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Effects of re-stripping on the seminal characteristics of pacu (*Piaractus mesopotamicus*) during the breeding season

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ABSTRACT

Seminal characteristics in teleost fish with an annual reproductive period, such as pacu (*Piaractus mesopotamicus*), may vary during the breeding season. The sperm formed before the beginning of the spawning period may be stored for a long time, causing damage to the cells. Therefore, re-stripping may be an important way to eliminate the "old" and allow for the collection of "new" spermatozoids. In this study, we analyzed the seminal characteristics of hormonally induced pacu at the beginning, middle and end of the breeding season, and we analyzed samples from re-stripped males (stripped first at the beginning, re-stripped in the middle, and re-stripped again at the end of the season) during two breeding seasons. The sperm density, ionic composition, pH, and osmolality were similar among the groups. The semen volume, seminal plasma protein concentration and incidence of morphologically anomalous sperm increased over time. In addition, some parameters that are associated with good-quality semen decreased, such as sperm motility, viability and DNA integrity. Moreover, we observed a positive association among motility, viability and DNA integrity for sperm with elevated 11-ketotestosterone, but there was no such association for *fshb* or *lhb* mRNA levels in the pituitary. The semen that was obtained earlier (at the beginning) or from re-stripped males exhibited better characteristics than the other samples collected. In conclusion, collecting semen from pacu at the end of breeding season should be avoided; it is preferable to strip early and then re-strip later in the season, and this approach may be used for diverse aquaculture purposes.

1. Introduction

Variations in teleost seminal characteristics during the breeding season, such as volume, sperm density and motility, duration of motility, morphology, and pH, have been previously reported for marine (Wang and Crim, 1997; Mylonas et al., 2003; Cabrita et al., 2006; Lanes et al., 2010) and freshwater species (Büyükhatipoglu and Holtz, 1984; Koldras et al., 1996; Luz et al., 2001; Alavi and Cosson, 2006), as well as masculinized females (Nynca et al., 2012).

According to Bobe and Labbé (2010), many factors can affect semen quality, such as stress, nutrition, photoperiod, salinity, temperature, reproductive season, genetic material, or even hormonal induction. Semen and sperm quality evaluation is complex, and in teleosts, the milt volume, sperm concentration, osmolarity, pH, ions, ATP (adenosine triphosphate) concentration, motility, and morphology are the most frequently used parameters for this purpose (Rurangwa et al., 2004). Among these parameters, motility and the duration of motility are the most important because they are directly related to fertilization capacity (Alavi and Cosson, 2005).

In diverse teleost fish species, spermatozoids are produced several months before the reproductive season and remain stored in the testes for a long period of time (Grier and Taylor, 1998; Brown-Peterson et al., 2002; Batlouni et al., 2006). Although the approximate time for spermatozoid formation (from preleptotene) depends on the species and temperature, the formation time is

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5–14 days in typical teleosts (Nóbrega et al., 2009). As production begins several months before the reproduction period (Batlouni et al., 2006; Oliveira et al., 2010), re-stripping may be a useful technique to eliminate "old" spermatozoids and to selectively favor collection of "new" spermatozoids (which would be expected to be of better quality). Indeed, re-stripping has been reported in trout and may be performed more than once during the breeding season (Hajirezaee et al., 2009; Heyrati et al., 2010). In addition, in South American rheophilic teleost fish, spermatogenesis is not interrupted during the spermiation period because a portion of the germinal epithelium of the testes remains "continuous" (Batlouni et al., 2006; De Souza et al., 2015).

The endocrine system coordinates spermatogenesis in teleost fish, and dopamine (DA) and gonadotropin-releasing hormone (GnRH) inhibit and stimulate, respectively, spermiation and reproduction (Dufour et al., 2010). In males, both gonadotropins (GtHs), follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), induce steroidogenesis (Planas and Swanson, 1995; Weltzien et al., 2003; García-López et al., 2009). In zebrafish (*Danio rerio*), Fsh is able to stimulate both spermatogenesis and steroidogenesis, whereas Lh appears to have a sharper action on spermatogenesis and steroidogenesis, likely at times when peak amounts of steroids are needed (García-López et al., 2010). Among steroid hormones, 11-ketotestosterone (11-KT) is the primary hormone that is secreted in response to GtHs in males (Nagahama and Yamashita, 2008; Mylonas et al., 2010; Schulz et al., 2010). Other steroid hormones with lesser-known functions, such as estradiol (E_2) and testosterone (T), are involved in the renewal and proliferation of spermatogonia (Miura and Miura, 2001; Kobayashi et al., 2011) and regulate sperm output (Mangiamele and Thompson, 2012).

In this context, fluctuations in the gene expression of GtHs in teleost fish males during breeding season have been reported in males of *Dicentrarchus labrax* (Mateos et al., 2003), *Anguilla japonica* (Yoshiura et al., 1999), *Gasterosteus aculeatus* (Hellqvist et al., 2006) and *Odontesthes bonariensis* (Elisio et al., 2015). In *Oncorhynchus mykiss* (Gomez et al., 1999) and *Fundulus heteroclitus* (Shimizu et al., 2012) males, in addition to fluctuations in gene expression plasma levels, changes in GtHs were also reported during breeding season. Although the precise regulatory mechanisms and functions of GtHs on spermatogenesis progress in teleost fish males is still not completely understood, in general, single or group-synchronous spawners have a temporal gene expression pattern with respective increased levels of *follicle-stimulating hormone beta polypeptide* (*fshb*) during spermatogenesis and *luteinizing hormone beta polypeptide* (*lhb*) at spermiation.

Circulating steroid levels may exert positive feedback by increasing pituitary Lh content and *lhb* mRNA levels and negative feedback by decreasing *Fshb* mRNA levels (Mateos et al., 2002). In *Argyrosomus regius* (Schiavone et al., 2012), increased levels of 11-KT, E2 and T during the pre-spawning period and a subsequent decrease through the spawning period have been reported. Fluctuations in steroid hormones concentrations are not restricted to endocrine control over the GtHs feedback system and spermatogonial renewal and proliferation, but also over the milt quality (Mateos et al., 2002; Schiavone et al., 2012). For instance, in *Clarias gariepinus* males (Nyina-wamwiza et al., 2012), a positive association of higher androgens levels and lower estrogen levels with good-quality sperm has been observed. In addition, in *Carassius auratus*, T rapidly increases ejaculate volume and sperm density via an aromatase-dependent mechanism, through the activation of estrogen receptors α and β (Mangiamele and Thompson, 2012). Moreover, in *A. regius*, the highest values of % motility and motility duration were recorded in the middle of the spawning period, concomitantly with a peak in the 11-KT plasma level (Schiavone et al., 2012).

Pacu (*Piaractus mesopotamicus*) is a rheophilic (migratory) species that has a seasonal reproductive cycle (Zaniboni-Filho and Weingart, 2007). Breeding occurs in November and January, but the gonad maturation process becomes more evident in September and October, reaching more advanced stages of maturation between October and January (Lima et al., 1991). Pacu is a large tropical herbivorous/frugivorous characiform fish used in aquaculture. The species is intensively cultivated in different regions of South America (Abimorad et al., 2008; Jomori et al., 2008). Pacu is a total spawner, and hormonal stimulation is required for spawning in captivity. Therefore, most problems with pacu reproduction in captivity are associated with unsuccessful ovulation (Romagosa et al., 1993; Criscuolo-Urbinati et al., 2012). In contrast, males do not tend to have problems releasing satisfactory amounts of semen; thus, they are used indiscriminately without any selection criteria. When problems with the quality of embryos and larvae occur, the causes are usually attributed to females and their oocytes. In this context, the objectives of the present study were to determine the characteristics of the semen and sperm of *P. mesopotamicus* during the breeding season and after re-stripping. Moreover, during these periods, we determined the average levels of gonadal steroids and pituitary *luteinizing hormone-β* (*lhb*) and *follicle-stimulating hormone-β* (*fshb*) gene expression.

