www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 17: 111-120 (2017) DOI: 10.4194/1303-2712-v17_1_13

PROOF RESEARCH PAPER

Effect of the Activation Media with Different Osmolality and Cool Storage on Spermatozoa Motility Parameters over Time in Zebrafish, *Danio rerio*

Sara Sadeghi*, Javier Nuñez, Carles Soler, Miguel Angel Silvestre

[1] Departamento de Biología Funcional y Antropología Física, Universitat de Valencia, Burjassot, 46100 Valencia, Spain.

* Corresponding Author: Tel.: +34.671 844000;
E-mail: sara.sadeghi1@gmail.com

Received 04 May 2016
Accepted 13 August 2016

Abstract

In the present work, the effects of the activation media with different osmolality and storage conditions on sperm motility and kinetic parameters over time after activation, using CASA system (ISAS®) in Danio rerio were analysed. The effect of pooled and individual ejaculate samples was also studied. Spermatozoids activated with high osmolality medium showed higher values in motility parameters and kinematic parameters in comparison with low osmolality medium. Respect to the individual ejaculates or the pool, although significant differences were observed in some parameters, no relevant changes were observed in total motility neither in kinetic parameter. Total motility and velocity of sperm showed significant lower values after sperm storage for 24 h at 4 ºC.

Keywords: Sperm motility, activation, osmolality, zebrafish, CASA, kinetic parameters

Introduction

Guaranteeing the conditions in which spermatozoa motility is optimal, is important, both in terms of *in vivo* and *in vitro* fertilizing of eggs for optimizing the biotechnology of fish reproduction (Cejko *et al.*, 2013). Sperm motility constitutes the basis for evaluating milt and controlling the ability of sperm to fertilize eggs. Bozkurt *et al.* (2006) confirmed a positive correlation between fertilization rates and spermatozoa motility. The spermatozoa of most fish species are immotile in the testis and seminal plasma. Motility is induced after the spermatozoa are released into aqueous environment during natural reproduction or in to the medium with different osmolality during artificial reproduction. There are clear relationship between seminal plasma composition, osmolality and the duration of fish sperm motility. Seminal plasma has a composition of ions, which affects spermatozoa function and motility. The osmolality of seminal plasma usually prevent sperm motility (Alavi and Cosson, 2005; Islam and Akhter, 2011). This shows the importance of using adequate osmolality to activate fish species spermatozoa. Many authors suggested that, as for several fresh water fish species, the primary signal for initiation of sperm motility in zebrafish *Danio rerio* (Hamilton, 1822), is a change in osmolality (Alavi and Cosson 2006; Wilson-Leedy *et al.*, 2009). Generally a decrease in the medium osmolality triggered sperm motility. *D. rerio* spermatozoa could be activated in hypotonic solutions at a wide range of osmolalities, ranging from 25 to 270 mOsm/kg, but the highest motility was observed at a range from 150 to 210 mOsm/kg (Jing *et al.*, 2009). Other parameters such as ion concentrations (K^+, Na^+ and Ca^{2+}), osmotic pressure, pH, and temperature and dilution rate can also affect sperm motility. As these conditions depolarize the cell membrane may affect the capacity of flagella motility (Alavi and Cosson, 2006; Morisawa and Suzuki, 1980).

Once motility of spermatozoa has been initiated, its duration is typically short, often less than one minute in fresh water species, with hypotonicity-associated cell swelling and lysis as a limiting factor (Alavi and Cosson, 2006; Wilson-Leedy *et al.*, 2009). It is worth considering that some characteristics, such as oocyte size, spermatozoa swimming distance, sperm motility duration and micropyle closing time are determinants in the procedures of application of insemination doses (Billard and Cosson, 1992). Several studies have described the effect of different factors like osmolality, ions, temperature on sperm motility initiation and duration, using CASA system, in species such as medaka *Orizias latipes* (Temminck and Schlegel, 1846), sturgeon, carp *Cyprinus carpio* or salmonids. However, there is still a lack of information in *D. rerio*. (Alavi *et al.*, 2009; Bastami *et*

al., 2009; Jing *et al.,* 2009; Wilson-Leedy *et al.,* 2009; Yang and Tiersch, 2009; Li *et al.,* 2012; Butts *et al.,* 2013; Cejko *et al.,* 2013; Dziewulska and Domagała, 2013). There are not enough studies about motility and sperm kinetics in D. *rerio.*

Zebrafish is an animal model in ecotoxicology and developmental biology for other vertebrate species (Seok *et al*., 2008). Moreover, zebrafish could be an adequate model to other fish species. An important factor in fishery and aquaculture is the ability of preserving semen for a short or long period, using cryopreservation or short storage techniques. The effect of storage temperature on *D. rerio* sperm motility revealed a rapid decline of motility after 2 h when samples were stored at "room temperature" (about 25°C) , and suggested cooling of sperm sample at 4°C for prolonged storage (Jing *et al.,* 2009). Generally, the storage has negative effect on sperm viability and plasma membrane integrity and motility in several species (Perez-Cerezales *et al.,* 2009). Also, variability within males and the condition of semen storage are critical factors that determine the viability of sperm after short-term storage. In salmon, diluted semen stored for several days at 4ºC presented higher motility rates than undiluted semen (Trigo *et al.,* 2015). However, the spermatozoa motility and its duration can change not only among different males but also in each individual ejaculate (Bozkurt *et al.,* 2006).

