Sperm contamination by urine in Senegalese sole (*Solea senegalensis*) and the use of extender solutions for short-term chilled storage.

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Highlights

Urine contamination of sperm samples appears inevitable due to the proximity of male reproductive and urinary systems.

Urine contamination increased seminal plasma osmolality, decreased pH and reduced sperm quality.

The spermatozoa cell concentration was similar in samples that appeared to be uncontaminated or contaminated with urine.

The dilution of sperm in modified Leibovitz or Marine Freeze®, preserved sperm quality for 24 hours.

Abstract

Methods are needed to manage the sperm of Senegalese sole (*Solea senegalensis*), which will enable the industry to use artificial fertilisation to reproduce hatchery raised sole and implement breeding programs. The present study aimed to (a) describe the male reproductive and urinary system, (b) describe the effects of urine contamination on sperm quality and (c) examine the use of extenders for short term chilled storage of sole sperm. Nine males were dissected to describe the male reproductive and urinary system. A total of 49 males were examined and 32 (65.3%) provided adequate sperm samples of the study. Initially the samples were described by appearance (colour, transparency and fluidity) and sub-samples analysed for sperm quality, urea concentration, osmolality, pH and protein concentration. Cell concentration and sperm quality parameters, percentage motility, curvilinear velocity (VCL) and average path velocity (VAP), were measured using ImageJ CASA. Control samples and samples diluted (1:3) in six different extender solutions (modified Leibovitz, Ringer, NAM, Sucrose, Stor Fish® and Marine Freeze®) were stored short-term (4ºC) and tested zero, three, six and 24 hours after collection. The close proximity of the reproductive and the urinary systems, especially the sperm ducts being attached to the urinary bladder makes obtaining sperm without urine contamination appear difficult. All the samples appeared to be contaminated with urine. Samples that appeared to be contaminated with urine (yellow colour) had similar spermatozoa cell concentration and urea concentration as samples that appeared not to be contaminated with urine (whitish colour), although motility was significantly lower in yellow samples. Seminal plasma urea concentration was positively correlated with osmolality. Cluster analysis grouped samples with significantly higher sperm quality and pH and significantly lower urea concentration and osmolality to indicate that urine contamination negatively affected sperm quality by increasing osmolality and decreasing pH. Amongst the six extender solutions Leibovitz and Marine Freeze® preserved significantly higher percentage motility 24 hours after collection. Ringer, NAM and Stor Fish® were intermediate and Sucrose was similar to control samples that significantly decreased motility three hours after collection. Taken together all sole sperm samples probably had urine contamination, which is difficult or impossible to avoid especially if all the sperm available needs to be collected. The extenders, Leibovitz and Marine Freeze® were used to maintain sperm quality and mitigate the negative effects of urine contamination. The collection and short term chilled storage in extenders of sole sperm from the majority of males in a broodstock (65.3%) can provide a valid sperm management system for industrial application for artificial fertilisation, however, further work is needed.

Keywords: *Solea senegalensis*, sperm motility, urine, extender solutions, chilled storage.

Introduction

Senegalese sole (*Solea senegalensis*) is a marine flatfish of important commercial value that is emerging as an aquaculture species. In five years, aquaculture production of Senegalese sole has increased from 95t in 2012 to 1818t in 2017 (FAO 2019). Nevertheless, the control of Senegalese sole reproduction in captivity has not been fully successful as hatchery reared males have a reproductive behavioural dysfunction and do not fertilize the eggs released by females (Guzman *et al*., 2009; Carazo 2013; Martin 2016; Martin *et al*., 2019). Currently, sole production is based on wild broodstocks that spawn spontaneously in captivity and, therefore, the industry relies on the capture of wild breeders, which is unsustainable (Morais *et al*., 2016). A possible solution to this problem has been the development of artificial fertilisation methods using gametes stripped from mature cultured Senegalese sole (Liu *et al*., 2008; Rasines *et al*., 2012; 2013). However, the development and application of artificial fertilisation protocols at industrial scale has been frustrated by the low volumes of sperm, poor sperm quality and high variability in sperm quality among individuals (Cabrita *et al*., 2006; 2011; Beirão *et al*., 2009; 2011; Chauvigné *et al*., 2016; 2017). Therefore, solutions are required to address these problems.

Low sperm volumes are probably related to the small testes size, the semi-cystic spermatozoa development and the spawning behaviour. Males have two small testes and low gonadal somatic index (Gracía-López *et al*., 2005), which produce low volumes of sperm. Spermatogenesis in sole is semi-cystic, which is different to the cystic development observed in most aquaculture species and which may be another factor implicated in low sperm production (Gracía-López *et al*., 2005, Mylonas *et al*., 2017). This low sperm production may be related to low sperm requirements considering the mating behaviour of Senegalese sole (Carazo *et al*., 2016). During spawning, males hold the urogenital pore in close proximity to the oviduct and sperm are introduced to the eggs at the point of release from the oviduct, which probably reduces the requirement for large numbers of sperm to achieve a successful fertilisation. Initial attempts to increase sperm volume with hormones doubled sperm production (Agulleiro *et al*., 2006; 2007; Guzman *et al*., 2011), however, recent studies with species-specific recombinant gonadotropins have increased sperm production by four times (Chauvigné *et al*., 2017; 2018).

A second aspect that affects both sperm volume and quality is the contamination with urine. In Senegalese sole, the spermatic ducts and the urinary system share the same urogenital pore (Gracía-López *et al*., 2005), thus it is difficult to avoid contamination with urine when sperm is collected. In other species, urine contamination has been determined by measuring urea in the seminal plasma (Dreanno *et al*., 1998) and contamination by urine or the presence of urea has been shown to negatively affect the quality of sperm in various species (Król *et al*., 2018; Cabrita *et al*., 2001; Rurangwa *et al*., 2004). The urine contamination changes the environment of the spermatozoa by altering aspects of the seminal plasma such as osmolality and pH (Cosson *et al*., 2008). Urine induced changes in osmolality and ion content, may cause the activation of spermatozoa during the collection of sperm. In freshwater fish the hypo-osmotic urine may reduce the seminal plasma osmolality to activate the spermatozoa (Alavi *et al*., 2007), whilst in marine fish the variable, but similar iso-osmotic urine (Fauvel *et al*., 2012) may change ion balance or even vary the osmolality of the seminal plasma to also activate the spermatozoa (Cosson *et al*., 2008; Valdebenito *et al*., 2009). This early activation reduces the percentage of motile spermatozoa, spermatozoa swimming speed and, therefore, the ability of the sperm to fertilize eggs (Poupard *et al*., 1998; Rurangwa *et al*., 2004; Linhart *et al*., 2003; Alavi *et al*., 2006; Cejko *et al*. 2010). In addition, urine contamination has caused a decrease in pH (acidification) (Ciereszko *et al*., 2010; Fauvel *et al*., 2012), which has been observed to also reduce motility (Nynca *et al*., 2012). Therefore, sperm samples contaminated with urine are usually discarded (Dreanno *et al*., 1998; Poupard *et al*., 1998; Król *et al*., 2018) and most studies with Senegalese sole only use what was considered by appearance to be only sperm and samples that appeared to be contaminated were not used (Agulleiro *et al.,* 2006; Cabrita *et al*., 2006; 2011; Beirão *et al*., 2008; 2009; 2015; Martinez-Pastor *et al*., 2008; Valcarce *et al*., 2016; Riesco *et al*., 2017; 2019; Fernandez *et al*., 2019). To date, no studies have examined the effect of urine contamination on the quality of Senegalese sole sperm.

Extender solutions have been used to preserve contaminated sperm and maintain sperm quality. These extender treatments have been developed to prevent the activation and damage of the spermatozoa by urine contamination (Rodina *et al*., 2004; Sarosiek *et al*., 2012; Gallego *et al*., 2013; Beirão *et al*., 2019). Generally, the sperm is diluted with the extender solution that lengthens the storage period and maintains sperm quality parameters. Extender solutions have been made from a combination of ions, antioxidants, amino acids, sugars and antibiotics and are species-specific. Extenders solutions have become an essential aspect for sperm conservation (short or long term storage), which ensures the availability of sperm for artificial fertilisation (Rodina *et al*., 2004; Bobe and Labbé 2009; Cabrita *et al*., 2010; Gallego *et al*., 2013; Beirão *et al*., 2019). Cryopreservation protocols have been studied for Senegalese sole (Rasines *et al*., 2012; Valcarce and Robles 2016; Riesco *et al*., 2017) and used also to have availability of sperm for artificial fertilisation (Rasines *et al*., 2012; 2013). These cryopreservation protocols used only what was considered uncontaminated sperm. Short term chilled storage of sperm using extenders have the possibility to work with contaminated sperm and are also useful for artificial fertilisation protocols (Bobe and Labbé 2009; Beirão *et al*., 2019; Ramos-Júdez *et al*., 2019). In addition, short term chilled storage of sperm is easier, cheaper and a more practical method to preserve sperm in the hatcheries. However, no studies have been published on the use of extender solutions for the short-term storage of Senegalese sole sperm.

