October), middle (January) and end (April) of the reproductive season.

$n = 15$) of the reproductive season. Before stripping, fish were anaesthetized with benzocaine (50 ppm). Fish bladders were emptied by abdominal pressure and the urogenital areas were blotted dry with a paper towel in order to avoid urine and faecal contamination. Sperm was hand stripped and collected in a graduated syringe without a needle (1 mL) and immediately transferred to 1.5 mL microtubes.

Evaluation of sperm motility

Spermatozoa motility was estimated subjectively under an optic microscope (Olympus CX-41, 400 ×) immediately after sperm was activated with seawater (35‰ ≈ 1050 mOsmol kg^{-1}). Dilution was equal to 1:50 (1 μL sperm and 49 μL seawater). Motility was estimated using an arbitrary scale, ranging from 0 to 5, where 0 represents no motility; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–90%; and 5, 91–100% of motile spermatozoa (Borges, Siqueira, Jurinitz, Zanini, Amaral, Grillo, Oberst & Wassermann 2005).

Progressive, vibratory and total sperm motility durations were estimated. These intervals were subjectively studied, considering progressive motility from the time of activation up to at least when 50% of the cells were in movement, vibratory motility from this time point until no more vibrating cells could be observed and total sperm motility duration was the sum of progressive and vibratory motility duration. The experiments were carried out in triplicate and room temperature was maintained at 20 °C, the temperature at which Radonic, Müller, López, Bambill, Spinedi and Boccanfuso (2007) obtained the best results for natural spawning. All evaluations were conducted by the same observer in order to reduce variability.

Physical characteristics of sperm

The volume of ejaculated sperm was measured directly in graduated plastic syringes. Spermatozoa concentration was determined using an improved Neubauer Bright line counting chamber (0.00025 mm^3 $square^{-1}$). Sperm was fixed in 4% formalin and diluted in distilled water (1:2000). Two dilutions were performed for each sample. Each dilution was counted once and the mean of the two counts was utilized to calculate spermatozoa density. Before counting, samples were left undisturbed for about 10 min in the Neubauer chamber, allowing cells to sediment. Cells were counted under a microscope at × 400 magnification.

Spermatocrit was determined using glass microhaematocrit capillary tubes (75 mm length, 1.1–1.2 mm internal diameter). Semen was drawn into the tubes until 60–80% of the volume was filled. One end of the tube was sealed with clay and tubes were centrifuged for 40 min at 12 000 *g*. All spermatocrits were determined within 2 h of sperm collection.

Sperm production (SP) was calculated using the formula $SP = D \times V$, where D is the sperm density and V is the volume of milt collected.

Analyses of seminal plasma

Sperm of each flounder was centrifuged for 20 min at 12 000 *g* and the seminal plasma was removed and maintained at − 20 °C until the ionic composition analyses. The pH was immediately determined by pH indicator strips between the range from 6.0 and 8.0 with an increment of 0.2 U (Macherey-Nagel; Düren, Germany). The Na^+, K^+ and Mg^{2+} (mmol L^{-1}) concentrations were established using an atomic absorption spectrophotometer (Model – GBC 932 plus; Dandenong, Vic., Australia). The Ca^{2+} concentration was determined using flame photometry (Digimed NK-2004; Campo Grande, SP, Brazil), while Cl^- ion was analysed using a colorimetric kit (Doles; Goiás, Brazil). A semi-micro-osmometer (Knauer; Berlin, Germany) was used to determine the osmolality (mOsm kg^{-1}) of seminal plasma.

Statistical analyses

Percentage values were arc-sine transformed before analysis. One-way ANOVA was used to compare the parameters of sperm quality during the reproductive season. Differences were considered to be significant at $P < 0.05$. Tukey HSD multiple range test comparisons were used if significant differences existed among the periods. Linear regression analysis was carried out in order to determine the correlation between spermatocrit and spermatozoa density. Data are presented as mean ± SE. All analyses were conducted using software R version 2.4.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Running milt was available from all individuals, except for two males at the beginning of the breeding season.