The aim of this work was to study the effect of the activation media with different osmolality and cooled storage for 24 h at 4ºC of diluted semen on the spermatozoa motility parameters analysed by a computer assisted sperm analysis (CASA) over time in *D. rerio*. Moreover, the effect of pooled or individual stripping samples on sperm motility was studied.

Materials and Methods

Animals and Semen Collection

Five adult *D. rerio* were obtained from Universidad Politécnica de Valencia. Animals were kept in a recirculating system that continuously filtered the system water to maintain the water quality required for a healthy aquatic environment. The tank temperature was generally maintained between 26-28.5 °C and the lighting conditions were 14:10 h (light: dark). The animals were fed with commercial dry fish food.

Sperm was initially diluted without activation, in Hank's balanced salt solution (HBSS). The solution was composed of 1.5 g of bovine serum albumin (BSA; Sigma-Aldrich H8264) and 0.1 g NaCl dissolved in 100 mL Hank's solution (Sigma-Aldrich A7906) with an osmolality of approximately 300 mOsm/kg. The osmolality was measured by an osmometer (The Fiske® Micro-osmometer 210).

The semen collection process was done approximately after 15 minutes the light period starting. Fishes were slightly anaesthetized with natural clove oil (0.18 mL in 1 L of fresh water. Rinse in fresh water, and dried gently with paper towels, and place belly up in a slit in a damp sponge. The semen was collected with glass capillary tubes after applying gentle pressure to the abdomen, from the top to the genital pore. Each ejaculate was diluted with 75 µL of HBSS solution into an Eppendorf microtube. In this study, two groups of sperm samples were used. Sperm samples stripped from at least from 4 males and/or pooled in equal amounts. Samples were stored into a fridge at 4° C until their use. Samples used on the same day of collection were stored for less than 2 h in the fridge and they were considered as fresh diluted semen. Samples were stored for 24 h, were considered cooled diluted semen.

Computer-Assisted Sperm Analysis (CASA)

Spermatozoa motility parameters were obtained by a CASA system (ISAS® v1.2; PROISER S.L., Paterna, Spain). The microscope used was a triocular UOP equipped with a negative contrast phase objective (lens Plan 10xPHN, PROISER), and recordings were made by a digital camera (Basler, A780-54fm, Ahrensburd, Germany). Then, each recording was analysed with the following acquire and track settings: Image per second: 25; image fields max: 25; with the area surface of $10<90$ µm^2. The following values were determined by CASA: the percentage of motile sperm (MOT,%), percentage of sperm moving with straightness (STR,%), curvilinear velocity (VCL, µm/sec), average velocity path (VAP, µm/sec), straight-line velocity (VSL, µm/sec), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL,%), linearity movement (LIN,%) and beat cross frequency (BCF, Hz).

Activation of Spermatozoa

Two sperm activation solutions with different osmolality were studied:

- Medium H composed of 9 mL of HBSA plus 3 mL of deionized water (~ 90 to 110 mOsm/kg).
- Medium L composed of fresh water (~ 15 to 20 mOsm/kg).

Sperm were activated at room temperature by mixture of 3 µL of diluted semen sample to 15 µL of activation medium (H or L) on a microslide (26x76 mm, Deltalab), using a micropipette followed by 2 seconds of gentle stirring with the micropipette tip.

Approximately 3 µL of this mixture of activated sperm was quickly applied to the sperm counter chamber (Spermtrack®, 10 µm depth; PROISER S.L., Paterna, Spain). Sperm motility was estimated in the same field and captured each 5 seconds until 2.5 minutes, then, each 30 seconds, until the 80% of spermatozoids became immotile.

Experimental Design

Study 1:The effect of two sperm activation media with different osmolality on motility and kinetic parameters of pooled fresh semen.

It was studied the effect of two activation media (H and L) on sperm motility and kinetic parameters over time in pooled fresh samples. Each pool sample obtained from five adult male. Four replicates were used.

Study 2:The effect of type of sperm sample (individual ejaculates or pooled samples) on the evolution of sperm motility and kinetics over time.

It was studied the effect of the pool *vs*. individual semen samples. For the individual group, we use an arithmetic mean of five adult males per replicate. For that, data from sperm activated with media H and L from study 1 (for pooled samples) were analysed together. Four replicates were used.

Study 3: The effect of short-term cool storage of pooled semen on sperm motility and kinetic parameters.

It was studied the effect of diluted sperm storage at 4ºC for approximately 24 h on sperm motility and kinetics over time in pooled samples, and compared with pooled fresh samples. A pool from five adult males and four replicates were used.

Statistical Analysis

All the statistical analysis was performed using SPSS software (IBM SPSS statistics V21). Results of sperm motility rate were analysed by a binary logistic GLM procedure. Results of kinetic parameters (VLC, VSL, STR, LIN, WOB, VAP and BCF) were analysed by GLM procedure following a linear model. A probability of $P<0.05$ was considered to be statistically significant. For Study 1, a model with two factors, activation media osmolality (2 levels: H or L) and video capture (20 levels; 20 consecutive video captures), and "two-way interaction" were used. For Study 2, a model with two factors, sperm sample (2 levels: I or P) and video capture (20 levels; 20 consecutive captures), and "two-way interactions" were used. For Study 3, a model with two factors, storage conditions (2 levels: fresh or cooled for 24 h at 4ºC) and video capture (16 levels; 16 consecutive captures) and "two-way interactions" were used.