2. Materials and methods

All of the fish were treated and euthanized according to protocols accepted by the Comissão de Ética no Uso de Animais from the Faculdade de Ciências Agrárias e Veterinárias/UNESP (Jaboticabal, SP, BR).

2.1. Maintenance, hormonal induction and sampling

The four-year-old breeders in Experiment 1 (Exp1) and three-year-old breeders in Experiment 2 (Exp2) were reared in 200-m^3 outdoor earthen ponds with 0.25 fish/m^3 density at the Aquaculture Center, Sao Paulo State University (Jaboticabal, SP, BR), during two breeding seasons: 2010/2011 (Exp1) and 2013/2014 (Exp2). The earthen ponds received constant running water at ≅20 L/min, and the water temperature ranged from 26 to 32 °C (mean ± SD = 29.5 ± 1.0 °C) during Exp1 (from November 2010 to February 2011) and from 25 to 31.5 °C (mean ± SD = 28.6 ± 1.2 °C) during Exp2 (from November 2013 to late January 2014). The fish were fed a commercially available feed (Guabi, Campinas, SP. BR – composed of 32.0% crude protein, 6.5% fat, 10.0% ash and 7.0% crude fiber) twice a day *ad libitum*.

Broodfish were netted from the earthen ponds and transported to the laboratory for acclimatization in 1000-L tanks before hormonal induction treatment. Spermiation induction consisted of a dose of 10 μg/kg buserelin acetate – Sincroforte® (Ourofino Agronegócio, Cravinhos, SP, BR) plus 5 mg/kg domperidone (Ranbaxy, Dewas, MP, IN). For each sample, non-stripped males were used. Males were sampled at the beginning (Beg; 11/17/2010 and 11/12/201; Exp1 and Exp2, respectively), middle (Mid; 12/21/2010 and 12/18/2013) and end (End; 02/12/2012 and 01/23/2014) of the breeding season. The "*re-stripped*" group in Exp1 (RS1) consisted of males that were first stripped at Beg and re-stripped in Mid ($n = 4$). The "re-stripped" group of Exp2 (RS1) consisted of males that were first stripped at Beg and re-stripped in Mid ($n = 6$), but in Exp 2, males were re-stripped for a second time (RS2) at End ($n = 5$) Table 1). In the previous experiment, the re-stripping procedure was effective and improved the characteristics that are associated with good-quality semen (CAGQS), as reviewed by Rurangwa et al.

Table 1
The experimental design diagram and timeline of this study.

	Breeding season	Date	*n*	Biometrics	Testes histology	Plasma steroid hormones	Gonadotropins gene expression	Seminal characteristics
Experiment 1	Beginning	11/17/2010	7	+	–	+	–	+
	Middle & first re-stripping	12/21/2010	5 & 4	+	+	+	+	+
	End	02/12/2011	4	+	+	+	+	+
Experiment 2	Beginning	11/12/2013	6	+	–	–	–	+
	Middle & first re-stripping	12/18/2013	5 & 4	+	–	–	–	+
	End & second re-stripping	01/23/2014	6 & 4	+	–	–	–	+

(2004) and Cabrita et al. (2014). Thus, we repeated the experiment to confirm the repeatability of re-stripping and to test the possibility of re-stripping the same male twice during the same breeding season. The experimental diagram and the timeline of the preset study are summarized in Table 1.

The levels of serum steroid hormones were determined during Exp1. For this measurement, fish were netted from the tanks, immediately anaesthetized with 100 mg/L of benzocaine, weighed (body mass – BM) and measured (standard length – SL). The condition factor (*K*) was then determined ($K = [(BM\ (g) \times SL^3\ (cm)) \times 100]$). In addition, blood samples (3 ml) were collected from the caudal vessels before hormonal induction and after stripping using EDTA-treated syringes (containing ≅75 μL of 10 mM EDTA solution). The plasma was separated by centrifuging the samples at 1500×*g* for 15 min at 4 °C and then stored at −80 °C until measurement of the steroid hormone concentrations.

After stripping, the breeders were euthanized by an anesthetic overdose (300 mg/L benzocaine). The testes, liver and viscera were dissected and weighed to calculate the following indices: gonadosomatic (GSI), hepatosomatic (HSI), and viscerosomatic (VSI). All of the indices were calculated using the following formula: Index = [(organ weight (g)/BM (g)) × 100]). The cranial, central, and caudal fragments of the testes tissue were fixed with phosphate-buffered (0.05 M) 2.5% glutaraldehyde (pH 7.3) for 24 h at 4 °C and embedded in glycol methacrylate resin. The entire pituitary glands were preserved in RNAlater™ solution (Ambion, Austin, TX, USA) following the manufacturer's instructions and stored at −80 °C until total RNA extraction.

2.2. Histological analysis and serum steroid hormone assays

Fixed fragments of the testis samples were dehydrated in a graded ethanol series and embedded in glycol methacrylate resin (Leica historesin embedding kit, Leica Microsystems, Nussloch, DE); the sections were cut to a thickness of 2.0 μm and stained with Hematoxylin-Phloxine B. The spermatogenesis stages were determined according to the differentiation stages of the germ cell types and their abundance in the seminiferous tubules based on a previously described method (Utoh et al., 2004; Huertas et al., 2006; Peñaranda et al., 2010): stage I – presence of type A and/or type B spermatogonia; stage II – presence of spermatogonia and spermatocytes; stage III – presence of spermatids; stage IV – presence of spermatozoa; stage V – increased number of mature spermatozoa compared with stage IV, with a low proportion of other germ cells; stage VI – presence of only spermatozoa and few spermatogonia; and stage VII – nearly all of the spermatozoa are phagocytosed by Sertoli cells, leaving early spermatogonia and the remainder of the sperm in the testis. All of the testes regions were evaluated and used to establish the spermatogenic stage of each fish, and the data were expressed as the frequency of fish in each spermatogenic stage.

The plasma levels of 17β-estradiol (E_2), testosterone (T) and 11-ketotestosterone (11-KT) were quantified using hormone enzyme-linked immunosorbent assays (ELISA) using commercial kits (Cayman Chemical Company, Ann Arbor, MI, EUA for 11-KT and BioCheck, Inc., Foster City, CA, EUA distributed by Interteck Internacional Imp. e Exp. Ltda. Sao Paulo, SP, BR for all others) according to the manufacturer's instructions. The E_2 and T plates were read at 450 nm, and the 11-KT plate was read at 405 nm using an ELISA plate reader Spectramax 250 (Molecular Devices, Inc., Sunnyvale, CA, USA); all of the samples were read in duplicate. For validation of the kits, intra- and inter-assays were performed with the following variations: 0.31–24.11% and 0.35–3.75% for intra- and inter-assays, respectively, for E_2; 0.75–19.99% and 1.75–9.31%, respectively, for T; and 0.33–12.67% and 3.09–9.23%, respectively, for 11-KT.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the pituitaries using the RiboPure™ Kit (Ambion®, Austin, TX, USA) following the manufacturer's instructions. The total RNA and the presence of DNA were quantified and confirmed using a Qubit™ fluorometer (Invitrogen™) with the Quant-iT™ RNA Assay Kit (Life Technologies™, Eugene, OR, USA) and Quant-iT™ dsDNA High-Sensitivity (HS) Assay Kit (Life Technologies™, Eugene, OR, USA), respectively. The calibrations and quantification were performed following the manufacturer's instructions; no samples with DNA contamination were observed. The Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Pico Kit (Agilent Technologies, Palo Alto, CA, USA) were used to evaluate the RNA quality, and RIN values greater than 8.5 were accepted. Approximately 2 μg of total RNA was used as a template to synthesize the first-strand cDNA with SuperScript™ III Reverse Transcriptase (Invitrogen™, Carlsbad, CA, USA) following the manufacturer's instructions, and these cDNA strands were used for cloning purposes. For reverse transcription quantitative PCR (RT-qPCR), SuperScript™ II Reverse Transcriptase (Invitrogen™, Carlsbad, CA, USA) was applied using random hexamer primers (Invitrogen™, Carlsbad, CA, USA) and 2 μg of total RNA as a template.