The smallest sperm volume was obtained at the beginning of the breeding season (250 ± 71 μL)

($P < 0.05$). During the middle and the end of the season, the highest volume of semen was observed ($P < 0.05$) (Fig. 2a). The highest spermatozoa density were verified in samples collected at the beginning ($12.7 \pm 0.92 \times 10^9$ cells mL^{-1}) and at the end ($11.8 \pm 0.39 \times 10^9$ cells mL^{-1}) of the breeding season ($P < 0.05$) (Fig. 2b). However, sperm production increased during the monitoring period, reaching the maximum value at the end ($5.17 \pm 0.44 \times 10^9$ spermatozoa) of the breeding season ($P < 0.05$) (Fig. 2c).

Regarding spermatocrit, the lowest value was verified in a sample collected in the middle of the reproductive season, reaching only 12%; however, several fish exhibited spermatocrit values up to 100% independent of the season. The highest spermatocrit average was obtained at the beginning ($88 \pm 5\%$) and at the end of the breeding season ($76 \pm 4\%$) ($P < 0.05$; Fig. 3). A positive correlation was found between spermatocrit and spermatozoa density ($n = 29$, adjusted $r^2 = 0.67$, $P < 0.0001$) (Fig. 4).

No difference was found throughout the reproductive season regarding the percentage of motile spermatozoa ($P > 0.05$). The values were 3.05 ± 0.14, 3.19 ± 0.22 and 3.33 ± 0.12 for the beginning, middle and end of the breeding season, respectively, indicating a percentage of sperm motility superior to 50%, according to the motility scale used in this study (Fig. 5). Sperm progressive motility duration increased throughout the breeding season ($P < 0.05$). The highest progressive motility duration was found at the end of the reproductive season, reaching 12 ± 1 min (Fig. 5). In the middle of the reproductive season, the lowest values of vibratory movement and total duration of spermatozoa motility were found, reaching 86 ± 6 and 96 ± 5 min respectively ($P < 0.05$) (Fig. 6).

No difference was observed regarding the concentrations of K^+, Mg^{2+} and Cl^- ions. Osmolality and pH also remained unchanged throughout the reproductive period ($P > 0.05$) (Table 1). The concentration of Na^+ ion increased during the breeding season, reaching the highest concentration at the end of the reproductive season ($P < 0.05$). The lowest concentration of Ca^{2+} ion was observed in the middle of the breeding season and no difference was observed at the beginning and at the end of the reproductive period ($P > 0.05$) (Table 1).