Results

Effect of Osmolality of Sperm Activation Media On Motility and Kinetic Parameters of Pooled Fresh Semen

Results from the Study 1 were showed in Table 1 and Figures 1A-H. Spermatozoa activated with medium H showed significant higher total motility rate than L (75.7 ± 0.4 vs. 63.7 ± 0.6 respectively, Table 1). Respect to spermatozoa kinetics, activation media affected almost all studied parameters except WOP. Sperm activated with medium H showed higher values than medium L. The total motility x time capture interaction was statistically significant, nevertheless motility drop over time capture, were similar up to the first 7 video captures in both H and L media, (approximately 55 seconds; Figure 1A). In reference to kinetic parameters, time affected respectively VCL, VSL, VAP and BCF (Table1). For VCL, VSL VAP, and BCF, values began to decrease for L media starting from 2rd-3rd time capture (Figure 1B, 1C, 1E and 1H respectively). The LIN, STR and WOB parameters presented higher values in medium L until 6th time capture and from this moment these values were lower than in medium H (Figure 1D, 1F and 1G).

Effect of Type of Sperm Sample (Individual Ejaculates or Pooled Samples) on Sperm Motility and Kinetics Over Time

Total motility of the pooled samples was slightly higher than individual (Table 2). However, individual samples maintained the motility better than pooled samples (Figure 2A). Respect to kinetics of sperm motility, all the parameters was significantly different except BCF. Otherwise, all the velocity dependents parameters like, VCL, VSL and VAP showed an increase (Table 2, Figure 2B, 2C and 2E).

Effect of Short-Term Cool Storage on Sperm Motility and Kinetics

In 3rd study, according to the results present in Table 3, significant differences were observed between fresh and cooled stored samples in total motility rate (78.1 ± 0.45 vs. 41.4 ± 0.8 for fresh and cooled samples respectively). Respects to kinetics of spermatozoa motility, the values are lower for cool stored samples than fresh pooled samples. The interaction term between captures and storage conditions of samples was also significantly different (Figures 3A-H). The LIN, STR and BCF parameters of cooled samples presented higher values until 5th time capture, and then it showed a decrease, with lower values than fresh pool samples (Figure 3D, 3F and 3H).

Discussion

Sperm motility is a key factor to determine semen quality and clearly is related to osmolality which having important relevance in fertilizing capacity and the duration of sperm motility of freshwater fish species (Ingermann *et al.*, 2011). Fish sperm is immotile in the testis or in an osmolality similar to its blood plasma or the seminal plasma osmolality (*300*-315 mOsm/kg).

The initiation of motility can be controlled by changes in ion concentration of the external medium