2.3.1. Cloning of genes encoding partial follicle-stimulating hormone beta polypeptide (fshb), luteinizing hormone beta polypeptide (lhb) and elongation factor 1-a (ef1a)

For partial pacu *lhb* gene cloning, first-strand cDNA was utilized as a template for PCR, with degenerate primers that were designed by Parhar et al. (2003) to clone *lhb* of *Oreochromis niloticus*. To clone the partial region of the pacu *fshb* and *ef1a* genes, PCR was performed with primers that were designed based on other species sequences as deposited in the National Center of Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/genbank) and the unpublished *Salminus hilarii* partial *fshb* gene sequence. The sizes of the expected fragments were 361, 220, and 660 bp for the *fshb*, *lhb* and *ef1a* cDNA, respectively. The primers are listed in Table 2.

The *ef1a* and *lhb* genes were cloned using Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions in a 25.0-μl reaction. The PCR

cycling conditions consisted of 2 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 68 °C; and a final extension step of 5 min at 68 °C, followed by the addition of 1 U/per reaction of Platinum® *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA), after which the mixture was kept for 10 min at 72 °C. For *fshb*, the Advantage 2 DNA polymerase kit (Clontech, Mountain View, CA, USA) was used, and the cycling conditions were 15 s at 95 °C; 35 cycles of 10 s at 95 °C, 15 s at 54 °C and 40 s at 68 °C; and a final extension step of 1 min at 68 °C. PCR products of the expected size were gel-purified, cloned into the pCR® 2.1-TOPO® (Invitrogen, Carlsbad, CA, USA) vector for the *ef1a* and *lhb* PCR products and into the pcDNA™ 3.1/V5-His TOPO® (Invitrogen, Carlsbad, CA, USA) vector for the *fshb* PCR product, and then sent for DNA sequencing.

2.3.2. Real-time quantitative PCR

Quantitative real-time PCR assays were performed in duplicate using 96-well optical plates on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) with default settings. For each 20 µl PCR reaction, 5 µl of cDNA (diluted 2-fold) was mixed with 900 nM sense primer and 900 nM antisense primer in 2 × SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA – see Table 2 for the primer sequences). Target (*fshb* and *lhb*) gene expression was normalized to *ef1a* gene expression and calibrated using the mean of the pituitary *ef1a* expression levels from the Mid sampling period. PCR efficiency was evaluated using the equation $Ct = m \times (\log Q) + c$, where *Ct* is the threshold cycle, Q is the initial copy number, *c* is the intercept on the *y*-axis, and *m* is the slope (Bogerd et al., 2001). A 4-fold serial dilution of the pituitary cDNA mixture was performed, and all of the primer sets had a standard curve with a slope value of approximately 2, indicating exponential PCR amplifications, assuming that 2 cycles are required to generate a 4-fold increase in the PCR product. To calculate gene expression levels, the ΔΔCt method was used, as previously described (Bogerd et al., 2001),

2.4. Stripping, milt quantitative and qualitative characteristics

In both experiments (Exp1 and Exp2), approximately 12 h after the hormonal induction, the fish were netted from the tanks and anesthetized; the urogenital papilla were cleaned and thoroughly dried, avoiding contamination of milt with feces, urine and water; and the milt was stripped through gentle abdominal pressure and collected in a graduated tube (ml) to record the milt volume (MV). Then, an aliquot of milt was dripped onto a slide and observed through an optical microscope to confirm that the sperm were activated. If the sperm were not activated, the sperm motility, duration, and density and the milt pH were assessed.

The sperm motility was estimated subjectively by activating the cells with distilled water (1:5 dilution milt:water). Motility was specified using an arbitrary scale, ranging from 0 to 5, where 0 represents no motility; 1 ≅ 1–25%; 2 ≅ 25–50%; 3 ≅ 50–75%; 4 ≅ 75–90% and 5 ≅ 90–100% of motile spermatozoa (Borges et al., 2005). The sperm motility duration was considered to be the time in seconds between activation and when 50% of the spermatozoa had stopped swimming.

The sperm concentration was determined using a hemocytometer; before counting, the sperm were fixed and diluted (1:2000 milt-fixative ratio) in formol-citrate (2.9 g of trisodium citrate dihydrate and 4 ml of a 35% solution of formaldehyde in q.s. distilled water to 100 ml) *in duplicate*. After leaving the dilutions undisturbed in the Neubauer chamber for 10 min, each dilution was counted under a microscope at 40× magnification. The mean of the counts was considered the sperm density (SD) and was expressed as the number of spermatozoa × 10^{10}/ml. The sperm production (SP) was calculated by the milt volume × sperm density. The pH of the milt was measured in freshly collected milt using a pH meter, model HI 4211 (HANNA® instruments, São Paulo, SP, BR).

The sperm motility, duration and pH were measured in triplicate, and the mean was used. Subjective observations were collected by the same observer to decrease the degree of variation.

2.4.1. Assessment of sperm viability, morphology and seminal plasma

To analyze sperm viability, 10 µl of milt was gently mixed with 90 µl of a solution of eosin/nigrosin (5% eosin Y and 10% nigrosin). This dilution was used to prepare smear slides (*in duplicate*) and examined under a light microscope. The cells that stained pink or red were considered dead, whereas those that stayed unstained were considered living. In total, 200 spermatozoa per slide were counted and classified as alive or dead. The averages were calculated for each period and used to indicate the viability, which was expressed as the percentage of living cells relative to the total cells counted.

To estimate the abnormalities index (sperm morphology), a 15-µl aliquot of fixed sperm in formol-citrate (as described in topic 2.2) was added to 0.5 µl of 3% Rose Bengal stain on a glass slide, and smear slides (*in duplicate*) were generated. The slides were dried and examined under a microscope (100× magnification). A total of 100 spermatozoids were observed on each slide, and the number of anomalous sperm was expressed as a percentage of the total number of cells evaluated. Based on Maria et al. (2012), morphologically anomalous sperm were defined by the following characteristics: detached head, macrocephalic, microcephalic, degenerated head, proximal and distal cytoplasmic drops, degenerated mid-piece and bent, broken, coiled or short tail.

To determine the ionic composition and osmolarity of the semen, each pacu milt sample was centrifuged at 10,000×*g* for 20 min at 4 °C. The seminal plasma was then separated and used to determine the concentrations of Ca^{+2}, Na^{+} and K^{+} (mmol/l) using an electrolyte analysis system (AVL 9180 Electrolyte Analyzer, Roswell, GA, USA). The Mg^{+2} and Cl^{-} concentrations were determined using colorimetric test kits (Labtest, Lagoa Santa, MG, BR). The seminal plasma osmolarity (mOsmol/kg) was measured on a

Table 2
Sequences of degenerate primers for cloning partial pacu (*Piaractus mesopotamicus*) *follicle-stimulating hormone beta polypeptide* (*fshb*), *luteinizing hormone beta polypeptide* (*lhb*) and *elongation factor 1-a* (*ef1a*) cDNAs and sequences of primers for real-time quantitative PCR (RT-qPCR).