The aim of the present study was to: (a) describe the anatomy of the urinary and male reproductive system to understand why Senegalese sole sperm is usually contaminated; (b) describe the characteristics of Senegalese sole sperm in relation to urine contamination; (c) examine the use of a range of extender solutions for chilled short-term storage to maintain the sperm quality parameters, motility and velocity.

Materials and methods

2.1 Animals and sample collection

The Senegalese sole broodstock used in the present study was kept in the facilities in IRTA Sant Carles de la Rápita (Catalonia, Spain). The broodstock was kept in two tanks (14 m^3) connected to a recirculation system (IRTAmar®) with a controlled natural temperature cycle (9-20 ºC) and under natural photoperiod (9-14 hours light). The fish were fed with 0.75% of wet feed (polychaetes and mussels) and 0.55% dry feed (balance diet) of total biomass, four days a week.

Trials were carried out during the two natural periods of reproduction of the sole, in autumn and in spring. Individual males (mean weight = 559 ± 193 g) were chosen randomly and anesthetized with 60 mg L^{-1} tricaine methanesulfonate (MS-222; Sigma-Aldrich, Spain) and weighed. Semen samples were obtained by applying gentle abdominal pressure towards the urogenital pore and collected with a 1 mL syringe. First, the testes were located by touch and gently massaged and then, the sperm duct was gently stripped from the testes towards the urogenital pore. This testes massage followed by sperm duct stripping was repeated to obtain the sperm sample. The volume collected was recorded and the sperm was placed in Eppendorf tubes above crushed ice.

The structure of the sole male reproductive and urinary system was examined in nine specimens. Males were sacrificed with an overdose of MS-222 (120 mg L−1). The reproductive and urinary system was dissected and the morphology and organization of both systems was examined and described. The length of seminal ducts and testis size were measured with a Vernier calliper and the testes weighed. The sperm ducts were fixed in Bouin´s solution, dehydrated in a series of alcohol baths, embedded in paraffin, cut into 5 µm sections and stained with H&E (Hematoxylin and eosin) for histological examination.

The broodstock was handled (routine management and experimentation) in agreement with European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA, http://www.felasa.eu/).

2.2 Assessment of sperm parameters

When collected, each sperm sample obtained was described according to the features such as tonality (sample colour: yellow, whitish yellow or whitish), transparency (translucent or opaque feature of the sample) and consistency (viscosity or fluidity of the sample) (Fauvel *et al*., 1999; 2012). All samples were divided into three sub-samples, the first subsample (100 µL) was used to assess the sperm quality in the short-time storage and diluents, the second subsample (20 µL) was used to measure the pH and cell concentration and the third sub-sample (80 µL) was centrifuged to perform different analysis. All samples were stored at 4 °C until assessment. During storage, the Eppendorf tubes were kept open for gas exchange. The following parameters: pH, cell concentration, osmolality and protein concentration were measured for each sample.

The pH was measured with a Hach electrode and CyberScan Instruments (Eutech Ins. pH510). To determine cell concentration (spermatozoa mL^{-1}), fresh sperm was diluted 1:500 in 10% formalin and 10 µL of this dilution was placed into a Thoma cell counting chamber that was left 10 minutes for spermatozoa to sediment. The sedimented sample was observed under the microscope Olympus BH with a 10x objective and a picture taken with a GigE digital camera (model: DMK 22BUC03 Monochrome, The Imaginsource, Bremen, Germany). Images of three different fields from each sample were taken with IC Capture Software (www.theimagingsource.com). The number of cells were counted with the image processor; ImageJ software (http://imagej.nih.gov/ij/); and processed by analysing the particles in each captured field. The mean from the triplicate measures was used to calculate the mean cell concentration. Seminal plasma was obtained by taking the supernatant after a sperm sub-sample was centrifuged (15 min, 4 ° C and 3000 rpm). To determinate the osmolality (mOsmol kg^{-1}), 10 µL of seminal plasma was put into Vapor Pressure Osmometer 5520 (Wescor, USA) and each sample was measured in triplicate. The protein concentration was measured in seminal plasma through Invitrogen Qubit 4 (Qubit Fluorometric Quantification. Thermo Fisher Scientific); 2 µL of seminal plasma were diluted in buffer solution mixed with the protein reagent (protein Assay kit. Thermo Fisher Scientific) and incubated for 15 min at room temperature before quantification of proteins in a Qubit fluorometer. The principle of the method is the fluorescence from the binding of fluorescent dyes to proteins is quantified with a Qubit Fluorometer, previously calibrated with standard solutions.

2.3 Evaluation of sperm quality

In all trials, the spermatozoa were activated and their paths recorded, until the motion ceased, using the IC Capture software and GigE digital camera (described above) connected to the microscope Olympus BH with a 20x objective. For sperm activation, either 1 µL of diluted sperm (extender trails, see below) was added to 20 µL of natural seawater with bovine serum albumin (BSA) prepared at 30% or 1 µL of undiluted sperm (control) added to 60 µL of seawater with BSA and gently mixed. One microliter of activated sperm was placed in a counting chamber ISAS R2C10 (Proiser *R+D, S.L.* Paterna, Spain) and the sperm motility was recorded. The videos obtained (AVI format) were processed with Virtual Dub 1.10.4 software (http://www.virtualdub.org/) to convert the video into image sequences in format *.jpeg. The files of image sequences were imported to ImageJ software and the sperm kinetics parameters were assessed at 15 seconds post-activation, using a computer-assisted sperm analysis (CASA) ImageJ plugin (http://rsb.info.nih.gov/ij/plugins/). The settings to analyse the videos were set as follows: brightness and contrast, -10 to 15/224 to 238; threshold, 0/198 to 202; minimum sperm size (pixels), 10; maximum sperm size (pixels), 400; minimum track length (frames), 10; maximum sperm velocity between frames (pixels), 30; frame rate, 30; microns/1000 pixels, 303; Print motion, 1; the additional settings were not modified. The parameters assessed during 2 seconds were the percentage of motile cells (% sperm motility), Curvilinear Velocity (VCL, µm/s) and Average Path Velocity (VAP, µm/s). Each sample was analysed in triplicate.

2.4 Urine Contamination

To determine the urine contamination, the urea concentration was measured, in the seminal plasma, using a urea kit (Urea-LQ urease –GLDH. Kinetic. Liquid, Spinreact, Sant Esteve de Bas, Spain). The principle of the method is two simultaneous enzymatic reactions, which are dependent on urea content. The reactions cause a change in the concentration of reagents, which is measured through absorbance at 340 nm. The urea concentration is calculated from the absorbance and expressed in units of mmol L^{-1}.

In addition, urine samples from females (n=3) were collected to compare the urea concentration, pH and osmolality between urine and seminal plasma. Samples were obtained from female fish in order to avoid contamination with sperm. After collection, the urine was kept on ice until the analysis. The urea concentration was measured with the same method as seminal plasma.

2.5 Extender trials

Samples that had motility lower than 10% were not used in this analysis. The samples were evaluated at 0, 3, 6 and 24 hours after being collected. Portions of each sample were diluted in the different extenders (see composition table 1) at a 1:3 dilution, ratio semen (20 µL): extender (40 µL) and one portion was conserved without adding extender solution as a control sample. At each time interval (0, 3, 6 and 24 hours) spermatozoa from each sample were activated and evaluated as described above.

In the first trial during the autumn, 12 samples were used and four extenders tested: modified Leibovitz (Fauvel *et al.*, 2012), Ringer (Chereguini *et al*., 1997; Rasines *et al.*, 2012), NAM (Fauvel *et al.*, 1999) and Sucrose (Cabrita *et al.*, 2006). The second trial was performed during the spring when ten samples were used and two extenders solutions tested: modified Leibovitz (Fauvel *et al*., 2012), and Stor Fish® (Haffray and Labbé, 2008). In the third trial, the extenders solutions of modified Leibovitz (Fauvel *et al.*, 2012) and Marine Freeze® (IMV Technologies) were tested during the autumn on six sperm samples. The procedures were the same in all trials.

All extenders osmolality and pH values where adjusted to fish semen parameters. Initially, the extenders medium had an osmolality range between 200 and 310 mOsmol kg^{-1} which was adjusted to 300 mOsmol kg^{-1} in order to avoid early activation of spermatozoa (Nynca *et al.*, 2012; Król *et al.*, 2018). A NaCl (5 M) solution was added to increase the osmolality and distilled water to decrease. With respect to pH, the range was between 7.7 and 8.06 among the different extenders and pH was adjusted to 8.0. An HCl (1 M) solution was added to lower the pH and NaOH (0.5 M) to increase the pH.