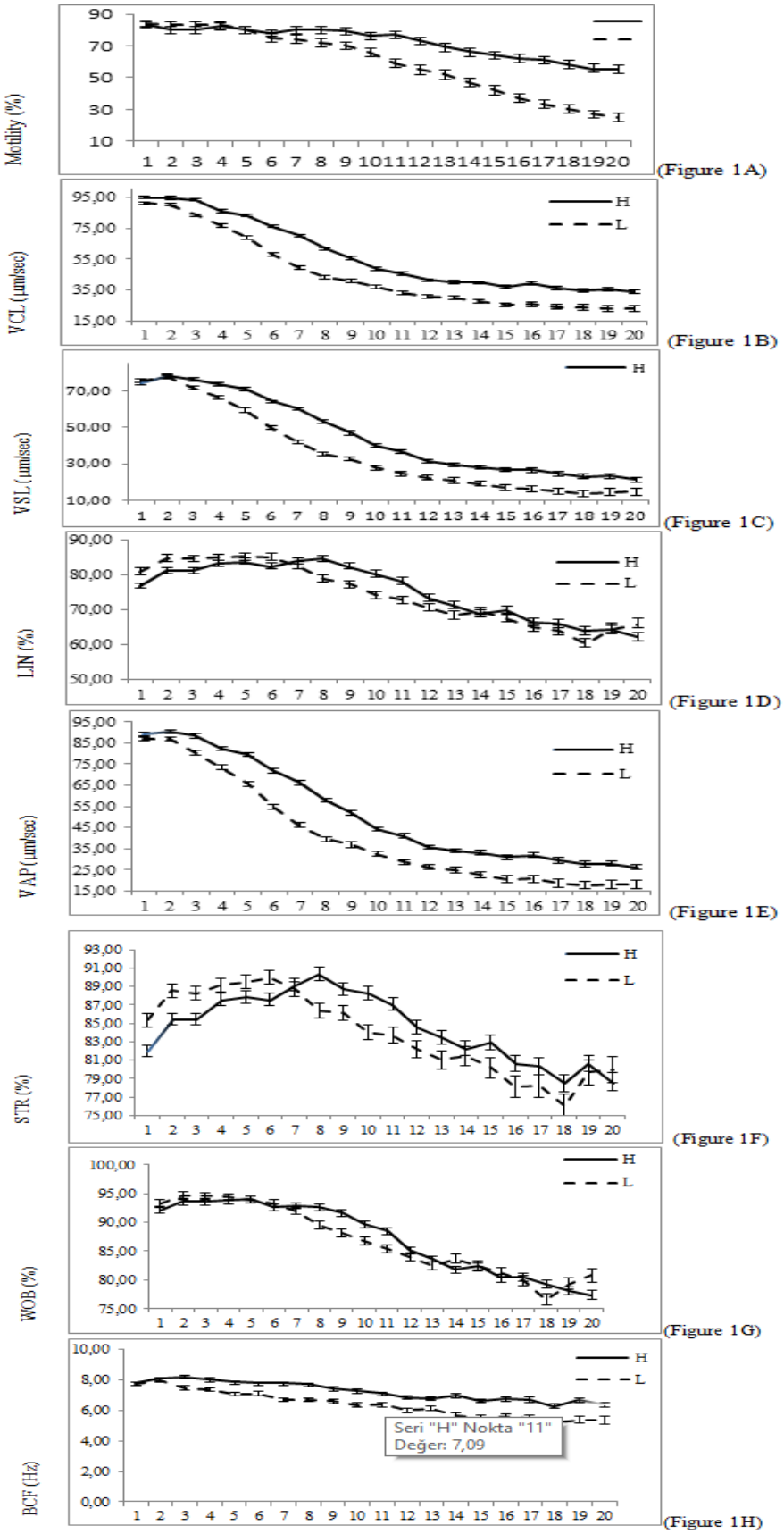


Figure 1.Effect of activation media H or L on motility and kinetic parameters during 20 captures. Motility (%) (Fig. 1A), VCL: curvilinear velocity (µm/sec)(Fig 1B), VSL: straight-line velocity (µm/sec)(Fig 1C), LIN: linearity of the curvilinear trajectory (%)(Fig 1D), VAP: average path velocity (µm/sec)(Fig 1E), STR: straightness (%)(Fig 1F), WOB: wobble (VAP/VCL)(%)(Fig 1G), BCF: beat-cross frequency (Hz)(Fig 1H), from sperm parameters as measured at different video capture rate (20 seconds post activation) each capture presented 5 seconds.

(Alavi and Cosson, 2005, 2006). Hypotonic medium initiates the motility of freshwater fish spermatozoa such as common carp *Cyprinus carpio* and goldfish *Carassius auratus auratus* (L). In salmonids (Cuvier, 1816) and sturgeon *Acipenser sturio* (Linnaeus, 1758), the decrease in K^+ concentration upon dilution is a key factor for sperm initiation, whereas in the cyprinids, a decrease in osmolality is the basis of this sperm activation (Islam and Akhter, 2011; Reinardy *et al.*, 2013).

In experiment 1, sperm motility rate was greater and duration was prolonged in time with the activation medium with the highest osmolality. To the best of our knowledge there is still lack of information focused on the effect of osmolality of sperm activation media on total motility and kinetic parameters evaluated by CASA in *D. rerio*. Jing *et al.* (2009) indicated that the osmolality of activation medium is the first signal for sperm motility, by using two different activation solutions (ionic and ion-free). However, they assessed sperm motility by subjective visual estimation, and no data showed about sperm kinetics.

Tejerina *et al.* (2008) discussed that sperm motility parameters analysed using CASA system provided a more objective assessment of sperm motility in mammals. CASA technology has several advantages as compared to manual counting, such as, fast analysis; increase consistence when the same setting is used, and high statistical power due to objective analysis of numerous sperm (Schleh and Leoni, 2013; Lammers *et al.*, 2014). Unlike Jing and collaborators' work, we used CASA system for evaluating the sperm motility, contributing with more objective and additional data such as kinetic parameters. Moreover, we studied the effect of different ejaculates or pooled samples. Rurengwa *et al.* (2004) stablished with the CASA system, that the progressive motility velocities correlated better with fertilization rates than the other parameters. Recently, using CASA system, Wilson-Leedy *et al.* (2009) studied the effect of several osmolality ranges on *D. rerio* sperm motility and observed that moderate NaCl concentration (80 mM) remained the highest sperm motility rate, but not with low or high NaCl concentrations (0 or 120 mM). In addition to osmolality, the pH and temperature are also important factors that involved with sperm motility in fresh water species (Alavi and Cosson, 2005). In other freshwater species, the motility activation may also depend on osmolality rather than extracellular ions concentration (*Prochilodus lineatus* and *Brycon orbignyanus*, medaka *Orizias latipes*, medaka *Orizias latipes)* (Yang and Tiersch, 2009).

On the other hand, curvilinear velocity (VCL) was not affected by osmotic concentration of the activation medium, and velocity has a better response to intermediate osmotic concentration (Wilson-leedy *et al.*, 2009). The linearity movement (LIN) and straightness of swimming (STR) did not show a relevant effect by activation media with different osmolality. However, values of VCL, VSL, and VAP showed a significant increase in high osmolality medium. Cejko *et al.* (2013) showed that carp *Cyprinus carpio* sperm activation in distilled water resulted in a decrease in not only the motility parameter but also the VSL and BCF compared with a medium with high osmolality. In contrast with our

Table 1. The effect of two sperm activation media with different osmolality on motility and kinetic parameters of pooled fresh semen along time

	Total Motility Rate	VCL	VSL	LIN	VAP	STR	WOP	BCF
Medium H	75.7 ± 0.4^a	57.3 ± 0.2^a	45.4 ± 0.2^a	75.2 ± 0.2^a	52.0 ± 0.2^a	84.6 ± 0.2^a	87.1 ± 0.1	7.2 ± 0.0^a
Medium L	63.7 ± 0.6^b	45.1 ± 0.3^b	35.8 ± 0.3^b	74.3 ± 0.3^b	40.9 ± 0.3^b	$83.8\ 0.2^b$	83.8 ± 0.2	6.4 ± 0.0^b

[1] Medium H was composed by 9 mL of HBSS plus 3 mL of deionized water (~ 90 to 110 mOsm/Kg); Medium L composed by aged water (~ 15 to 20 mOsm/Kg). Values are expressed as the means ± SEM. VCL: curvilinear velocity, VSL: straight-line velocity, LIN: linearity of the curvilinear trajectory, VAP: average path velocity, STR: straightness, BCF: beat-cross frequency WOB: wobble (VAP/VCL). [ab]Numbers within columns with different superscripts differ (P<0.05)

Table 2. The Effect of type of sperm sample (individual ejaculates or pooled samples) on the evolution of sperm motility and kinetics over time.