	Genes	Sense	Antisense
Cloning	lhb	5′ - TCTSTGGAGAAGGADGGCTG - 3′	5′ - CGAWGGTRCAGTCGGANGTG - 3′
	fshb	5′ - CCATGGTGTTGCTGTTGCC - 3′	5′ - CAGMTRCTBGGCTGCATGCT - 3′
	ef1α	5′ - TCGTTGCTGCTGGTGTTGG - 3′	5′ - TGGGTGGGTCGTTCTTGCT - 3′
RT-qPCR	lhb	5′ - CCTGGTGTTTCAGACCAGCAT - 3′	5′ - CGTGCTGATAGATGGTGGAGAA - 3′
	fshb	5′ - AAGAATGCGGGAGCTGCA - 3′	5′ - GGTCTCATACGTCCACTCCCTAAA - 3′
	ef1α	5′ - TCTGGATGGCACGGAGACA - 3′	5′ - CAATCTTCCATCCCTTGAACCA - 3′
	(B = T + C + G; D = A + G + T; S = G + C; M = A + C; N = A + C + G + T; R = A + G; W = A + T)		

Vapro™ Vapor Pressure Osmometer – Model 5500 (Wescor, Logan, UT, USA).

The total protein in the seminal plasma was quantified using a Qubit™ fluorometer (Invitrogen™, Carlsbad, CA, USA) and a Quant-iT™ Protein Assay Kit (Life Technologies™, Eugene, OR, USA) following the manufacturer's instructions. The resulting values were expressed in mg/ml.

2.4.2. Sperm DNA integrity

Single-cell gel electrophoresis (comet assay) was used to assess DNA integrity following Cabrita et al. (2005). The slides were covered with 0.5% agarose one day before being used. The milt samples were prediluted ($\cong 10 \times 10^6$ spermatozoa/ml) in a non-activating solution (phosphate buffer – NaCl 8.0 g; KCl 0.2 g; Na_2PO_4 1.15 g; KH_2PO_4 0.2 g; q.s. 1000 ml). The dilution (27 μl) was mixed with a 0.5% low-melting agarose solution (273 μl) and was then laminated (85 μl) onto a pre-prepared agarose-veneered slide (all of the samples were assayed in triplicate). The slides was immersed in cell lysis buffer (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1% Triton X-100, 1% N-Laurylsarcosine) for 90 min at 4 °C, after which 10 mM dithiothreitol was added for 1 h at 4 °C. The slides were incubated at room temperature for 30 min. Before electrophoresis, the slides were placed in a horizontal electrophoresis system in contact with the alkaline solution (0.3 M NaOH, 1 mM Na_2-EDTA, pH > 13) for 20 min at 4 °C. The electrophoresis was performed for 20 min at 25 V, 300 mA and 4 °C. The slides were then placed three times with a neutralization solution (0.4 M Tris, pH 7.5 at 4 °C) for 5 min each, drained, fixed in 100% ethanol for 5 min and stored until further observation. Control slides were included in each electrophoresis, and DNA fragmentation was induced by UV exposure. An aliquot (5 ml) of prediluted milts was UV-irradiated with a germicidal lamp at 254 nm (TUV 15W/G15T8 – Philips Co., Eindhoven, NL) in a Petri dish for 20 min at room temperature, at a distance of 20 cm from the lamp.

To visualize the comets, a fluorescent stain (GelRed™ – Biotium, Hayward, CA, USA) was applied, and 50 μl of stain solution (1:3000 – GelRed™/Milli-Q water) was dripped onto the slide and covered with a cover slip. Then, the slides were assessed at 40× magnification with a fluorescence microscope (Leica DM 5000 B) coupled to a digital camera (Leica DFC300 FX), and the images were acquired using the Leica Application Suite (LAS v2.7.1). The comets were classified by visual inspection and divided into five categories: 0 representing undamaged cells (comets with barely detectable or no tails) and 1–4 representing increasing DNA damage (tail length and intensity). A total of 100 comets were used to generate an overall score of between 0 and 400 (Collins et al., 2008). The DNA damage index was calculated following the equation below:

DNA damage index = 0 × (total number of "score 0" comets) + 1 × (total number of "score 1" comets) + 2 × (total number of "score 2" comets) + 3 × (total number of "score 3" comets) + 4 × (total number of "score 4" comets).

2.5. Statistical analysis

The statistical analyses were performed using statistical software (STATISTICA – StatSoft, Inc., Tulsa, OK, USA) or Excel (Microsoft, Redmond, USA). Assumptions of normality and homoscedasticity were tested using Shapiro–Wilk and Levene's tests, respectively. Parametric variables (morphological data, morphometric indexes, plasma and seminal characteristics, hormone levels and sperm characteristics except for the class of sperm motility and the abnormalities index) were assessed using an analysis of variance (ANOVA), followed by Fisher's least-significant difference (LSD) test. The hormonal levels during hormonal treatment were analyzed using Paired Student's *t*-test. For the non-parametric variables (class of sperm motility and abnormalities index), the non-parametric Kruskal–Wallis and Mann–Whitney *U* tests were used. The Pearson's correlation coefficient was calculated, and a linear regression was used to model the relationship between the abnormalities index and the DNA damage index. Significance ($\alpha = 0.05$) was determined for all of the datasets, and the results are shown as the mean ± standard error of the mean (SEM).

3. Results

3.1. Experiment 1

3.1.1. Morphometric, sperm and seminal data

The mean values of the morphological and morphometric indexes are shown in Table 3. The mean values of the SL, BM, K, GSI, VSI and HSI variables were similar among the groups ($p > 0.05$). All of the fish responded positively to hormone treatment by releasing some milt, including RS1.

The mean values of the qualitative and quantitative semen and seminal plasma variables are presented in Table 4. The sperm motility categorization, duration of sperm motility, sperm viability, DNA damage index, abnormalities index and seminal plasma protein mean values are shown in Fig. 1.

The MV of the Beg group (3.58 ± 0.48 ml) was lower ($p < 0.05$) than that of the End group (7.75 ± 1.29 ml) (Table 4). The MV, SD and SP values from the RS1 group were similar to those of the other groups. Furthermore, no significant differences were found ($p > 0.05$) for the SD, SP, seminal plasma ions (Ca^{+2}, Na^+, K^+, Mg^{+2} and Cl^-), osmolarity or pH (Table 4).

The sperm motility, duration of sperm motility and sperm viability values of the End group were reduced. Moreover, the rates of abnormalities were higher in the End group than in the other groups ($p < 0.05$) (Fig. 1). The seminal plasma protein and the DNA damage index (Fig. 1) increased gradually over the investigated period ($p < 0.05$). However, the mean values of all of the evaluated parameters of the RS1 group were similar to those of the Beg group (Fig. 1). Moreover, the RS1 DNA damage index and seminal plasma protein mean values were reduced compared with those of animals that were stripped the first time during the same collection period (Fig. 1) ($p > 0.05$).

We found a strong positive correlation (Pearsoń s correlation coefficient $r = 0.82$; $p < 0.05$) between the DNA damage index and the abnormalities index. Moreover, the linear regression equation $y = 72.3164 + 3.097 * x$ (*Adj.* $R^2 = 0.70$; $p < 0.05$) (Fig. 2) shows that for every one percentage increase in the abnormalities index, the expected DNA damage index increased by 3.10 points.

Table 3
Morphometric values and indexes (mean ± SEM) from pacu (*Piaractus mesopotamicus*) during different times of the breeding season: at the beginning – Beg (11/17/2010), in the middle – Mid & re-stripped – RS1 (12/21/2010), and at the end – End (02/12/2011). Standard length (SL), body mass (BM), condition factor (K), gonadosomatic index (GSI), viscerosomatic index (VSI) and hepatosomatic (HSI).

	Sampling times			
	Beg	Mid	End	RS1
$SL_{(cm)}$	41.33 ± 0.72[a]	41.06 ± 1.06[a]	44.07 ± 1.42[a]	38.42 ± 0.98[a]
$BM_{(Kg)}$	2.10 ± 0.07[a]	2.38 ± 0.22[a]	2.54 ± 0.21[a]	1.87 ± 0.10[a]
K	2.09 ± 0.13[a]	2.48 ± 0.18[a]	2.44 ± 0.32[a]	2.29 ± 0.09[a]
GSI	–	0.76 ± 0.25[a]	0.80 ± 0.14[a]	0.84 ± 0.17[a]
VSI	–	5.84 ± 0.64[a]	5.91 ± 0.24[a]	5.54 ± 0.33[a]
HSI	–	0.80 ± 0.06[a]	0.64 ± 0.03[a]	0.92 ± 0.10[a]
n	7	5	4	4

Different superscript letters denote significant differences among sampling times, for each variable ($p < 0.05$).