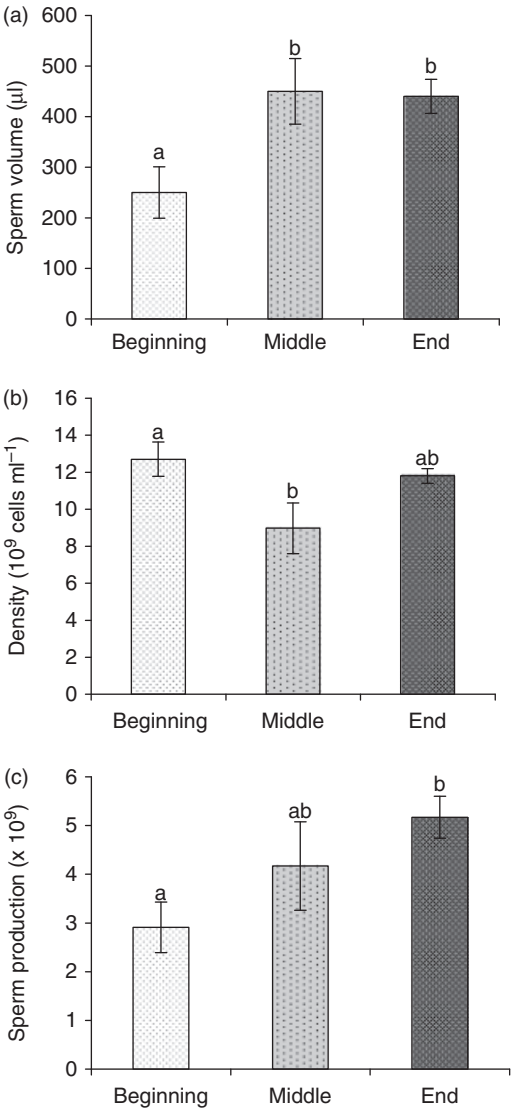

Figure 2 Changes in the characteristics of Brazilian flounder *Paralichthys orbignyanus* sperm during the reproductive season. (a) Sperm volume collected (µL); (b) Sperm density (spermatozoa mL^{-1}); and (c) sperm production (total volume of sperm collected by striping x sperm density). Different letters above each bar indicate a significant difference during the monitoring periods ($P < 0.05$). Data are represented as mean ± SE.

Discussion

Sperm production and its quality have been reported for several marine fish species (Suquet, Billard, Cosson, Dorange, Chauvaud, Mugnier & Fauvel 1994; Mylonas *et al.* 2003; Babiak *et al.* 2006; Cabrita *et al.* 2006; Cosson, Groison, Suquet & Fauvel 2008). However, this is the first study on sperm production and quality throughout the breeding season for Brazilian flounder.

The volume of semen produced by *P. orbignyanus* was always small, similar to other Pleuronectiformes

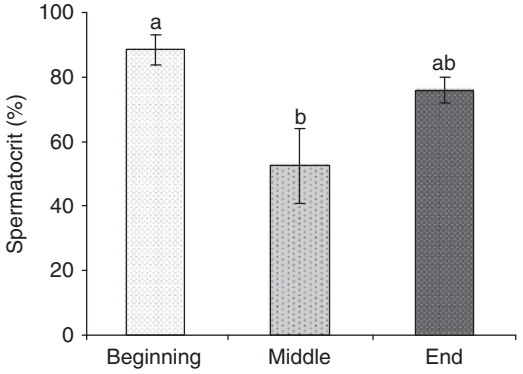

Figure 3 Variation of spermatocrit throughout the reproductive season of Brazilian flounder *Paralichthys orbignyanus*. Different letters above each bar indicate a significant difference during the monitoring periods ($P < 0.05$). Data are represented as mean ± SE.

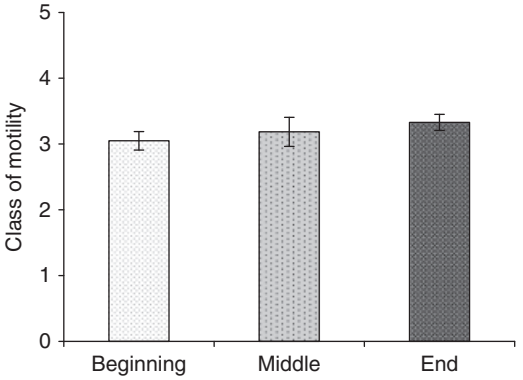

Figure 5 Sperm motility of Brazilian flounder *Paralichthys orbignyanus* during the reproductive season. There were no significant differences among the monitoring periods ($P > 0.05$). Data are represented as mean ± SE.