Group[2]	Total Motility Rate	VCL	VSL	LIN	VAP	STR	WOP	BCF
Individual	65.2 ± 0.2^b	62.8 ± 0.2^a	48.1 ± 0.2^a	75.0 ± 0.1^b	57.6 ± 0.2^a	82.4 ± 0.1^b	89.4 ± 0.1^a	6.8 ± 0.0^a
Pool	67.3 ± 0.4^a	55.7 ± 0.3^b	45.5 ± 0.3^b	77.8 ± 0.2^a	51.5 ± 0.3^b	85.8 ± 0.2^a	89.1 ± 0.1^b	6.9 ± 0.0^b

[2] Values are expressed as the means ± SEM. VCL: curvilinear velocity, VSL: straight-line velocity, LIN: linearity of the curvilinear trajectory, VAP: average path velocity, STR: straightness, BCF: beat-cross frequency WOB: wobble (VAP/VCL). [ab]Numbers within columns with different superscripts differ (P<0.05)

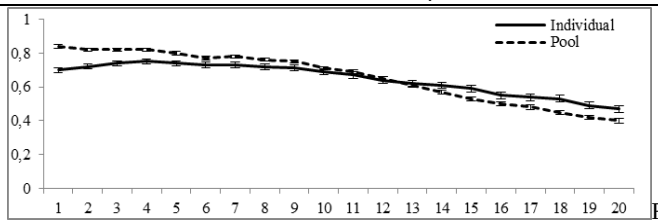


Figure 2A

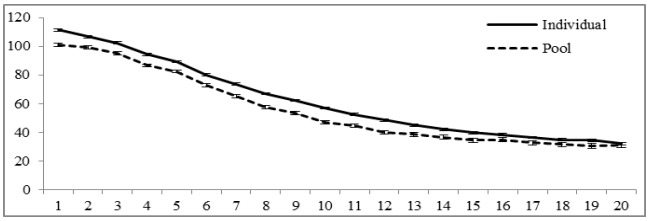


Figure 2B

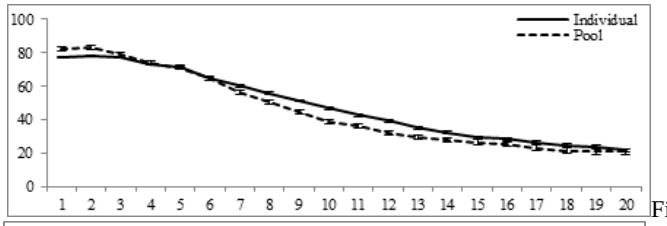


Figure 2C

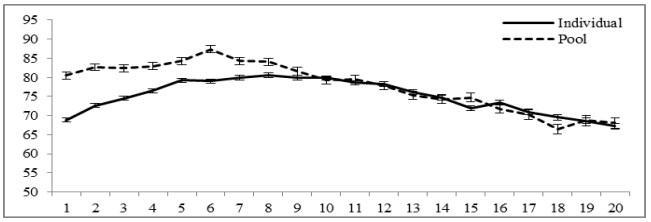


Figure 2D

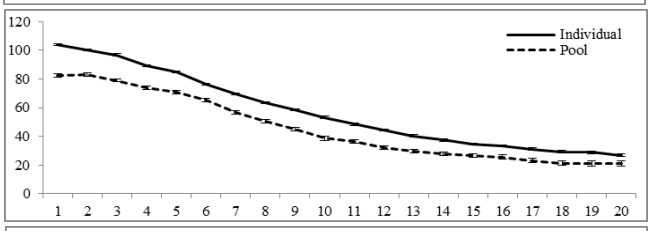


Figure 2E

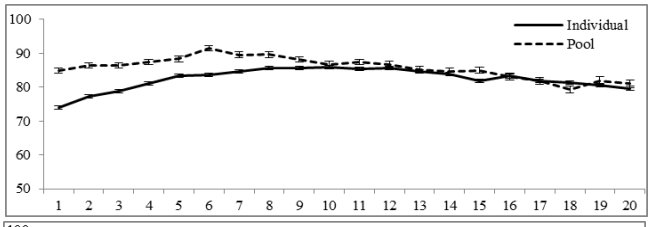


Figure 2F

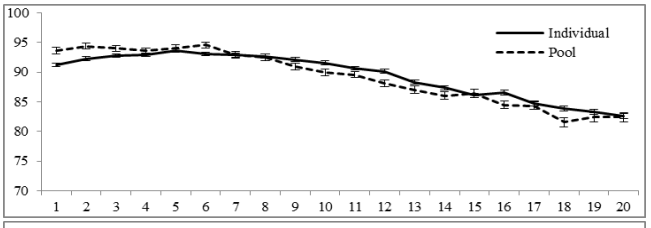


Figure 2G

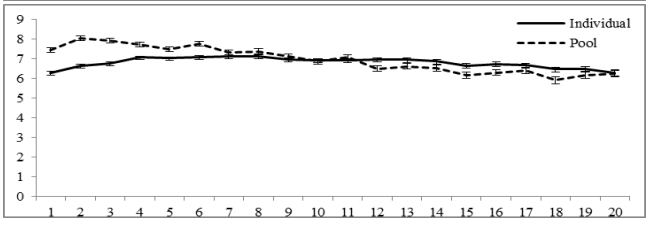


Figure 2H

Figure 2. Effect of type of samples (Individual vs. Pool) on motility and kinetics of spermatozoa during 20 captures. Motility (%) (Fig. 1A), VCL: curvilinear velocity (μm/sec)(Fig 1B), VSL: straight-line velocity (μm/sec)(Fig 1C), LIN: linearity of the curvilinear trajectory (%)(Fig 1D), VAP: average path velocity (μm/sec)(Fig 1E), STR: straightness (%)(Fig 1F), WOB: wobble (VAP/VCL)(%)(Fig 1G), BCF: beat-cross frequency (Hz)(Fig 1H), from sperm parameters as measured at different video capture rate (20 seconds post activation) each capture presented 5 seconds.

results, Wilson-Leedy *et al.* (2009) concluded that, initial velocity (VCL) was insensitive to osmolality of activation medium, but velocities were better maintained over time by activation media with intermediate osmotic concentrations in *D. rerio.* Butts *et al.* (2013) demonstrated higher osmolality in activation medium, higher velocity (curvilinear) in redside dace *Clinostomus elongates* spermatozoa for the first 10 s. Cosson (2010) linked a higher initial velocity with lower duration of sperm motility but in our case, we observed a higher initial velocity in high osmolality medium was maintained with longer swimming period and total duration of motility in VCL parameter. The reason to explain these results could is the quantity of stored ATP. In this sense, it is known that the primary source that supports spermatozoa motility in the quantity of stored ATP in sperm cells (Ingermann *et al.,* 2011). It also has been reported that the ATP content was lower at low osmolality medium at the end of the carp sperm motility (Billard *et al.,* 1995). In carp, a decrease in MOT, VCL, and BCF values was associated with using up the intracellular ATP supplies (Perchec *et al.,* 1995). Moreover, the decrease in sperm motility duration in low osmolality medium may be caused by damage of the flagellum after plasma membrane alteration (Alavi *et al., 2009).*