Table 4
Chemical characteristics of the seminal plasma (mean ± SEM) of pacu (*Piaractus mesopotamicus*) in the beginning - Beg (11/17/2010), middle – Mid & re-stripped – RS1 (12/21/2010), and end - End (02/12/2011) of the breeding season. Milt volume (MV), sperm density (SD) and sperm production (SP).

	Sampling times			
	Beg	Mid	End	RS1
$MV_{(ml)}$	3.58 ± 0.48[a]	6.33 ± 2.49[ab]	7.75 ± 1.29[b]	6.00 ± 0.54[ab]
$SD_{(spz\times10^{10}/ml)}$	6.76 ± 1.58[a]	6.94 ± 0.34[a]	4.74 ± 0.29[a]	5.42 ± 0.26[a]
$SP_{(spz\times10^{11})}$	2.69 ± 0.93[a]	4.54 ± 1.93[a]	3.75 ± 0.73[a]	3.26 ± 0.35[a]
pH	8.05 ± 0.10[a]	8.29 ± 0.02[a]	8.27 ± 0.02[a]	8.17 ± 0.03[a]
$Ca^{+2}_{(mmol/l)}$	0.65 ± 0.02[a]	0.70 ± 0.03[a]	0.66 ± 0.03[a]	0.71 ± 0.02[a]
$Na^{+}_{(mmol/l)}$	93.83 ± 2.69[a]	97.00 ± 3.51[a]	92.50 ± 3.20[a]	95.25 ± 1.49[a]
$K^{+}_{(mmol/l)}$	42.67 ± 2.64[a]	36.00 ± 2.08[a]	38.25 ± 0.48[a]	40.50 ± 2.63[a]
$Mg^{+2}_{(mmol/l)}$	0.31 ± 0.04[a]	0.59 ± 0.10[a]	0.46 ± 0.10[a]	0.51 ± 0.08[a]
$Cl^{-}_{(mmol/l)}$	118.42 ± 3.69[a]	115.20 ± 1.02[a]	110.98 ± 3.89[a]	124.25 ± 3.62[a]
$Osmolality_{(mOsmol/kg)}$	208.50 ± 17.36[a]	190.00 ± 4.36[a]	194.00 ± 5.05[a]	204.75 ± 7.47[a]
n	6	4	4	4

Different superscript letters denote significant differences among sampling times for each variable ($p < 0.05$).

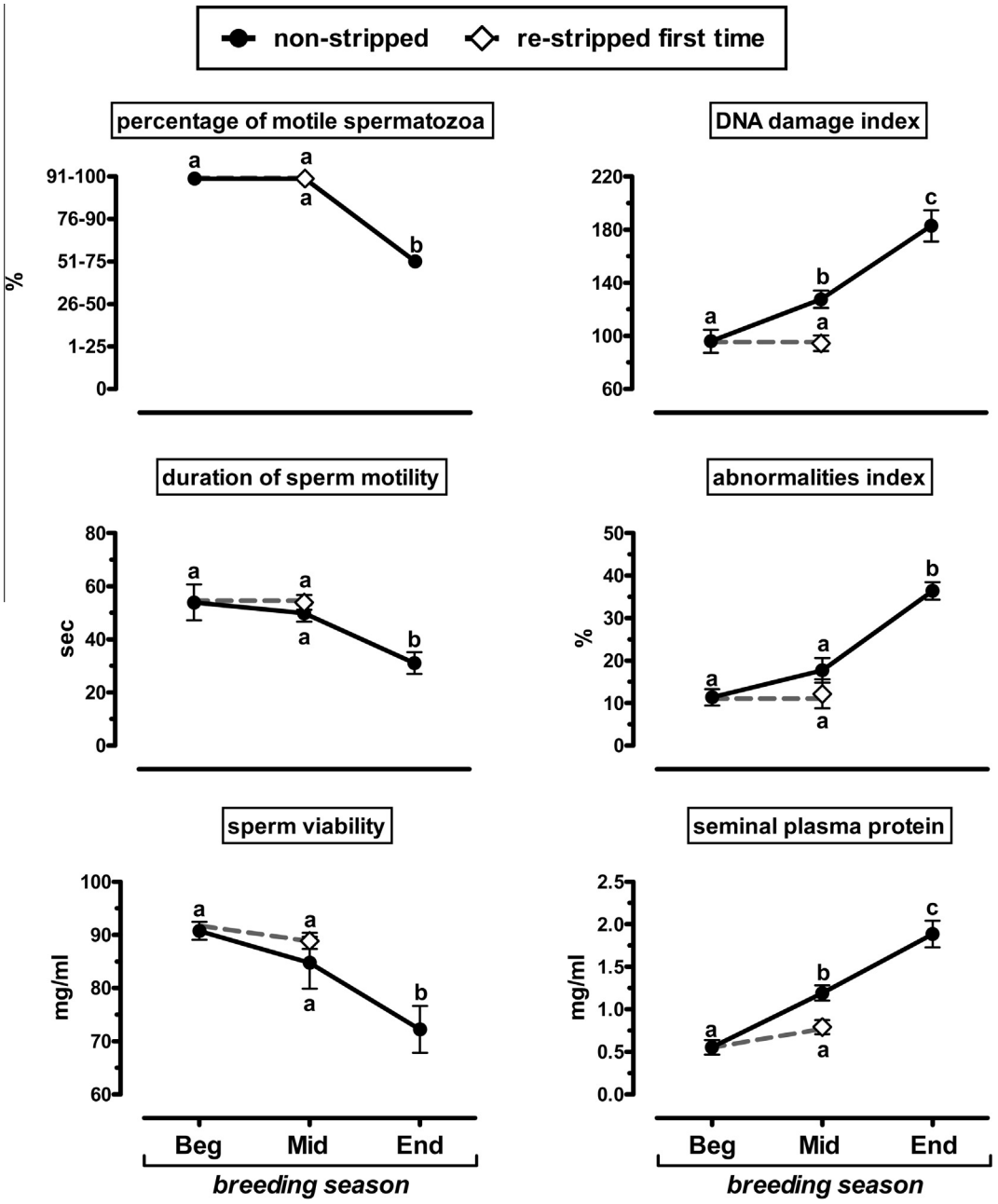

Fig. 1. Sperm characteristics (mean ± SEM), class of sperm motility, duration of sperm motility, sperm viability, DNA damage index, abnormalities index and seminal plasma protein during the beginning – Beg (11/17/2010), middle – Mid & re-stripped – RS1 (12/21/2010) and end – End (02/12/2011) of the breeding season. Different letters denote significant differences among the sampling days for each variable ($p < 0.05$).

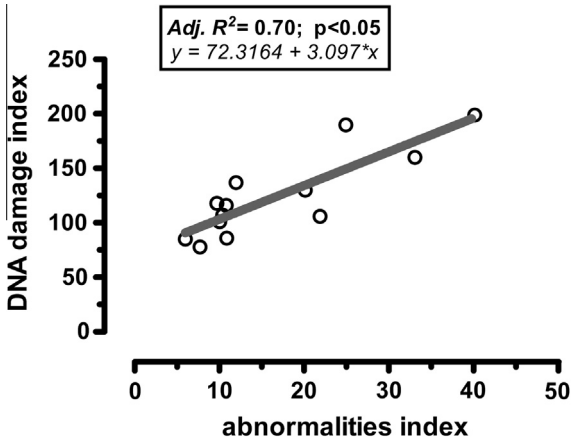

Fig. 2. Linear regression analysis between the DNA damage and abnormalities indexes. A strong, significant positive Pearson's correlation was found between the two indexes ($r = 0.82$; $p < 0.05$).