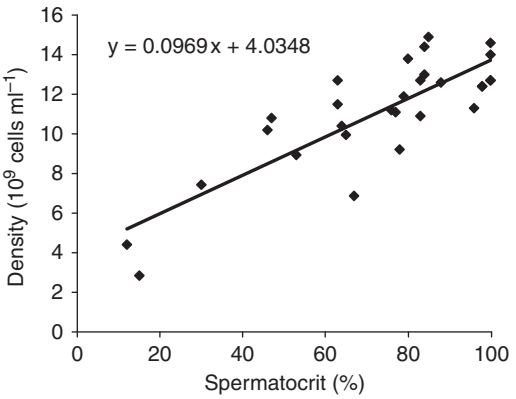

Figure 4 Correlation between spermatocrit (%) and spermatozoa density (cells mL^{-1}) for Brazilian flounder *Paralichthys orbignyanus* sperm ($n = 29$, adjusted $r^2 = 0.67$, $P < 0.0001$).

such as the turbot *Psetta maxima* L. and the yellowtail flounder *Limanda ferruginea* (Storer), which produce < 1 mL of semen (Suquet *et al.* 1994; Clearwater & Crim 1998). According to Silveira *et al.* (1995), mature spermatozoa are present in gonads of Brazilian flounder even during the winter. However, the volume of semen is smaller at the beginning of the breeding period, in spring, when two of the individuals, analysed in October, failed to release sperm after being hand stripped. The volume of sperm produced in summer and early fall was higher and all males produced sperm. Sperm production followed the same trend, increasing from the beginning of the breeding season towards its end. These results suggest that the reproductive peak of this species occurs between January and April.

The spermatozoa density for *P. orbignyanus* ranged from 8.9 to 12.7 × 10^9 cells mL^{-1}, within the same range reported for other Pleuronectiformes, such as *P. maxima*, starry flounder *Platichthys stellatus* (Pallas) and *H. hippoglossus* (Suquet, Omnes, Nomant & Fauvel 1992; Lim, Han & Chang 2002; Babiak *et al.* 2006). Changes in sperm density throughout the breeding season have been reported for several marine species, including *P. maxima* and *H. hippoglossus*, whose spermatozoa concentration increased during the reproductive season (Suquet, Dreanno, Dorange, Normant, Quemener, Gaignon & Billard 1998; Babiak *et al.* 2006). However, the opposite was observed for seabass *Dicentrarchus labrax* L. and seabream *Sparus aurata* L. (Kara & Labed 1994). Conversely, no difference was verified for sperm of *P. pagrus* along its breeding season (Mylonas *et al.* 2003). In the present study, it was found that a decrease in sperm density occurred in the middle of reproductive season; however, it increased again towards its end.

Sperm production has been used to characterize the contribution of males in each stripping in several species (reviewed by Suquet *et al.* 1994). Suquet *et al.* (1992) registered sperm production ranging from 0.2 to 12 × 10^9 spermatozoa for *P. maxima*. However, Cabrita *et al.* (2006) observed very low values, ranging from 5 to 11 × 10^7 spermatozoa, for *S. senegalensis*. In the present study, sperm production was similar to that observed for *P. maxima* and reached the maximal value (5.17 × 10^9 spermatozoa) at the end of the reproductive season.

Table 1 Characteristics of the seminal plasma of Brazilian flounder *Paralichthys orbignyanus* during the beginning ($n = 15$ males), middle ($n = 8$ males) and end ($n = 15$ males) of the reproductive season

Parameters	Beginning	Middle	End
Na^+ (mmol L^{-1})	112.46 ± 6.66[a]	146.85 ± 28.25[ab]	174.62 ± 12.68[b]
Mg^{2+} (mmol L^{-1})	8.30 ± 1.57[a]	10.47 ± 2.45[a]	4.61 ± 0.70[a]
K^+ (mmol L^{-1})	44.71 ± 0.22[a]	28.89 ± 12.28[a]	42.7 ± 4.55[a]
Ca^{2+} (mmol L^{-1})	20.70 ± 1.13[a]	12.31 ± 3.08[b]	19.55 ± 0.59[a]
Cl^- (mmol L^{-1})	100.15 ± 4.58[a]	113.59 ± 4.41[a]	142.25 ± 10.87[a]
pH	6.77 ± 0.02[a]	6.67 ± 0.11[a]	6.77 ± 0.03[a]
Osmolality (mOsmol kg^{-1})	301.67 ± 1.67[a]	312.5 ± 7.72[a]	325.5 ± 7.33[a]

Data are represented as mean ± SE. Values sharing different letters in each line indicate a significant difference among the monitoring periods ($P < 0.05$).