2.7 Statistical analysis

The data was expressed as mean ± standard deviation (SD). All analyses were performed at a significance level at $P < 0.05$. Pearson´s correlation test was used to determine the existence of a correlation between urine contamination and the parameters analysed, as predictors of semen quality. The samples classified according to appearance (colour, transparency and consistency) were compared through a multivariate General linear model to determine if there were differences in quality parameters. In addition, a Principal Component Analysis (PCA) was used in order to examine linear correlation amongst parameters and to obtain principal components using the Kaiser criterion, where the components PC1 and PC2, were chosen. A Clusters analysis was performed on the variables of sperm quality and seminal plasma characteristics, in order to classify the samples into groups with homogeneous features. The samples were clustered into three groups using Ward's method established on Euclidean distances. The means of different parameters of the three clusters were compared with a one-way analysis of variance (ANOVA) and a Games-Howell post-hoc test was applied to determine significant differences between clusters. The effect of short-time storage and extenders on sperm motility parameters were assessed by a Repeated Measures Designs and a Bonferroni test with multiple comparisons between the means. Statistical analysis was carried out using SPSS Statistic 20 for Windows (SPSS Inc. Chicago, IL, USA).

Results

During three sampling periods, a total of 49 cultured male sole were examined to obtain sperm samples for the study. From these 49 males, a total of 32 (65.3%) samples were obtained with the characteristics required for the study. The rejected males either had no sperm (n=3) or low volumes with low initial motilities that were not sufficient for all the proposed analysis (n=14). Although these 17 males were rejected, 13 did have motile sperm and, therefore, 45 (91.8%) from 49 randomly selected males had motile sperm. The initial values of sperm quality parameters exhibited high variation amongst the 32 males used in the study and in particular spermatozoa concentration followed by motility, urea and protein concentration were highly variable (table 2).

3.1 Morphology of male reproductive and urinary systems

As previously described by García-López *et al*. (2005), the male reproductive system of Senegalese sole is located in the abdominal cavity and is formed by two asymmetric testicular lobes. The abdominal cavity is divided, in the posterior region, into upper (ocular side) and lower (blind side) cavities by a central skeletal dividing wall. The testes are located close to the anterior edge of the skeletal division on either side of the division (Fig. 1). The largest testis is located on the upper ocular side of the division and the smallest testis, on the lower blind side. The upper testis is adhered to the upper side of the skeletal division and the lower testis is adhered to the lower (blind side) wall of the abdominal cavity. The urinary bladder is located anteriorly to the skeletal division and extends along the anterior edge of the division from the position of the testes to where the skeletal division connects with the abdominal cavity wall. The urinary bladder continues along the abdominal wall and ends where the urinary duct emerges and enters the abdominal wall. The urinary bladder appeared to be full of urine in all the males examined. From each testis, the spermatic duct emerges and travels along the length of the urinary bladder to the point where the urinary duct emerges from the urinary bladder and enters the wall of the abdominal cavity. The spermatic duct from the upper testis is adhered to the upper ocular side of the urinary bladder and the spermatic duct from the lower testis is adhered to the lower blind side of the urinary bladder. All three ducts, two spermatic ducts and the urinary duct enter the abdominal wall at the same point as separate ducts. Within the abdominal wall, the ducts combine and emerge on the outside of the fish as a single urogenital pore (Fig. 1). The mean length of the spermatic ducts, from testicles to the urogenital pore, was 3.60 ± 0.91 cm in individuals with a weight of 791.3 ± 376.5 g and a length of 37.3 ± 6.3 cm. The spermatic ducts were entirely full of spermatozoa (Fig. 2A, 2B, 2C) as shown in a longitudinal section from a middle section between the testis and abdominal wall (Fig. 2A) and a cross section made close to the testis (Fig. 2B).

3.2 Contamination with urine and sperm quality

The sperm samples showed signs of contamination by urine, owing to the tonality or colour (yellow, whitish yellow or whitish), yellow samples had the appearance of sperm mixed with a lot of urine, samples described as whitish yellow had the appearance of sperm mixed with smaller amounts of urine and samples described as whitish had the appearance of sperm with little or no urine contamination. Transparency (transparent or opaque) and consistency (viscous or fluid) also exhibited variation, but did not seem related to sperm concentration. A total of 51.1 % of samples had a yellow tonality, 22.2% had whitish yellow and 26.7% had whitish tonality; whilst 65.7% of samples showed opacity and 34.3% were transparent; regarding consistency, 45.2% were fluent and 54.8% were viscous. The samples described based on the tonality (yellow, whitish yellow or whitish) showed significant differences amongst mean sperm motility ($P=0.001$), urea concentration ($P=0.04$) and osmolality ($P=0.011$) (table 3). The whitish samples had significantly higher sperm motility and urea concentration and osmolality were similar compared to yellow samples. Cell concentration was similar irrespective of sample colour ($P=0.772$) (table 3). The samples classified by different features of transparency and consistency did not have any differences indicating that these features did not differentiate between sperm quality or seminal fluid characteristics.

The level of urea concentration contained in seminal plasma samples ranged between 0.41 and 7.99 mmol L^{-1}. The urea concentration and osmolality of the seminal plasma had a significant positive correlation (R= 0.513; P< 0.004) (Fig. 3). However, no correlation was found between urea concentration and others parameters.

In addition, the following parameters were analysed in female urine samples: pH, osmolality and urea concentration in order to compare with seminal plasma; where the urea concentration and pH showed a significant difference between the samples (table 4).

3.3 PCA and Cluster analysis

The PCA defined two components, describing 54.36 % of the variability in the data. Velocity parameters were related in the first component (PC1), together with protein concentration, pH and cell concentration that were negative values; the second component (PC2) was loaded positively to urea concentration and osmolality, whilst motility was included as a negative value (Table 5) (Fig. 4A).

The samples were grouped through cluster analysis and three groups were obtained. Each clustered group was characterized according to the variables of seminal plasma, cell concentration and kinetic parameters that described the sperm quality (Fig. 4B). The cluster formation had a significant interaction amongst groups (P=0.005). Significant differences were found amongst the means of the groups for the following parameters: urea concentration (P=0.002), osmolality (P=0.000), VAP (P=0.000), VCL (P=0.000) and pH (P=0.036), whilst no differences were found for cell concentration, protein concentration, and percentage motility (Fig. 5). In general terms, group 1 had lower levels of sperm quality and higher levels of urine contamination, group 2 had intermediate values (between groups 1 and 3) and group 3 represented the samples with higher sperm quality and lower levels of urine contamination. Therefore, group 1 had significantly higher levels of urea and osmolality compared to groups 2 and 3 and a lower pH (acidification) compared to group 3 (Fig. 5). While group 3 had significantly higher levels of sperm velocity (VAP and VCL) and higher (not significant) percentage motility than groups 1 and 2 (Fig. 5).

3.4 Short-term storage

In all three short-term sperm storage trials, there were no differences in sperm quality parameters, percentage motility and velocity (VCL and VAP) when the samples were collected and diluted in the different extenders (T = 0) and mean percentage motility ranged between 24.73 ± 14.14 % (Leibovitz, trail 3) and 38.89 ± 25.32 % (NAM, trail 1). Significant ($P<0.05$) differences were found, for motility and velocity parameters (VCL and VAP), for groups over time and amongst groups within some time points (Figs. 6, 7 and 8). There were also significant interactions between the different extender solutions and storage time for motility ($P<0.05$), VCL ($P<0.05$) and VAP ($P<0.05$) with the exception of VAP ($P=0.102$) in trail 2 and VCL ($P=0.525$) in trail 3. In trial 1 (n=12), the rate of decrease in kinetic parameters in relation to storage time was different amongst the groups. The control ($P=0.026$) and Sucrose ($P=0.005$) groups had declined significantly three hours after collection. The Ringer group had declined significantly ($P=0.038$) six hours after collection. The NAM ($P=0.012$) and Leibovitz ($P=0.038$) groups did not decline significantly until 24 hours after collection. A similar trend was observed in relation to sperm velocities parameters. Velocities (VCL and VAP) declined significantly ($P<0.05$) in groups control and Sucrose six hours after collection, in Ringers and NAM 24 hours after collection and values were similar at all time points for the Leibovitz group. The comparison of the motility among all extenders revealed differences after six hours of storage when motility was significantly higher for sperm stored in modified Leibovitz compared to Sucrose ($P<0.005$) (Fig. 6). After 24 hours of storage, samples diluted with Leibovitz extender maintained a significantly ($P<0.005$) higher percentage motility, VAP, and VCL (Fig. 6) compared to controls and Sucrose. The motility of sperm stored in NAM and Ringer was intermediate with no significant differences compared to controls and other extenders.

In the second trial (n=10), after three hours of storage, a significant (P=0.016) decrease in motility was observed in control samples that were significantly lower than samples in Leibovitz and Stor Fish® (Fig. 7). After six hours of storage, a significant (P= 0.049) decrease in motility was observed in samples diluted with Stor Fish®. After 24 hours of storage, a significant (P=0.01) decrease in motility was observed in samples diluted with Leibovitz. At 24 hours, the sperm samples stored with Leibovitz showed significantly (P<0.05) higher motility rate, VCL and VAP (Fig. 7), compared to the control samples and the samples stored in Stor Fish®. In relation to the velocity parameters, the VCL exhibited a significant decrease at 24 hours of storage in control samples, (P=0.004) and samples diluted in Stor Fish® (P=0.006). However, in samples diluted in Leibovitz, the only significant (P=0.022) difference was between three hours and 24 hours of storage. Likewise, the VAP values decreased after 24 hours of storage for all samples, control (P=0.005), Stor Fish® (P= 0.001) and Leibovitz (P=0.003).