In many industrial and laboratory hatcheries large volumes of eggs were fertilized with small volumes of semen, which frequently originates from a small potentially ejaculation volume (Targonska *et al.,* 2008). Also, it was suggested that an option in order to decrease variability of *D. rerio* in vitro fertilization efficiency could be pooling gametes (Hagedorn and Carter, 2011) as it accrued in some mammals or avian livestock species mentioned that pooling of gametes from different individual might be contemplated an option to decrease variability in fertility (Donoghue *et al.,* 2003). Moreover, there was no effect on fertilization success using pooled male frozen/thawed sperm with individual female eggs in *D. rerio* (Hagedorn and Carter, 2011). Targonska *et al.* (2008) indicated that the use of the pooled semen obtained from many males, allowed obtaining better breeding results, because of the fertilization success of some males was significantly higher than the others. In the second experiment, the effect of each kind of sample, individual ejaculates or pooled semen from different males on total motility and kinetic parameters was studied. There are no previous studies about how the sort of sample (individual vs. pool semen samples) can be affecting the spermatozoa different motility parameters in *D.* rerio. We observed statistical differences between individual ejaculates or pooled samples, although these differences were not very relevant. Hagedorn and Carter (2011) mentioned the effect of pooling gametes (female and male) in fertilization rates, observing that the pooling sperm samples did not affect fertilization rates. In mammals, Batista *et al.* (2012) observed that differences in sperm motility between individual and pooled samples were evident after 8 h of preservation and depending of semen storage temperature. In the present work, the participation of males to the pool was not exactly the same since it was very difficult to adjust male contribution due to the sample size was smaller in comparison with other animal species. In this sense, the difference in sperm motility, were due to different male contribution to the pool. Other possibility could be due to the effect of the semen sample of the same ejaculate.

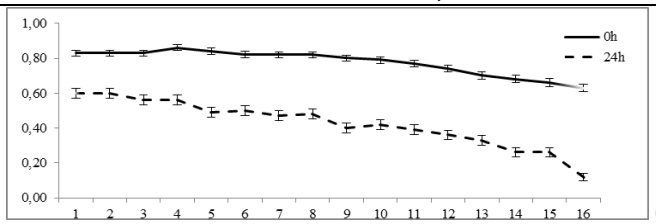
In the last study, we observed that cooled storage affected motility and kinetics in *D. rerio* sperm. We observed a higher sperm motility rates for the fresh semen samples, and the drop of total sperm motility over time seems more pronounced in the sperm samples stored at 4ºC. The effective short-term storage of semen is essential when processing multiple sperm samples use and semen must be transported far away to the collection sites, this make it possible to maintain viable spermatozoa for a short time (Bozkurt *et al.,* 2009; Aramli *et al.,* 2013; Trigo *et al.,* 2015). However, there is no data concerning analysis effect of cool storage (24 h - 4ºC) using CASA in D. *rerio.* Sahin *et al.* (2013) reported that sperm motility in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) was affected during preservation and motile cells decreased as the length of storage. Short storage (12-36 h) in 4ºC could be best period and temperature for cool short storage. Cardona-Costa *et al.* (2009) observed the same rate of larvae at 24 h after in vitro fertilization, using *D. rerio* sperm stored at 8ºC for 24 h in comparison with fresh sperm. They also observed the optimal motility after 48-72 h in some cases.