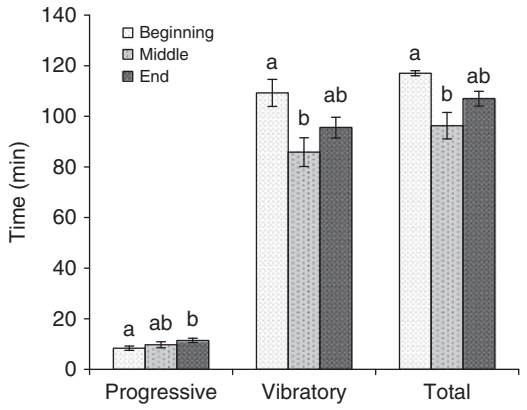

Figure 6 Duration of progressive and vibratory movement and the total duration of motility of Brazilian flounder *Paralichthys orbignyanus* spermatozoa during the reproductive season. Different letters above each bar indicate a significant difference during the monitoring periods ($P < 0.05$). Data are represented as mean ± SE.

In the present study, a positive correlation was found between spermatocrit and spermatozoa density, corroborating with data demonstrated for other teleosts (Rakitin, Ferguson & Trippel 1999; Rideout, Trippel & Litvak 2004; Borges *et al.* 2005; Rouxel *et al.* 2008). However, considering the Pleuronectiformes, this correlation may be controversial, because Suquet *et al.* (1992) did not find any correlation between spermatozoa density and spermatocrit for *P. maxima* sperm, while Tvedt, Benfey, Martin-Robichaud and Power (2001), working with *H. hippoglossus*, suggested that spermatocrit can be used as an easy, simple and fast technique for estimating sperm density.

Spermatozoa motility is considered to be the main factor related to sperm quality in fish (Rurangwa *et al.* 2004). In the present study, no differences in the percentage of motile cells were observed during the breeding season. A decrease in the percentage of sperm motility and a linear increase in cell density were observed throughout the reproductive season of *P. maxima* and *H. hippoglossus* (Suquet *et al.* 1998; Babiak *et al.* 2006). Both parameters are usually indicators of sperm ageing in several teleost fish (Billard, Cosson, Crim & Suquet 1995). Babiak *et al.* (2006) reported other alterations in the quality of halibut sperm during the breeding season, considering that these changes are related to spermiogenesis, hydration and the cell decomposition process, which regulates gamete maturation along the reproductive season.

In the present study, it was observed that the total duration of spermatozoa motility of *P. orbignyanus* is high. However, progressive motility duration decreased after 10 min, and for most of the period, spermatozoa remained only vibrating. Trippel and Morgan (1994) reported the same process for *G. morhua*, where progressive motility of spermatozoa was observed during a few minutes, while for a long period, only vibrating cells were observed (≅ 120 min). Rainis, Gasco and Ballestrazzi (2005) found that the total duration of sperm motility for *S. aurata* was about 50 min, while spermatozoa of rainbow trout *Oncorhynchus mykiss* (Walbaum) and brown trout *Salmo trutta* L. are motile for < 2 min. According to these authors, the differences in the duration of spermatozoa motility among the species are related to the type of environment where fecundation occurs. In the case of *S. aurata*, fertilization occurs in open sea, where hydrodynamic processes are unstable unlike freshwater environments. Therefore, spermatozoa need to swim for a longer period to reach the ovulum and fertilize it. This can illustrate why spermatozoa of Brazilian flounder showed a very long motility duration. Although mature male Brazilian flounder are

found in estuaries (Silveira *et al.* 1995), the reproduction of this species is likely to take place in coastal waters, because eggs sink to the bottom in brackish water (Sampaio *et al.* 2007).