In the third trial (n = 6), the control samples (P=0.014) and samples stored in Leibovitz (P=0.012) did not decline significantly until 24 hours after collection. Samples stored in Marine Freeze®, did not exhibit a significant decline in motility and maintained similar values during the 24 hours of storage. After 3 hours of storage, the samples diluted in Leibovitz solution had significantly (P=0.006) lower motility compared to samples stored in Marine Freeze®. However, after 24 hours of storage, the motility of samples stored in Marine Freeze® were significantly (P=0.008) higher than control samples (Fig. 8) and samples in Leibovitz were not different from control or Marine Freeze®. The velocity parameters (VCL and VAP) did not exhibit significant differences over time or amongst groups within time points (Fig. 8).

Discussion

All sperm samples used in the present study contained concentrations of urea that indicated the samples were contaminated by urine. Although urea is a natural metabolite found in most body fluids and tissues, the concentration is normally low as the toxic urea is removed, concentrated in urine and expelled. Urea concentration in uncontaminated sperm samples was 0.01 µmol L^{-1} in testicular sperm from rainbow trout (*Oncorhynchus mykiss*) (Billard and Menezo, 1984) and 48 µmol L^{-1} in sperm collected from the sperm ducts of Walleye (*Stizostedion vitreum*) (Gregory 1970), which are > 50 times lower than the mean of the samples (2.58 ± 1.60 mmol L^{-1}, table 3) obtained in the present study. Therefore, urea has been used and demonstrated to be an indicator of urine contamination in the present study as in other studies in marine fish (Dreanno *et al*., 1998) and other taxa (Althouse *et al*., 1989).

The description of the anatomy of the urinary and male reproductive systems clearly indicates why samples contained urine contamination. The spermatozoa are located in the testes lumen and the sperm ducts and sperm must be collected from the common urogenital pore (Garcia-Lopez *et al*., 2005). The present study demonstrated that sperm was obtained by applying gentle pressure, through the abdominal wall (lower blind side) or the abdominal wall and digestive system (upper ocular side), to the testes and along the sperm ducts towards the urogenital pore. However, the sperm ducts pass along the upper and lower side of the urinary bladder and, therefore, pressure applied to the sperm ducts was also applied to the urinary bladder to extract spermatozoa mixed with urine.

The mean urea concentration obtained in seminal plasma of Senegalese sole in the present study was similar to that obtained in turbot (*Psetta máxima),* where the samples were collected by a similar method (Dreanno *et al*., 1998). Dreanno *et al*. (1998) described two methods to extract sperm and found that emptying the urinary bladder before collection of sperm, which was impossible in Senegalese sole (see above), did not avoid concentrations of urea that indicated urine contamination. Various studies in other species have shown that urine contamination negatively influenced sperm quality, duration of motility, efficiency of movement after being activated and fertilisation ability in fresh water fish (Rurangwa *et al*., 2004; Rodina *et al*; 2004; Alavi *et al*., 2006; 2007; Sarosiek *et al*. 2016; Sadegui *et al*., 2017; Król *et al*. 2018) and marine fish (Dreanno *et al*., 1998; Linhart *et al*., 1999; Fauvel *et al*. 2012). Although the reduced sperm quality and even mechanisms affected were similar in fresh water and marine fish, the causes appear to be different, as for fresh water fish a decrease in osmolality and ions activates sperm and urine is hypo-osmotic (Król *et al*., 2018; Cejko *et al*. 2010; Linhart *et al*., 2003; Nynca *et al*., 2012; Poupard *et al*., 1998; Rurangwa *et al*., 2004) compared to marine fish where an increase in osmolality and ions activates sperm and urine is isosmotic (Cosson *et al*., 2008; Valdebenito *et al*., 2009). Therefore, in fresh water fish the premature activation of spz and reduced motility has been attributed to an osmotic shock when urine contamination lowers the osmolality (Perchec *et al*., 1995), whilst in marine fish although changes in osmolality have not been completely discounted, changes in ion balance, pH and ATP stores have been implicated in the premature activation of spz and reduced motility (Dreanno *et al*., 1998; Fauvel *et al*. 2012). In marine fish, urine contamination appeared to vary the composition of seminal plasma, decreasing significantly Na+, Cl^-, pH and intracellular ATP, which in turn modified the spz integrity to reduce motility percentage and spz velocity (Dreanno *et al*., 1998, Fauvel *et al*., 2012). In the present study, a significant positive correlation was obtained, between the urea concentration and the osmolality in seminal plasma and although not correlated, associations (PCA and cluster analysis) were found. Samples with significantly lower urine concentration, lower osmolality, higher pH and higher sperm quality (motility and velocities VAP and VCL) were clustered together. Therefore, as observed in other marine fish, in the present study, urine contamination appeared to reduce sperm quality probably due to an increase in osmolality and an associated decrease in pH (acidification).

The detrimental effect of urine on sperm quality reduces the possibility to use the sperm after a period of storage (Ciereszko *et al*., 2010; Sarosiek *et al*., 2012). An essential part of artificial fertilisation procedures is the storage of sperm for a short to long period to have sperm available when females ovulate and this has been achieved using extenders for short or long term storage (Chereguini *et al*., 1997; Dreanno *et al*., 1998; Rurangwa *et al*., 2004; Bobe and Labbe 2009; Cejko *et al*., 2010; Wang *et al.*, 2016; Beirão *et al*., 2019; Ramos-Júdez *et al*., 2019). Methods for the short term storage of sperm control the temperature and may also dilute the sperm in extenders to provide suitable conditions that maintain sperm quality during storage (Ciereszko *et al*., 2010; Fauvel *et al*., 2012; Gallego *et al*., 2013; Sadegui *et al*., 2017; Santos *et al*., 2018). Usually, cold storage of sperm (around 4 ºC), has been successfully used in order to lower metabolism and avoid damage to the sperm (Chereguini *et al*., 1997; Favuel *et al*., 2012; Santos *et al*., 2018). A temperature of 4ºC was used in the present work, however, chilled storage alone was not successful for sperm storage and the motility of the spz decayed within three-six hours after collection as has been observed in other species where extenders were required (Chereguini *et al*., 1997; Rodina *et al*., 2004; Berríos *et al*., 2010; Fauvel *et al*., 2012; Gallego *et al*., 2013; Santos *et al*., 2018). On the contrary, sperm samples that were diluted in immobilising solutions showed an increase in the storage time, reducing the loss of sperm quality and in addition, counteracted the negative effects of others factors such as urine contamination (Dreanno *et al*., 1998; Rodina *et al*., 2004; Bobe and Labbé, 2008; Fauvel *et al*., 2012, Gallego *et al*., 2013; Król *et al*., 2018).

In the trials in the present study, all sperm samples diluted in extenders with the exception of Sucrose solution prolonged sperm quality parameters during storage. Sucrose solution was ineffective and the decline in sperm quality parameters was similar to control samples. Samples in Ringer and Stor Fish® had decreased significantly six hours after collection and in NAM 24 hours after collection. On the contrary to sole, Cherenguini *et al*. (1997) found that the Ringer extender was suitable for short term storage of turbot (*Scophthalmus maximus*) sperm. Stor Fish®, has been successfully used for sperm storage in various species (Haffray and Labbé, 2008), including the Patagonia blenny (*Eleginops maclovinus*) (Contreras *et al*., 2017) and a range of salmonids, Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) (Merino *et al*., 2016; Risopatrón *et al*., 2017). However, the present study found that for sole sperm, Stor Fish® was not suitable for short term sperm storage. In the marine species, meagre (*Argyrosomus regius*), NAM was also found to be a poor extender for sperm storage (Santos *et al*., 2018).

Leibovitz and Marine Freeze® had significantly higher sperm quality parameters than control samples 24 hours after collection and while samples in Leibovitz declined significantly 24 hours after collection, samples in Marine Freeze® did not decline during 24 hours. Similarly, Fauvel *et al*. (2012) described that sperm samples from sea bass (*Dicentrarchus labrax*) that were diluted with cell culture medium Leibovitz L15 as an extender solution had improved motility when activated 24 hours after collection. The modified Leibovitz solution contained elements that had positive effects on the spz by providing a stable osmolality (different salts), stable pH, energy (pyruvate), aminoacids (glutamine), a shield for the plasma membrane (BSA) and an antibiotic was added to prevent bacterial growth (Bobe and Labbé, 2008; Niksirat *et al.*, 2011; Gallego *et al*., 2013). Marine Freeze®, according to the manufacturers (IMV Technologies) description, contains similar elements and had a similar effect as Leibovitz for sperm storage. Leibovitz and Marine Freeze® were the most successful in inhibiting the loss of motility and mitigating the detrimental effects of urine contamination.