3.1.2. Testis histology and plasma sex steroid levels

The classification of the testes of the Mid group was as follows (with their respective frequencies in parentheses): IV–V (8%), VI–V (84%) and VII–VI (8%) (Supplementary Figs. 1 and 2). In the End group, we found fish with testes that were classified into stages V–VI and VII (92% and 8%, respectively), indicating advances in the regression process. In the RS1 group, testes were observed only in stages IV–V and V–VI (50% and 50%) (Supplementary Fig. 1).

As shown in Fig. 3, the E_2 mean plasma levels in the End group (before hormone treatment) (0.208 ± 0.059 ng/ml, $p < 0.05$) were inferior to those of the other groups. The mean values of E_2 were similar among groups after induction. Significant increases ($p < 0.05$) in the E_2 mean concentrations within each group due to hormonal treatment were detected only in the Beg group (from 0.688 ± 0.170 to 0.963 ± 0.191 ng/ml) and RS1 (from 0.429 ± 0.188 to 0.853 ± 0.150 ng/ml) (Fig. 3).

The plasma concentration of T before hormonal induction was lower (0.063 ± 0.001 ng/ml, $p < 0.05$) in the End group compared with the other groups (Fig. 3). The values were similar among the groups after induction. Significant increases ($p < 0.05$) in T concentration after hormonal treatment were detected in the End group (from 0.063 ± 0.001 to 1.310 ± 0.285 ng/ml) and the RS1 group (from 0.488 ± 0.384 to 2.834 ± 0.388 ng/ml).

The values of 11-KT were similar among the groups before hormone treatment ($p > 0.05$). After stripping, the mean values of 11-KT of the Beg and RS1 groups were higher ($p < 0.05$) than those of the other groups. Among the analyzed steroids, the most pronounced differences during hormonal treatment were those relating to 11-KT increases. In the Beg and RS1 groups, these values increased ≅23 times (from 0.194 ± 0.050 to 4.431 ± 0.713 ng/ml) and ≅27 times (0.165 ± 0.036 to 4.448 ± 0.667 ng/ml), respectively, compared with the baseline level.

3.1.3. Partial cloning and gene expression

The partial sequences of *fshb*, *lhb* and *ef1a* cDNAs were isolated and sequenced and then used for BLAST analysis (Altschul et al., 1997). The deduced amino acid sequence of the 361-bp pacu *fshb* cDNA fragment (accession number KC867349) is 82%, 79% and 74% similar to the follicle-stimulating hormone beta subunit amino acid sequences of North African catfish (*C. gariepinus* – accession number AAN75753.1), southern catfish (*Silurus meridionalis* – AAY42270.1) and roach minnow (*Rutilus rutilus* – ACN79582.1), respectively. The deduced amino acid sequence of the 259-bp pacu *lhb* cDNA fragment (accession number KC867348) shows 96% similarity with the luteinizing hormone beta subunit amino acid sequences of marinka prenantova (*Schizothorax prenanti* – AEB33873.1), black carp (*Mylopharyngodon piceus* – AAK07414.1), grass carp (*Ctenopharyngodon idella* – ABM73670.1), bighead carp (*Hypophthalmichthys nobilis* – ABQ42715.1) and fathead minnow (*Pimephales promelas* – ABB51645.1). The pacu 660-bp *ef1a* cDNA fragment (accession number KC867347) is 100% similar to that of rock-bacu (*Lithodoras dorsalis* – AAQ62486.1), bacú (*Megalodoras uranoscopus* – AAQ62485.1), bagre hueso (*Rhinodoras boehlkei* – AAQ62489.1), bagre (*Anadoras grypus* – AAQ62485.1), sierra culata (*Trachydoras microstomus* – AAQ62525.1) and sierra paeña (*Orinocodoras eigenmanni* – AAQ62492.1).

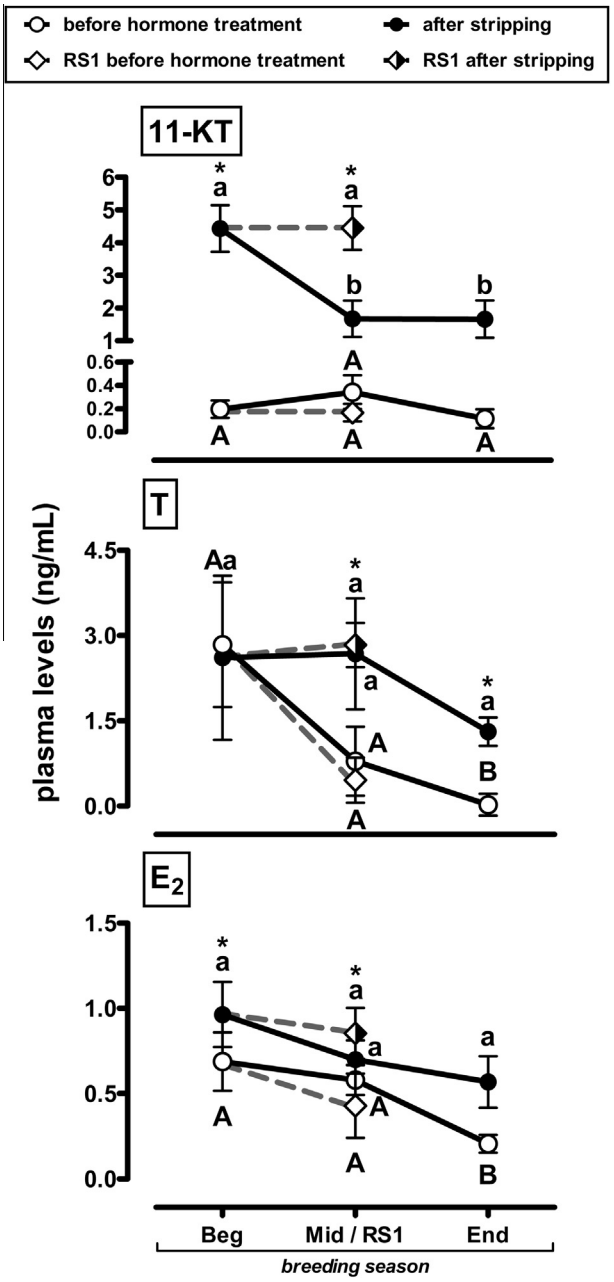

Fig. 3. Plasma 17β-estradiol (E_2), testosterone (T) and 11-ketotestosterone (11-KT) levels in pacu males (*Piaractus mesopotamicus*) during the beginning – Beg (11/17/2010), middle – Mid & re-stripped – RS1 (12/21/2010), and end – End (02/12/2011) of the breeding season. Different capital letters denote significant differences ($p < 0.05$) among the "before hormonal induction" samples for each variable during the breeding season and RS1; within the same mean, different small letters represent significant differences ($p < 0.05$) among "after stripping" samples. *Indicates a significant difference ($p < 0.05$) between the "before hormonal induction" and "after stripping" samples within the same sample data and for each variable.

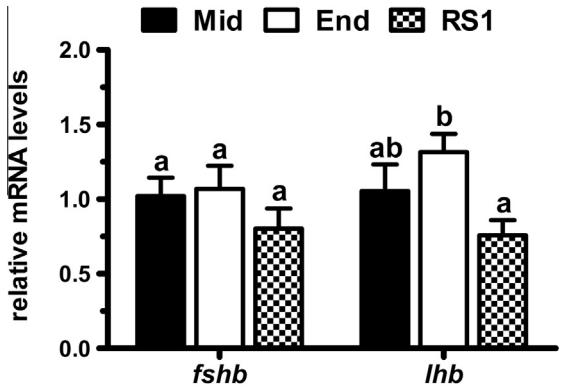

Fig. 4. Relative expression of *fshb* and *lhb* in the pituitary of pacu (*Piaractus mesopotamicus*) males that were collected after hormonal induction (after stripping) during the beginning – Beg (11/17/2010), middle – Mid & re-stripped – RS1 (12/21/2010) and end – End (02/12/2011) of the breeding season. Different letters denote significant differences ($p < 0.05$) among the sampling days for each gene.