Several parameters such as ionic concentration (Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^-), pH, osmotic pressure, temperature and dilution ratio affect the percentage of motile cells and the duration of sperm motility (Alavi, Cosson, Karami, Amiri & Akhoundzadeh 2004; Alavi & Cosson 2005, 2006). In the present study, alterations in the duration of sperm motility were observed throughout the reproductive season. The lowest motility duration was verified in the middle of the reproductive season and coincided with the smaller Ca^{2+} ion concentration in the seminal plasma. The K^+ ion concentration was low in this period, but no difference was observed among the monitored periods, because there was a high variability among the individuals. In contrast to Cyprinidae and Salmonidae, the literature on the effect of ions on sperm motility in marine fish is fairly limited. In salmonids, sturgeons and paddlefish, high concentrations of K^+ ion in seminal plasma favour inhibition of sperm motility (Cosson, Linhart, Mims, Shelton & Rodina 2000; Alavi *et al.* 2004; Alavi & Cosson 2006). However, in marine teleosts, K^+ has no inhibitory effect on sperm motility in the summer whiting *Sillago ciliata* (Cuvier), flounder *Platichtys flesus* L. and *G. morhua* (Morisawa 1985; Goodall, Blackshaw & Capra 1989). In freshwater species, Ca^{2+} influences the total time of activity, percentage of sperm motility and sperm velocity (Alavi & Cosson 2006). In some marine teleosts, recent studies demonstrated that the presence of Ca^{2+} allows motility to occur at a high osmotic pressure (Alavi & Cosson 2006). In the present study, a decrease in the Ca^{2+} and K^+ concentrations seems to be related to the duration of sperm motility.

The predominant ions in the seminal plasma of *P. orbignyanus* are Na^+ and Cl^-, which follow the same trend of osmolality, increasing during the reproductive season. Wang and Crim (1997) reported the same correlation among Na^+, Cl^- and osmolality for ocean pout. In general terms, the osmolality and concentration of Na^+, Cl^- and Mg^{2+} ions found in the seminal plasma of *P. orbignyanus* were similar to *P. maxima* (Dreanno, Suquet, Desbruyères, Cosson, Delliou & Billard 1998). However, K^+ and Ca^{2+} levels are higher than the seminal plasma of *P. maxima*. The K^+ and Ca^{2+} concentrations observed in the seminal plasma of *P. orbignyanus* are similar to some freshwater species (Alavi & Cosson 2006). Because *P. orbignyanus* is considered to be an estuarine-dependent species, this can influence the ionic composition of the seminal plasma of this species.

The pH values of Brazilian flounder seminal plasma throughout the reproductive season averaged 6.8, similar to the pH values recorded for the seminal plasma of *L. ferruginea* (6.8) (Clearwater & Crim 1998), but smaller than those recorded for *P. maxima* (7.1–7.6), *P. stellatus* (7.3–8.0) and *H. hippoglossus* (7.3–7.6) (Dreanno *et al.* 1998; Lim *et al.* 2002; Babiak *et al.* 2006).

In conclusion, changes in the sperm quality of Brazilian flounder occur during the reproductive season. The analyses of volume, cell density, sperm production and duration of progressive motility indicate that the reproductive peak of this species takes place between January and April. Plasmatic concentrations of K^+ and Ca^{2+} ions can be related to the duration of sperm motility. However, other studies should be carried out to better evaluate the relationship among these parameters. Moreover, the ageing phenomenon of spermatozoa throughout the reproductive season, common for other flatfish, is not so evident for *P. orbignyanus* within the sampling period of this study. The information reported in this study related to the physical and chemical characteristics of Brazilian flounder sperm should help to improve management and optimize the development of protocols for short-term storage and cryopreservation of semen.

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