Another factor that plays a role in short term storage in an extender is the dilution ratio that determines the reduction in sperm concentration, dilutes the urine contamination and influences the osmolality and pH control (Bobe and Labbé, 2008). In the present study, a dilution ratio of 1:3 was used after preliminary tests on different dilutions ratios. The same ratio has been successfully used with Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*) and rainbow smelt (*Osmerus mordax*) (Bobe and Labbé, 2008), while dilution ratios 1:4 and 1:9 were used for meagre (*Argyrosomus regius*) (Santos *et al*., 2018; Ramos-Júdez *et al*., 2019) and 1:5 for European seabass (Fauvel *et al*., 2012). However, some species may be sensitive to the dilution ratio and components of an extender and for this reason many studies on sperm storage have developed specific extenders for each species, trying to approximate extender composition to the species seminal fluid and secure osmotic balance between the extender solution and sperm (Bobe and Labbé, 2008; Gallego *et al*., 2013; Beirão *et al*., 2019). In the case of Senegalese sole sperm, the use of diluents is a tool that can help to maintain sperm quality during storage and improved tailor-made extenders may further improve storage.

Currently, Senegalese sole aquaculture production is based on wild broodstocks and the development of artificial fertilisation methods has been frustrated by the low volumes of poor quality sperm (Cabrita, *et al*., 2006; 2011; Beirão *et al*., 2009;

Rasines *et al*., 2012; 2013; Chauvigné *et al*., 2016; 2017). However, a contributing factor to these low sperm volumes may be that aquaculture technicians working with sperm and most published studies to date only use sperm samples that were considered subjectively by appearance to be uncontaminated sperm (Agulleiro *et al*., 2006; Cabrita *et al*., 2006; 2011; Beirão *et al*., 2008; 2009; 2015; Martinez-Pastor *et al*., 2008; Valcarce *et al*., 2016; Riesco *et al*., 2017; 2019; Fernandez *et al*., 2019) and contaminated samples were discarded. In the present study a subjective assessment was made to determine differences between samples that by appearance were considered uncontaminated (whitish) or contaminated (yellow). All samples grouped by colour (whitish, whitish yellow and yellow) contained high spz densities and exhibited motility. Whitish (uncontaminated) samples had significantly higher motility, but similar spz densities, urea concentration and osmolality as yellow (contaminated) samples. The mean motility of the whitish samples (45.75 ± 20.18 %) was similar to the mean motility reported in other studies working with uncontaminated samples from Senegalese sole that ranged from 20-30 % (seasonal baseline values in Cabrita *et al*., 2011) to ~80 % (Cabrita *et al*., 2006; Riesco *et al*., 2019). The yellow samples had a motility of 17.76 ± 9.81%, which was similar to the lowest motilities reported in other studies (Cabrita *et al*., 2008; 2011). The mean spz densities from yellow and whitish samples were similar to lower densities reported for uncontaminated sperm, which ranged from 1.0×10^9 spz mL^{-1} (0.7 to 1.2×10^9 spz mL^{-1} in cultured males in Cabrita *et al*., 2006) to 6.84×10^9 spz mL^{-1} (Fernandez *et al*., 2019). By weight densities in the present study, were four to 100-fold higher than densities per kg that have been reported, which ranged from 0.01 to 0.3×10^9 spz kg^{-1} (Cabrita *et al*., 2006; Agulleiro *et al*., 2006; 2007; Beirão *et al*., 2011). The sperm densities per kg in the present study were similar to densities reported by Chauvigné *et al*. (2017; 2018), who used similar methods to obtain all the sperm and assess the sperm production capacity of males. Therefore, the subjective analysis in the present study and comparisons of motility and spz densities within the present study and with other studies indicate that uncontaminated samples may actually be contaminated, that only collecting whitish sperm samples (or uncontaminated samples) will exclude or discard samples with high densities of sperm that had a degree of motility and underestimate spz densities per kg of male.

Cryopreservation protocols have been studied for Senegalese sole (Rasines *et al*., 2012; Valcarce and Robles, 2016; Riesco *et al*., 2017) and used to have availability of sperm for artificial fertilisation (Rasines *et al*., 2012; 2013).These cryopreservation protocols used only what was considered uncontaminated sperm. The present study found that only 26.7% of males had sperm that appeared to be uncontaminated (whitish samples) and therefore, few males appear to have the sperm quality required for methods that need uncontaminated sperm. The use of only uncontaminated sperm may make methods difficult or impossible to implement in the industry as it will be difficult to obtain enough sperm for large scale fertilised egg production or to have enough males to form sufficient families for a breeding program. The present study has demonstrated that contaminated sperm samples and short term chilled storage in extenders to mitigate the negative effects of urine contamination may represent a viable sperm management system that can be used by the sole aquaculture industry. In the present study, 91.8% of males had motile sperm and 65.3% had adequate samples for the present study. However, further work is need to improve sperm management using short term chilled storage for the sole culture industry.

Conclusions

The morphology of the urogenital system of Senegalese sole contributes greatly to the contamination by urine observed in the sperm samples collected by the stripping method. The proximity of the seminal ducts and the urinary bladder, makes it difficult or impossible to obtain sperm without urine contamination. Although, the colouration of the sperm sample may help identify samples with improved motility, all samples (yellow, whitish yellow and whitish) contained large numbers of motile spz and discarding samples that have a yellow colouration will discard large quantities of sperm. The effect of urine contamination, measured as urea, induced a reduction in sperm quality which may have been caused by a decrease in pH (acidification) and an increase in osmolality, which are known to activate sole sperm and reduce quality in marine fish. Urea contamination was positively correlated with the osmolality values in the seminal plasma. The tests carried out with extender solutions revealed that samples diluted with modified Leibovitz and Marine Freeze® extenders had significantly higher motility after 24 hours compared to control samples. In particular, the use of extender solutions is relevant to help to cushion the effect of urine contamination when the sperm is required for artificial fertilisation. However, although the present work is promising giving important insights for sperm management in sole, further work is required to determine the most suitable compounds to elaborate extenders that can further offset the negative effects of urine contamination as well as work to improve the methods to collect the sperm.

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Figure legends

Table 1. Composition of different extender solutions per litre.

Table 2. The initial values of sperm quality parameters. The values were measured from sperm samples: sperm volume (µL), sperm motility percentage, VCL (µm/s), VAP (µm/s), duration sperm activity (s), cell concentration (spz mL^{-1}) and spermatozoa per kg of body weight (spz kg^{-1}) and from seminal plasma: pH, osmolality (mOsmol kg^{-1}), Urea concentration (mmol L^{-1}) and Protein concentration (µg mL). All values were referred as mean ± SD.

Table 3. Comparative values of sperm motility percentage, osmolality (mOsmol kg^{-1}), Urea concentration (mmol L^{-1}) and cell concentration (spz mL^{-1}) amongst the samples described based on the tonality (yellow, whitish yellow or whitish). All values were referred as mean ± SD. Different letters indicate significant differences ($P<0.05$).

Table 4. Mean and standard deviation of urea concentration, pH and osmolality in urine from females (n=3) and seminal plasma from males (n=32). Different letters indicate significant differences ($P<0.05$).

Table 5. Proportion of variables descriptors to sperm quality used in the Principal Component Analysis.

Figure 1. Male reproductive system in Senegalese sole (*Solea senegalensis*)*;* 1A. Photograph of dissected sole showing testes and urinary system. 1B. Diagram from photograph showing, a, upper ocular testicular lobe; b, lower, blind side, testicular lobe; c, urinary bladder; d, spermatic ducts; e, urogenital pore. 1C Diagram of cross section to show the position of testes, sperm ducts and urinary system.

Figure 2. Longitudinal mid-section of spermatic duct (A), transverse section of spermatic duct close to testis (B) and longitudinal mid-section of spermatic duct (C) of Senegalese sole (*Solea senegalensis*) showing the ducts were full of spermatozoa.

Figure 3. Positive correlation ($R=0.513$; $P<0.004$) between osmolality (mOsmol kg^{-1}) and urea concentration (mmol/L) in seminal plasma from Senegalese sole (*Solea senegalensis*).

Figure 4A. Distribution of variables, descriptors of sperm quality and seminal plasma from Senegalese sole (*Solea senegalensis*) for the two principal components.

Figure 4B. Clusters obtained from Principal Component Analysis that formed three groups 1 (red), 2 (green) and 3 (blue) based on the parameters of sperm quality and seminal plasma from Senegalese sole (*Solea senegalensis*).

Figure 5. Mean value of clusters obtained from parameters of sperm quality and seminal plasma from Senegalese sole (*Solea senegalensis*). Different letters above each bar indicate significant differences (P<0.05) amongst groups.

Figure 6. Effect on percentage motility, VCL and VAP of storage time on Senegalese sole (*Solea senegalensis*) control sperm samples and sperm samples diluted in the extenders Leibovitz, Ringer, NAM and Sucrose. Different letters above each bar indicate significant differences (P<0.05) among treatments within the sample time.