Expression of the *fshb* gene was similar ($p > 0.05$) among the groups. In the expression profile of *lhb*, the mean values of the End group were greater than those in the RS1 group ($p < 0.05$), and the mean values of the Mid group were similar to those of the other groups ($p > 0.05$) (Fig. 4).

3.2. Experiment 2

3.2.1. Morphometric and seminal data

All of the fish responded positively to hormone treatment by releasing some amount of milt, including the RS1 and RS2 groups. The mean values of BM, MV, SD and SP and the statistical evaluations among groups are presented in Table 5. The MV (3.13 ± 0.48 ml) of the Beg group was less ($p < 0.05$) than that of the End group (5.57 ± 0.42 ml) and the RS1 group (5.45 ± 0.80 ml) (Table 5). Moreover, the Beg group had a lower ($P < 0.05$) mean SD than all other groups and a lower SP than the End group ($p < 0.05$). The MV, SD and SP of RS1 and RS2 were similar to those of other groups, except for the Beg group, as described above.

Concerning the sperm characteristics (Fig. 5), the Mid and RS1 groups presented higher categories of sperm motility ($p < 0.05$) than the Beg and End groups. The RS1 group had the highest duration of sperm motility ($p < 0.05$). The End group had reduced rates of sperm viability and the highest rates of abnormalities compared with the other groups ($p < 0.05$), whereas the Mid group had higher rates of abnormalities than all other groups except End ($p < 0.05$). In addition, the mean values of sperm viability and the abnormalities index in the RS1 and RS2 groups were similar to each other and to those of the Beg group ($p > 0.05$).

4. Discussion

Pacu sperm and seminal characteristics were evaluated throughout the spawning period using various parameters, including milt volume, class and duration of sperm motility, sperm viability, DNA damage index, abnormalities index and seminal plasma protein concentration. Except for a gradual increase in the concentration of seminal plasma protein during the breeding season, the analyzed seminal plasma characteristics (ion concentration, osmolarity and pH) were constant. In contrast, we observed a marked reduction in the CAGQS from the Beg period to the End period. Considering all of the evaluated parameters (Rurangwa et al., 2004; Cabrita et al., 2014), sperm with CAGQS were observed in the Beg (early spawning season, 11/17/2010 and 11/12/2013), RS1 (12/21/2010 and 12/18/2013) and RS2 (01/23/2014) groups, strongly suggesting that cellular aging may be related to decreased CAGQS. Our results suggest a decrease in pacu sperm quality throughout the spawning season and imply that using sperm from the end of the spawning period should be avoided.

Several studies have reported that poor sperm quality directly affects oocyte fertilization success, generating poor-quality embryos (Campbell et al., 1992, 1994; Wu et al., 2003; Bobe and Labbé, 2010; De Souza et al., 2015). Pacu farmers may be using lower-quality semen because the only criterion that is routinely used in hatcheries is the ability to release semen. In fact, males that release a higher milt volume are preferentially selected over those that release a lower volume. Our results indicate that the best strategy for this species would be to induce reproduction at the Beg period. Although a smaller volume of semen may be obtained, it will exhibit CAGQS and provide a greater number of viable sperm, ensuring the production of viable embryos and larvae.

Studies of semen quality have demonstrated that ion concentrations, osmolarity and pH may be correlated with motility (for review: Cosson, 2010), which is in turn associated with CAGQS (Rurangwa et al., 2004). However, in this study, the seminal physico-chemical characteristics were stable during the experiment, and the motility variables were reduced in the End group. The mean values for sperm motility and duration of motility were greater in the Beg group than in the End group in Exp1. Therefore, in this study, we did not find any association of ion content, osmolarity or pH with semen motility or CAGQS.

In Exp2, in contrast with Exp1, the sperm motility categorization and the duration of sperm motility in the Beg group were not different from those in the End group. This result can be explained, at least partially, by differences in each particular spawning season. Spawning is affected by abiotic parameters, such as temperature, photoperiod, and pluviosity (Costa and Mateus, 2009; Oliveira et al., 2010; Dala-Corte and Fialho, 2014; Giora et al., 2014), which can influence the spermatogenic process (Rurangwa et al., 2004).

Consistent with the Exp2 results, the common carp (*Cyprinus carpio*) has previously been found to have lower sperm quality (including motility) in the Beg and End periods (Christ et al., 1996). The lower motility in the Beg group in Exp2 was likely related to 'sperm maturation' or 'capacitation', which, according Schulz and Miura (2002), "*...is the development from non-functional gametes to mature spermatozoa fully capable of*

Table 5
The mean ± SEM of body mass (BM), milt volume (MV), sperm density (SD) and sperm production (SP) from pacu (*Piaractus mesopotamicus*) during different times of breeding season: at the beginning – Beg (11/12/2013), in the middle – Mid & re-stripped – RS1 (12/18/2013), at the end – End & re-stripped 2 – RS2 (01/23/2014).

	Sampling times				
	Beg	Mid	End	RS1	RS2
$BM_{(Kg)}$	1.79 ± 0.15^{a}	1.83 ± 0.15^{a}	1.59 ± 0.14^{a}	1.76 ± 0.20^{a}	1.58 ± 0.11^{a}
$MV_{(ml)}$	3.13 ± 0.48^{a}	4.06 ± 1.01^{ab}	5.57 ± 0.42^{b}	5.45 ± 0.80^{b}	4.40 ± 0.73^{ab}
$SD_{(spz \times 10^{10}/ml)}$	2.42 ± 0.23^{a}	4.19 ± 0.41^{b}	3.56 ± 0.38^{b}	3.69 ± 0.79^{b}	3.64 ± 0.44^{b}
$SP(spz \times 10^{11})$	0.81 ± 0.19^{a}	1.73 ± 0.47^{ab}	2.00 ± 0.27^{b}	1.80 ± 0.67^{ab}	1.35 ± 0.43^{ab}
n	6	5	6	4	4

Different superscript letters denote significant differences among sampling times, for each variable ($p < 0.05$).