Metabolic activity of sperm decreases during cooling storage of sperm samples in mammals, being possible to prolong their lifespan (Aurich, 2008). One explanation could be that there is decreasing trend in the intracellular ATP concentration during the storage

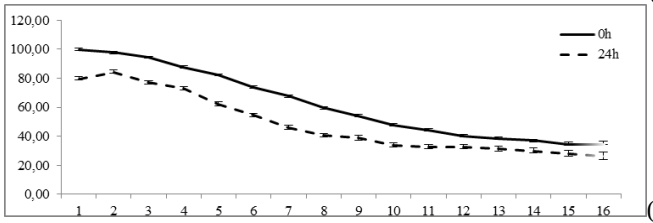
Table 3-The Effect of short-term and cool storage on sperm motility and kinetic parameters.

Group[3]	Total Motility Rate	VCL	VSL	LIN	VAP	STR	WOP	BCF
0h	78.1 ± 0.5^{a}	62.2 ± 0.2^{a}	50.5 ± 0.2^{a}	78.0 ± 0.2^{a}	57.6 ± 0.2^{a}	85.5 ± 0.2^{a}	89.6 ± 0.1^{a}	7.2 ± 0.0^{a}
24h	41.4 ± 0.8^{b}	48.3 ± 0.4^{b}	37.8 ± 0.4^{b}	72.4 ± 0.3^{b}	42.9 ± 0.4^{b}	83.0 ± 0.2^{b}	84.9 ± 0.2^{b}	7.0 ± 0.0^{b}

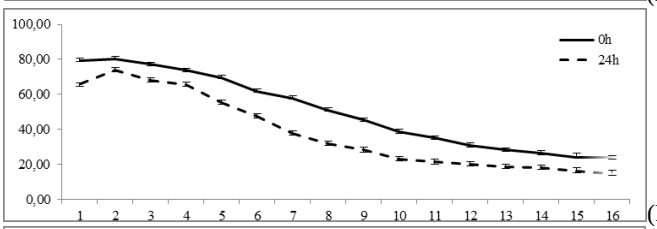
[3] The pool samples were stored at 4°C for 24 hours as the group of (24 h). Values are expressed as the means ± SEM. VCL: curvilinear velocity, VSL: straight-line velocity, LIN: linearity of the curvilinear trajectory, VAP: average path velocity, STR: straightness, BCF: beat-cross frequency WOB: wobble (VAP/VCL). [ab]Numbers within columns with different superscripts differ ($P<0.05$)



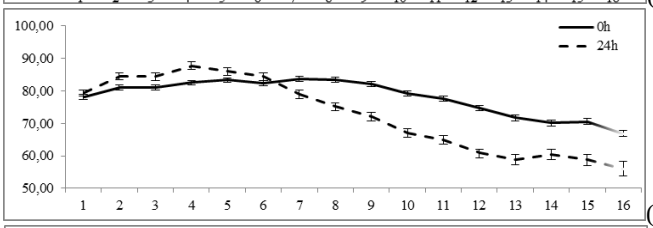
(Figure 3A)



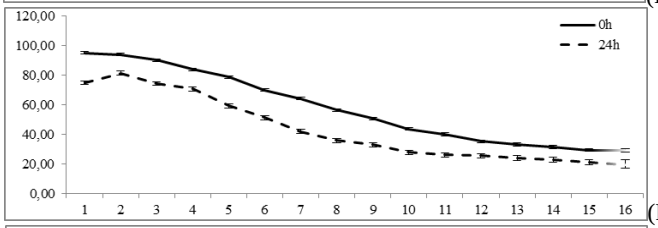
(Figure 3B)



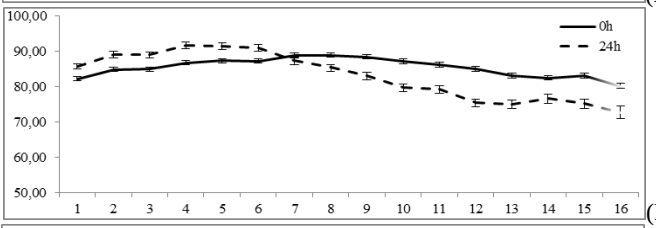
(Figure 3C)



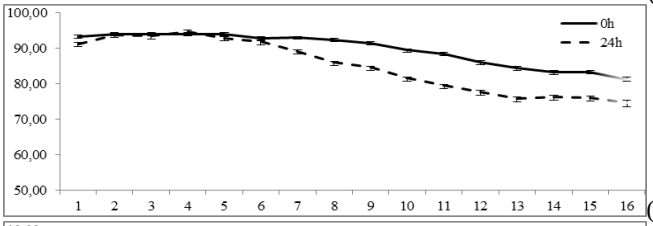
(Figure 3D)



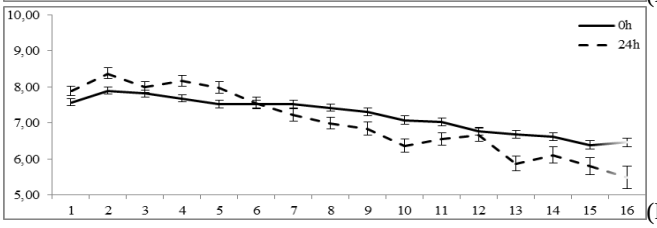
(Figure 3E)