Figure 7. Effect on percentage motility, VCL and VAP of storage time on Senegalese sole (*Solea senegalensis*) control sperm samples and sperm samples diluted in the extender, Leibovitz and Stor Fish®. Different letters above each bar indicate significant differences (P<0.05) among treatments within a sample time.

Figure 8. Effect on percentage motility, VCL and VAP of storage time on Senegalese sole (*Solea senegalensis*) control sperm samples and sperm samples diluted in the extenders, Leibovitz and Marine Freeze®. Different letters above each bar indicate significant differences (P<0.05) among treatments within the sample time.

Table 1

Composition	Ringer	Leibovitz	NAM	Sucrose	Stor Fish ®	Marine Freeze ®
Leibovitz L-15**		14.8 g				
NaCl	2.165 g		1.875 g			
KCl	1.000 g		0.05 g			
MgCl			0.615 g			
$CaCl_2$	0.099 g		0.195 g			
NaH_2CO_3	0.067 g		0.84 g			
Glucose			0.04 g			
Sucrose				51.35 g		
BSA***		20 mg mL^{-1}	10 mg			Yes*
Glutamine		300 µg mL^{-1}				Yes*
Sodium pyruvate		6 mg mL^{-1}				
Gentamycin		1 mg mL^{-1}			0.5 g	Yes*
Ultra-pure water	1 L	1 L	1 L	1 L	Yes*	Yes*
Biological buffer					Yes*	Yes*
Salts					Yes*	Yes*

*Manufacture only indicated what was present and quantities were not specified.

**Leibovitz L-15 medium, Sigma-Aldrich, Spain (product code: L-4386)

***Bovine Serum Albumine

Table 2

Parameter	Mean ± SD.	Minimum	Maximum	Coefficient of variation
Sperm volume (μL)	361.40 ± 173.40	130	700	48%
Initial sperm motility (%)	29.02 ± 20.42	4.54	77	70%
VCL (μm/s)	144.84 ± 64.51	57.35	277.81	45%
VAP (μm/s)	117.49 ± 64.89	42.07	255.30	55%
Duration sperm activity (s)	143.95 ± 5.33	85	240	4%
Cell conc. (spz mL^{-1})	1.48 ± 2.92 x 10^9	1.25 x10^8	1.38 x10^{10}	197%
Spermatozoa per kg (spz kg^{-1})	2.81 ± 5.21 x 10^9	1.82 x 10^8	2.45 x 10^{10}	185%
pH	6.91 ± 0.38	6.21	7.59	5%
Osmolality (mOsmol kg^{-1})	360.67 ± 138.46	185	713	38%
Urea conc. (mmol L^{-1})	2.58 ± 1.60	0.41	7.99	62%
Protein conc. (μg mL)	13.21 ± 8.14	3.45	24.30	62%

Table 3

Parameter	Whitish samples	Whitish yellow samples	Yellow samples
Sperm motility (%)	45.75 ± 20.18^{a}	30.83 ± 31.16^{ab}	17.76 ± 9.81^{b}
Urea conc. (mmol L^{-1})	1.95 ± 1.16^{a}	3.83 ± 1.25^{b}	2.94 ± 0.94^{ab}
Osmolality (mOsmol kg^{-1})	311.59 ± 59.64^{a}	464.66 ± 104.75^{b}	380.30 ± 46.84^{ab}
Cell conc. (spz mL^{-1})	$1.85 \pm 3.98 \times 10^{9\ a}$	$0.36 \pm 0.32 \times 10^{9\ a}$	$1.51 \pm 2.49 \times 10^{9\ a}$
Spz per kg (spz kg^{-1})	$1.41 \pm 0.83 \times 10^{9\ a}$	$0.12 \pm 0.58 \times 10^{9\ a}$	$1.19 \pm 0.58 \times 10^{9\ a}$

Table 4

Samples	Urea (mmol L^{-1})	pH	Osmolality (mOsmol kg^{-1})
Urine	7.60 ± 3.17[a]	6.23 ± 0.27[a]	289.44 ± 31.18[a]
Seminal fluid	2.58 ± 1.60[b]	6.91 ± 0.38[b]	360.77 ± 138.46[a]

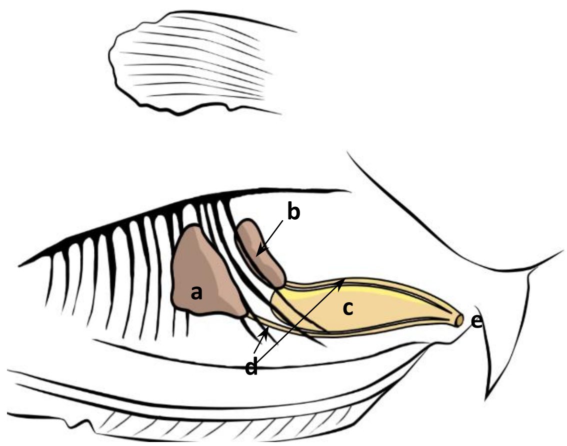
Table 5

Parameters	Component	
	1	2
VCL (µm/seg)	0.846	-0.0675
VAP (µm/seg)	0.840	-0.153
Protein concentration (µg ml^{-1})	-0.693	0.134
pH	-0.567	-0.329
Cell concentration ($x10^9$ spermatozoa ml^{-1})	-0.495	-0.067
Urea concentration (mmol L^{-1})	-0.229	0.830
Osmolality (mOsmol kg^{-1})	0.361	0.825
Motility %	0.283	-0.289

Figure 1

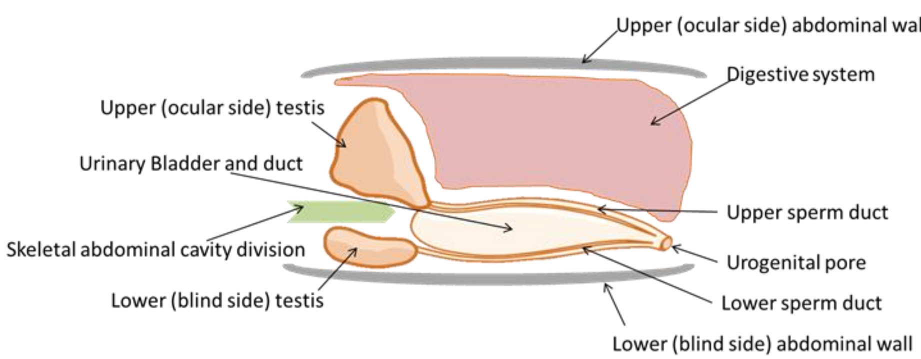
A.

B.



b
a
c
e
d

C.



Upper (ocular side) abdominal wall
Digestive system
Upper (ocular side) testis
Urinary Bladder and duct
Upper sperm duct
Skeletal abdominal cavity division
Urogenital pore
Lower (blind side) testis
Lower sperm duct
Lower (blind side) abdominal wall