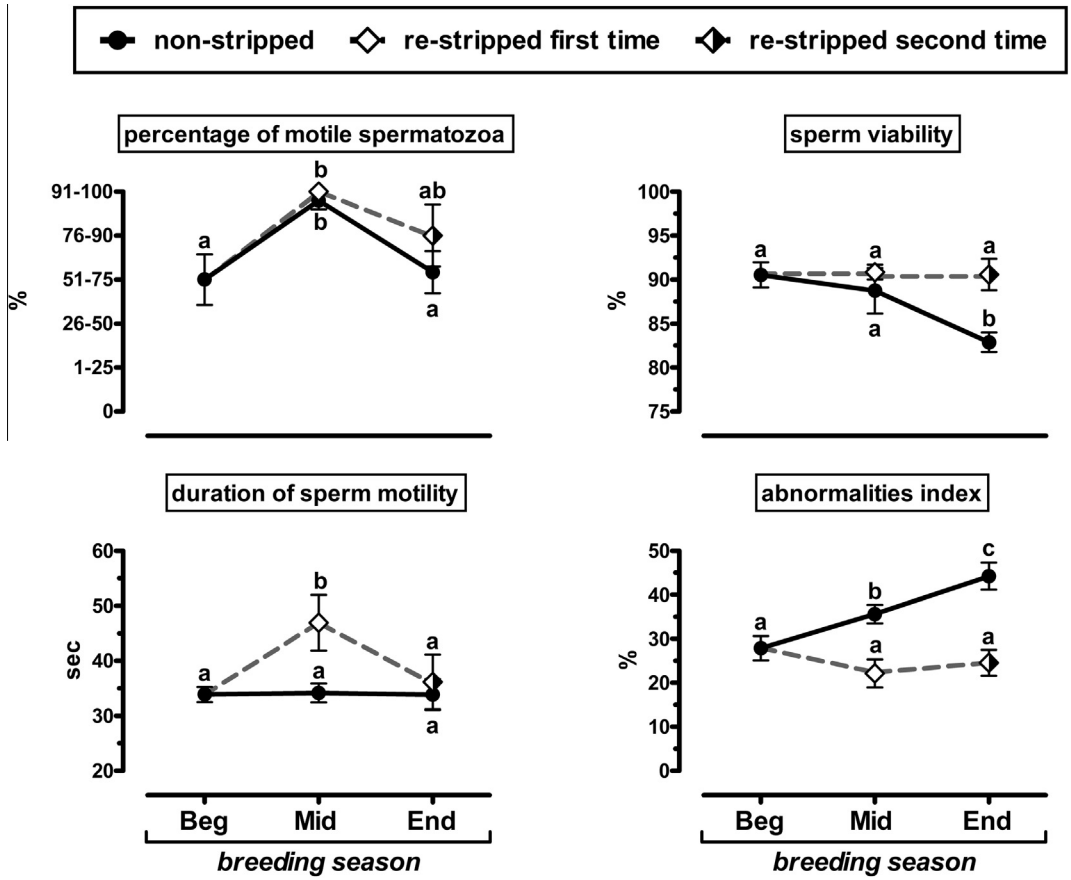

Fig. 5. Sperm characteristics (mean ± SEM), class and duration of sperm motility, sperm viability and abnormalities index during different times of the breeding season: at the beginning – Beg (11/12/2013), in the middle – Mid & re-stripped – RS1 (12/18/2013), and at the end – End & re-stripped 2 – RS2 (01/23/2014). Different letters denote significant differences between the sampling days for each variable ($p < 0.05$).

vigorous motility...” Additionally, maturation or capacitation is accompanied by sperm hydration, which involves production of efferent duct fluid, and the mixture of seminal fluid and sperm in the ducts is milt (Schulz and Miura, 2002). Furthermore, there were lower MV values in the Beg groups in both of the experiments (Exp1 and Exp2) and lower SD and SP values in Exp2, indicating a lower quantity of sperm in the lumen. The differences in the End group may be related to the “aging” of sperm. Reductions in sperm motility and in the duration of sperm motility from the beginning to the end of the breeding season have been reported in other species (Suquet et al., 1998).

In our study, we found an association between DNA damage, sperm morphological abnormalities and changes in seminal plasma proteins. In mammals (bull), sperm DNA damage is correlated with morphological sperm abnormalities (Enciso et al., 2011), but ours is the first report of a strong correlation between sperm DNA damage and morphological abnormalities in a teleost fish. In addition, relationships of seminal plasma proteins to sperm quality may occur (Lahnsteiner et al., 2004; Lahnsteiner, 2007; Li et al., 2009; Cecchini and Caputo, 2010; Butts et al., 2011). Unfortunately, there are as yet no rapid methods for verifying these characteristics in the field to avoid the use of this type of semen; such methods would be of great value to aquaculture of pacu and other fish species.

The expression of *lhb* in this study was induced at the End. In *O. mykiss* a higher expression of *lhb* at the End was associated with an elevation of Lh (Gomez et al., 1999). In *D. labrax* both Lh protein and *lhb* mRNA levels increase through spermatogenesis and reach maximum levels during the period of full-spermiation (Mateos et al., 2003). As GnRH-treated males can stimulate an increase in *lhb* mRNA expression and Lh plasma levels (Kitahashi et al., 1998; Mateos et al., 2002), GnRH treatment, not only during the breeding period, may also be associated with higher levels of pacu *lhb* expression in the End period. The interference of the GnRH treatment with the *lhb* levels and its importance in attempts to re-strip should be addressed in future studies.

The groups with CAGQS (Beg and RS1) were also those that experienced a significant increase in 11-KT ($p < 0.05$) following hormonal induction. The hormone 11-KT is the most potent androgen in fish (Borg, 1994; Nagahama and Yamashita, 2008; Schulz et al., 2010) and may increase during spermiation (Prat et al., 1990; Carragher and Pankhurst, 1993; Adebiyi et al., 2013) and after hormonal treatment in stellate sturgeon (*Acipenser stellatus* Pallas) (Semenkova et al., 2002). In this study, the elevation of the 11-KT levels was related to good-quality semen in pacu. A positive correlation between 11-KT and sperm motility characteristics (percentage and duration of motility) was also found in Persian sturgeon (*Acipenser persicus*) during the final testicular maturation induced by hormonal treatment (Hajirezaee et al., 2011). In addition, with respect to changes in the plasma levels of T during hormonal induction, differences were only found in the RS1 and End groups and were therefore not related to semen quality. Moreover, further research is required to understand the reasons for the induction of T in the End and RS1 groups.

We observed a reduction in the T and E_2 plasma levels in the End period compared with those during other periods. A similar profile of T was also found in rainbow trout (*O. mykiss*), in which the levels of T were similar at the end of the spawning season

(Baynes and Scott, 1985; Espinosa et al., 2011). The function of E_2 is still largely unknown in teleost males but may be associated with the proliferation of immature germ cells, which would explain the gradual reduction in E_2 concentration in pacu as regression approached. Similar reasoning can be developed for T (Nóbrega et al., 2009), which plays a role in spermatogenesis and in the production of E_2. Therefore, the decreased concentrations of both steroids appear to have marked the end of spermiation in pacu.

Because sperm production begins many months before sperm release and may require only a few days (Nóbrega et al., 2009; Schulz et al., 2010), "aged sperm" may be responsible for the low fertility rates in spawning in this and other species. The comet assay is an emerging tool for sperm analysis to measure the effects of aging, and DNA damage may be an oxidative stress marker because reactive oxygen species (ROS) that result from normal cell metabolism can damage DNA (Cabrita et al., 2014). "Aged sperm" may experience increased exposure to ROS. Indeed, ROS are harmful to cells, and consequently, spermatozoa may lose their DNA integrity, motility and vitality (Aitken et al., 2012). Re-stripping is therefore a relevant and efficient tool for the renewal and collection of freshly produced sperm. Re-stripped males remained in maturation, even 35 days after the first stripping, and it is therefore possible to re-strip again after more than 35 days. These results indicate that this management does not induce depletion, regression or rest in RS1 or RS2 males. Thus, it is possible that using GnRHa for hormonal induction may have influenced positively the milt quality in re-stripped pacu, as has been reported for *D. labrax* treated with GnRHa, which exhibited increased and prolonged spermiation (Sorbera et al., 1996). Hormonal stimulation of spermiation and re-stripping of pacu need to be explored in future studies.

Intervals of 90 and 7 days between stripping and re-stripping are possible in catfish and salmonids, respectively (Heyrati et al., 2010; Diyaware et al., 2010). In pacu, an interval of 35 days after first stripping allowed for testis recovery and generated an MV similar to that obtained from the first stripping in the same period (Mid or End). Given the differences between the characteristics of spermatogenic process among teleosts, such as the need for ablation in catfish (Diyaware et al., 2010), it is possible that stripping and re-stripping require a specific case study for each species. Pacu semen and sperm quality are gradually reduced throughout the breeding season. Hormonal induction should be performed as soon as the females are ready (normally later than males), early in the season when the semen is of superior quality, taking care not to strip too early, as in Exp2. We found that re-stripping is viable and also represents an interesting management method to maintain pacu semen and sperm quality throughout the breeding season, including permitting semen cryopreservation. Moreover, the first collection of semen for re-stripping should be performed from the middle to the end of the first third of the spawning period, which will ensure excellent-quality semen and allow sufficient time for additional stripping.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2015.06.007.

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