Figure 2

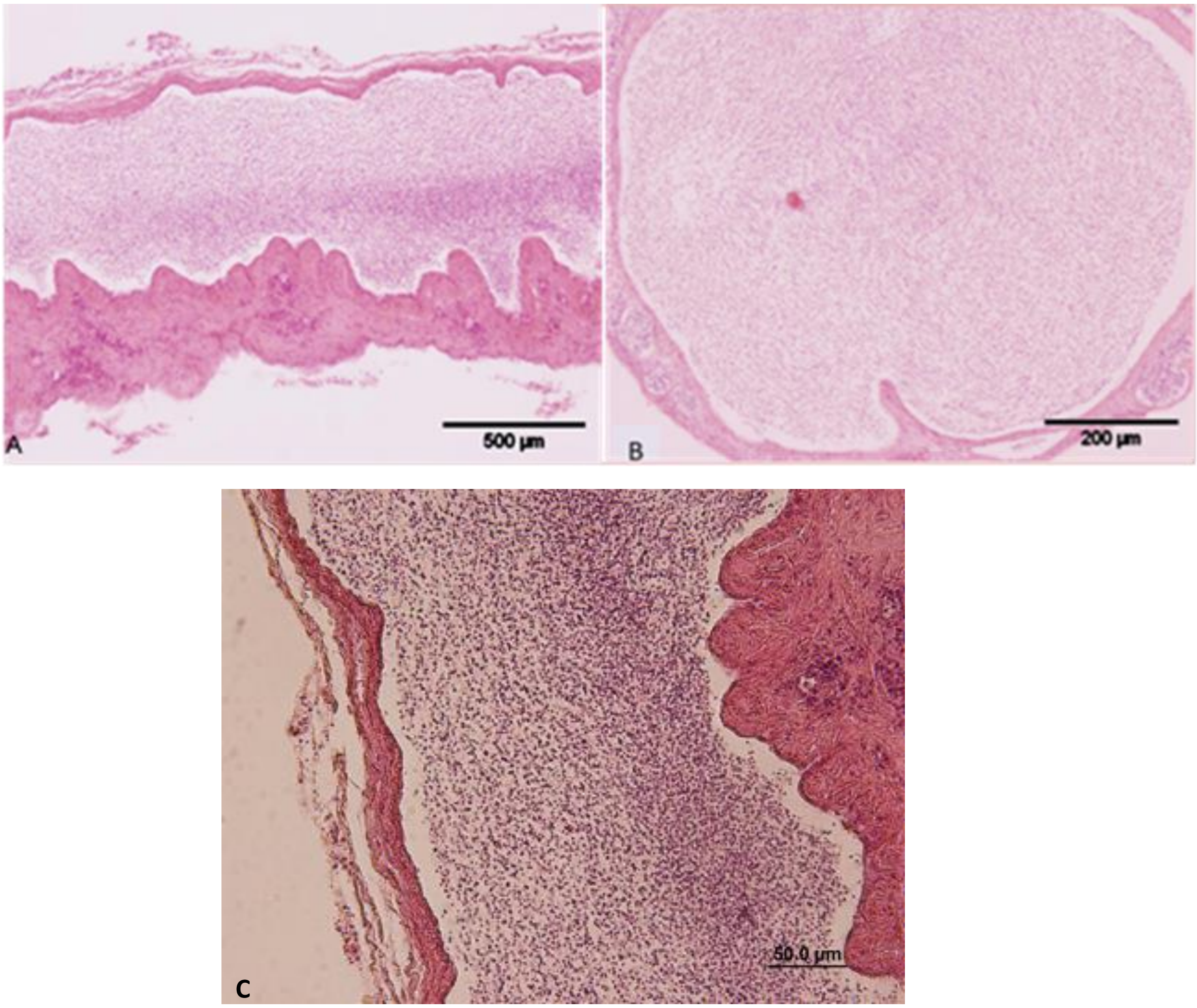
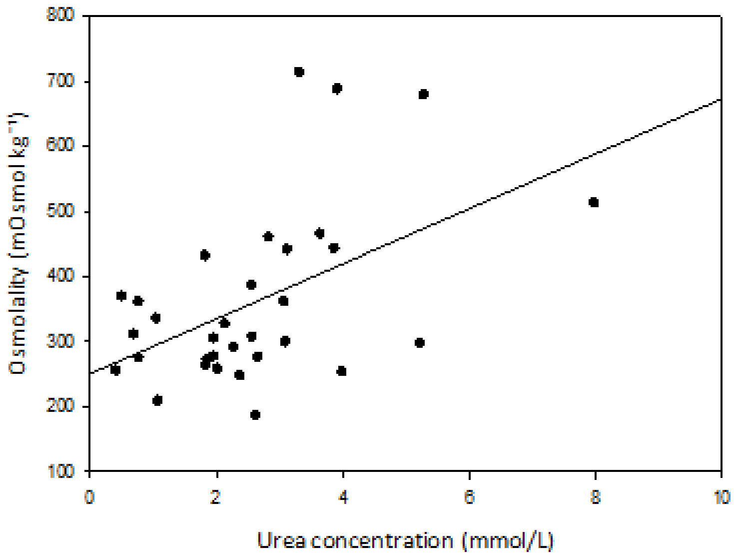


Figure 3



800
700
600
500
400
300
200
100
0
2
4
6
8
10
Osmolality (mOsmol kg^{-1})
Urea concentration (mmol/L)

Figure 4a

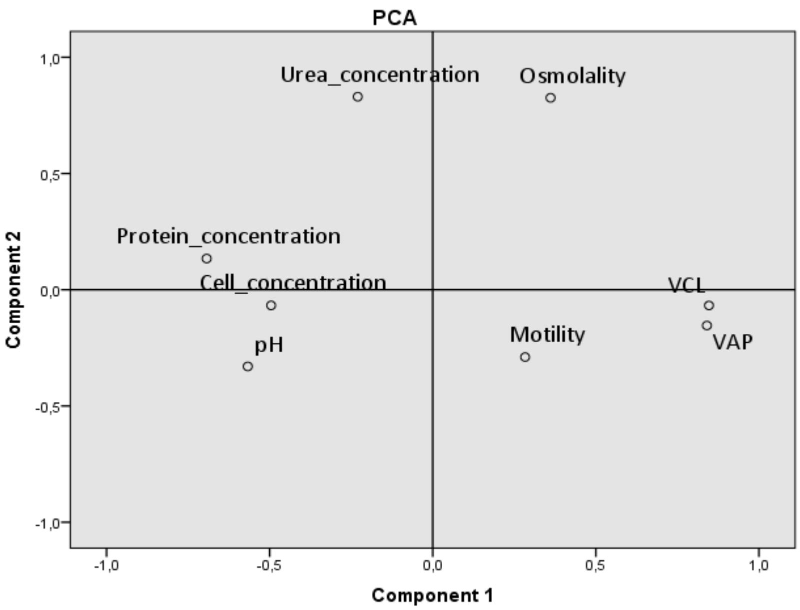

PCA
Urea_concentration
Osmolality
Protein_concentration
Cell_concentration
VCL
VAP
Motility
pH
Component 2
Component 1
1,0
0,5
0,0
-0,5
-1,0
-1,0
-0,5
0,0
0,5
1,0

Figure 4b

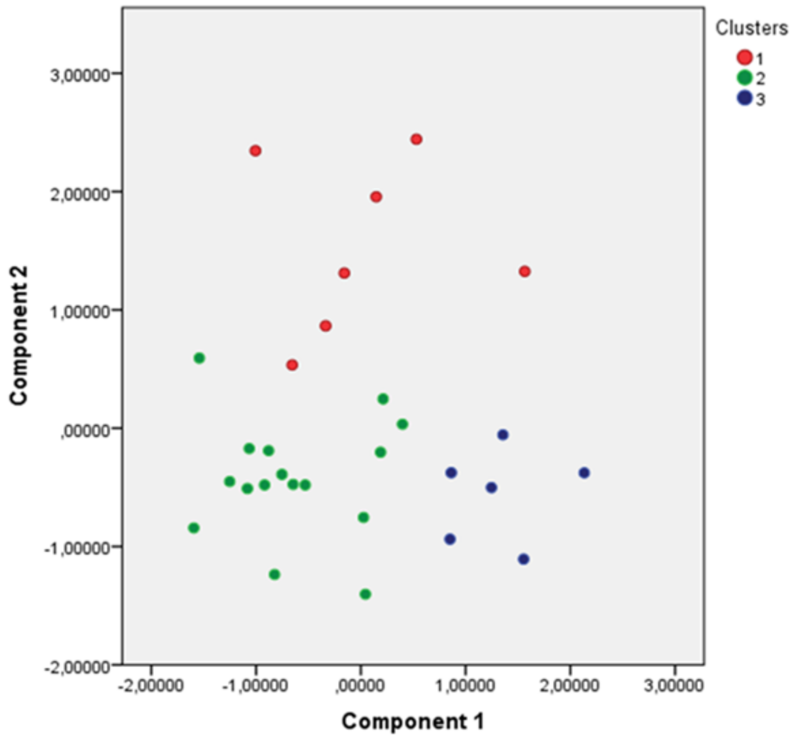
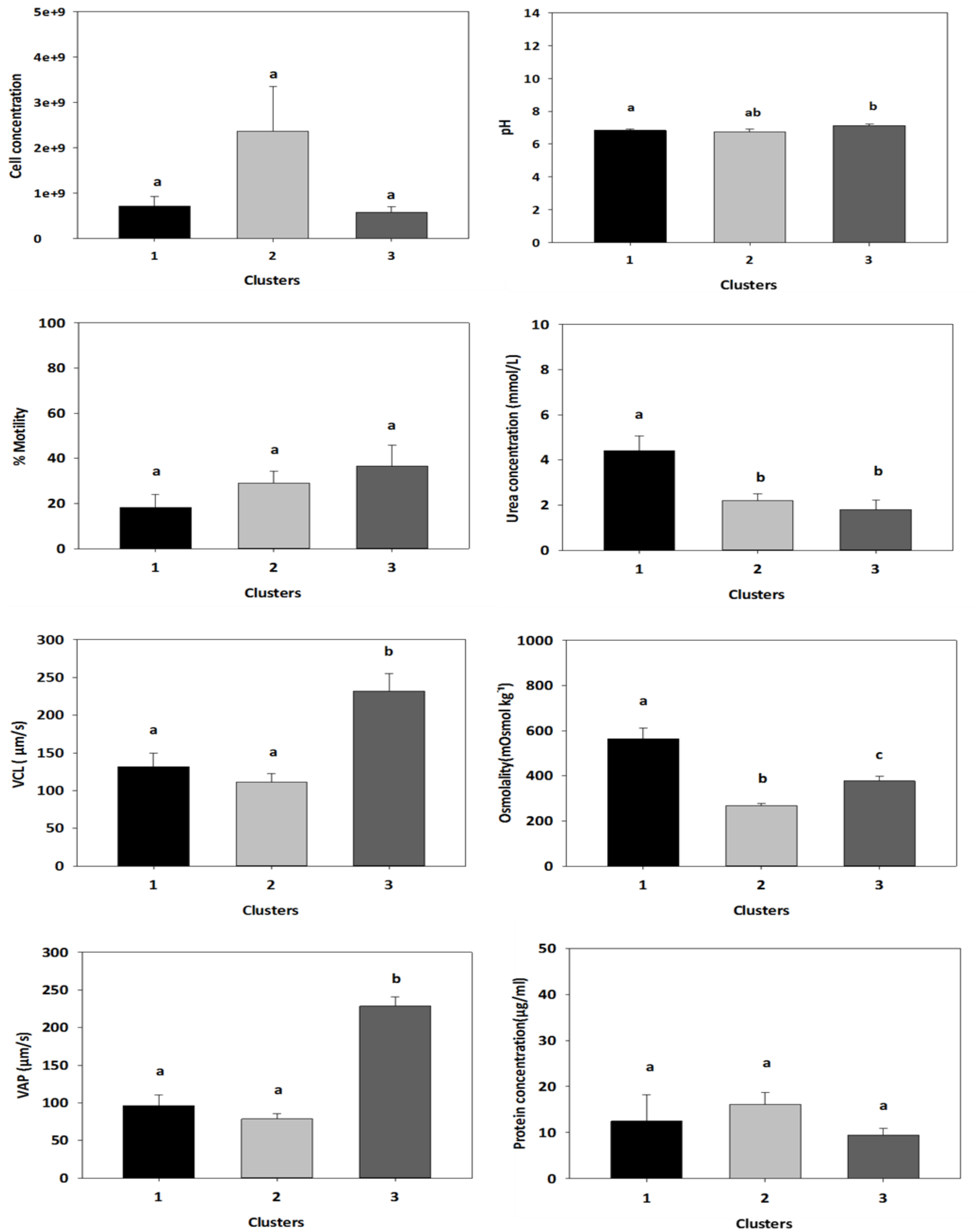

Clusters
1
2
3
3,00000
2,00000
1,00000
,00000
-1,00000
-2,00000
Component 2
-2,00000
-1,00000
,00000
1,00000
2,00000
3,00000
Component 1

Figure 5



Cell concentration
5e+9
4e+9
3e+9
2e+9
1e+9
0
a
a
a
1
2
3
Clusters
pH
14
12
10
8
6
4
2
0
a
ab
b
1
2
3
Clusters
% Motility
100
80
60
40
20
0
a
a
a
1
2
3
Clusters
Urea concentration (mmol/L)
10
8
6
4
2
0
a
b
b
1
2
3
Clusters
VCL (µm/s)
300
250
200
150
100
50
0
a
a
b
1
2
3
Clusters
Osmolality(mOsmol kg-1)
1000
800
600
400
200
0
a
b
c
1
2
3
Clusters
VAP (µm/s)
300
250
200
150
100
50
0
a
a
b
1
2
3
Clusters
Protein concentration(µg/ml)
50
40
30
20
10
0
a
a
a
1
2
3
Clusters

Figure 6

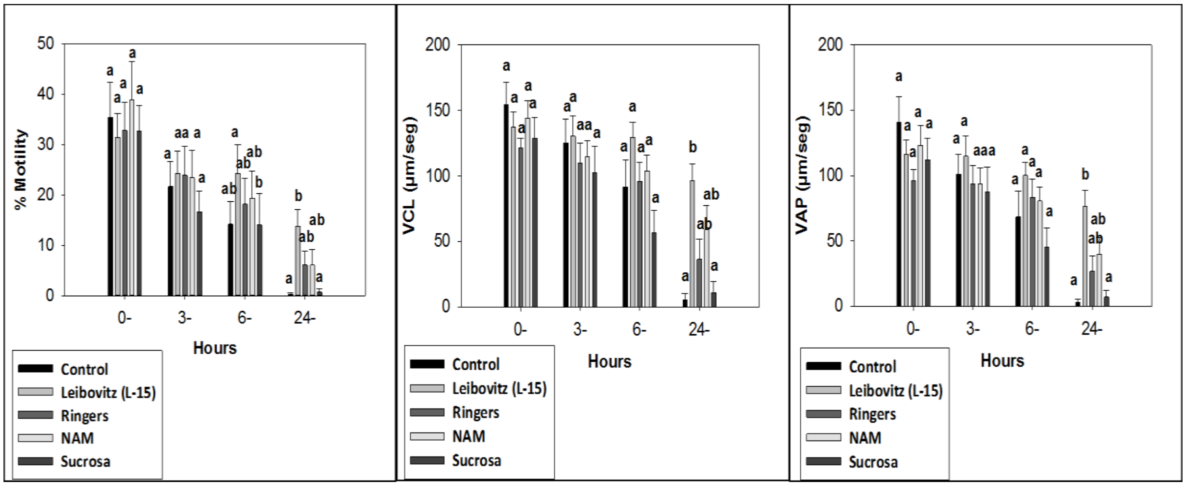
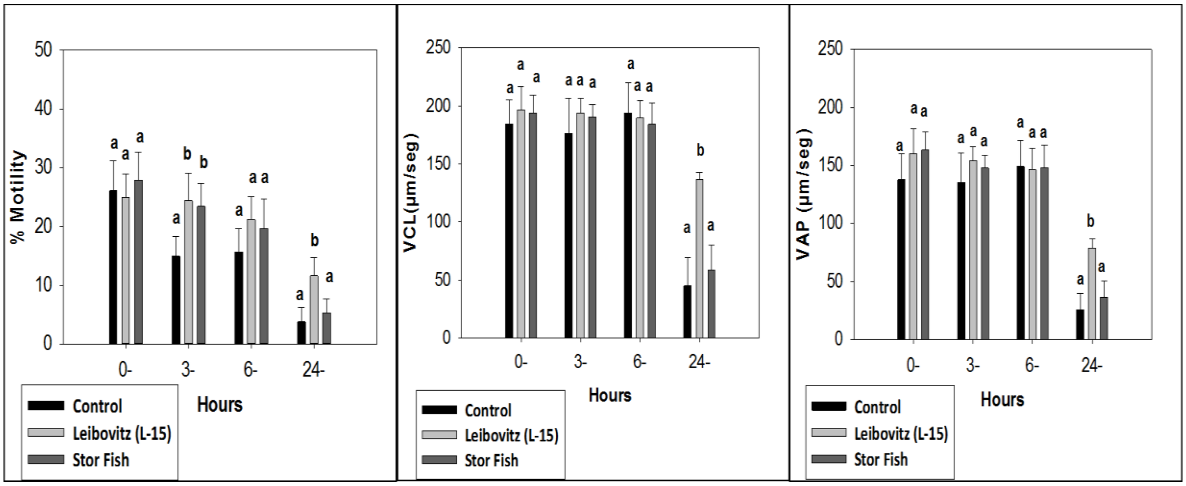
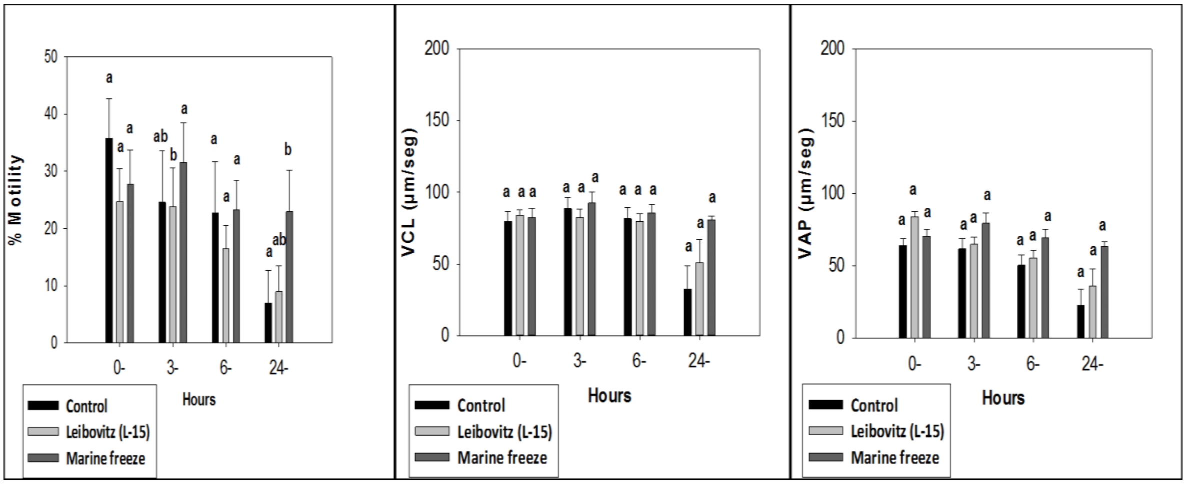

% Motility
VCL (µm/seg)
VAP (µm/seg)
Hours
0-
3-
6-
24-
Control
Leibovitz (L-15)
Ringers
NAM
Sucrosa

Figure 7



% Motility
VCL(µm/seg)
VAP (µm/seg)
Hours
0-
3-
6-
24-
Control
Leibovitz (L-15)
Stor Fish

Figure 8


% Motility
VCL (µm/seg)
VAP (µm/seg)
0-
3-
6-
24-
Hours
Control
Leibovitz (L-15)
Marine freeze