(Figure 3F)



(Figure 3G)



(Figure 3H)

Figure 3. Effect of storage time on motility and kinetics along 16 captures (24 hours at 4°C). Motility (%) (Fig. 1A), VCL: curvilinear velocity (μm/sec)(Fig 1B), VSL: straight-line velocity (μm/sec)(Fig 1C), LIN: linearity of the curvilinear trajectory (%)(Fig 1D), VAP: average path velocity (μm/sec)(Fig 1E), STR: straightness (%)(Fig 1F), WOB: wobble (VAP/VCL)(%)(Fig 1G), BCF: beat-cross frequency (Hz)(Fig 1H), from sperm parameters as measured at different video capture rate (16 seconds post activation) each capture presented 5 seconds.

period in salmonid semen (Trigo *et al.,* 2015). The declines in ATP contents and oxidative stress can cause metabolic or functional disorders, and reducing spermatozoa motility (Aramli *et al.,* 2013). In addition, the alteration of sperm motility after short cool storage may be caused by insufficient oxygen supply occurring during storage. In rainbow trout, the decrease of sperm motility and VSL, LIN was more pronounced than the other parameters (Dietrich *et al.*, 2005).

In the present work the steady decrease in motility duration and other kinetic parameters except from LIN, STR and BCF after 24 h storage time was observed. The decrease in sperm ATP content it could be correlated with affected parameters.

In summary, spermatozoids activated with medium with a high osmolality medium showed higher values in motility and kinetic parameters in comparison with low osmolality medium. Respect to the individual ejaculates or the pool, although significant differences were observed in some parameters, no relevant changes were observed in total motility neither in kinetic parameter. Total motility and velocity of sperm showed significant lower values after cool storage at 4ºC for 24 h.

Acknowledgments

This work was partially supported by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA grant RTA2013-00107-C03-00), COST Office (Food and Agriculture COST Action FA1205: AQUAGAMETE), and the European FEDER Fund.

References

Alavi, S.M.H. and Cosson, J. 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell biology international, 29: 101–110. doi:10.1016/j.cellbi.2004.11.021

Alavi, S.M.H. and Cosson, J. 2006. Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell biology international, 30: 1–14. doi:10.1016/j.cellbi.2005.06.004

Alavi, S.M.H., Rodina, M., Viveiros, A.T.M., Cosson, J., Gela, D., Boryshpolets, S. and Linhart, O. 2009. Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (*Esox lucius* L.). Theriogenology, 72: 32–43. doi:10.1016/j.theriogenology.2009.01.015

Aramli, M.S., Kalbassi, M.R., Nazari, R.M. and Aramli, S. 2013. Effects of short-term storage on the motility, oxidative stress, and ATP content of Persian sturgeon (*Acipenser persicus*) sperm. Animal reproduction science ,143: 112–117. doi:10.1016/j.anireprosci.2013.10.010

Aurich, C. 2008. Recent advances in cooled-semen technology. Animal Reproduction Science, 107: 268–275. doi:10.1016/j.anireprosci.2008.04.015

Bastami, K.D., Imanpour, M.R. and Hoseinifar, S.H. 2009. Sperm of feral carp Cyprinus carpio: optimization of activation solution. Aquaculture International, 18: 771–776. doi:10.1007/s10499-009-9299-6

Batista, M., Santana, M., Alamo, D., González, F., Niño, T., Cabrera, F. and Gracia, A. 2012. Effects of incubation temperature and semen pooling on the viability of fresh, chilled and freeze-thawed canine semen samples. Reproduction in domestic animals = Zuchthygiene, 47: 1049–55. doi:10.1111/j.1439-0531.2012.02014.x

Billard, R., Cosson, J., Perchec, G. and Linhart, O. 1995. Biology of sperm and artificial reproduction in carp. Aquaculture, 129: 95–112. doi:10.1016/0044-8486(94)00231-C

Billard, R. and Cosson, M.P. 1992. Some problems related to the assessment of sperm motility in freshwater fish. Journal of Experimental Zoology, 261: 122–131. doi:10.1002/jez.1402610203

Bozkurt,Y., Seçer S., and Bekcan S. 2006. "Relationship between Spermatozoa Motility, Egg Size, Fecundity and Fertilization Success in Salmo Trutta Abanticus." Agricultural Sciences 12 (4): 345–48.

Bozkurt, Y., Ogretman, F. and Secer, F.S. 2009. Effect of different extenders and storage periods on motility and fertilization success of Grass Carp (*Ctenophatyngodon idella*) sperm during spawning season.Tarim Bilimeri Dergisi, 15(3): 277-284

Butts, I.A.E., Alavi, S.M.H., Mokdad, A. and Pitcher, T.E. 2013. Physiological functions of osmolality and calcium ions on the initiation of sperm motility and swimming performance in redside dace, Clinostomus elongatus. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology, 166: 147–157. doi:10.1016/j.cbpa.2013.05.011

Cardona-Costa, J., Pérez-Camps, M., García-Ximénez, F. and Espinós, F.J. 2009. Effect of gametes aging on their activation and fertilizability in zebrafish (*Danio rerio*). Zebrafish, 6: 93–95. doi:10.1089/zeb.2008.0578

Cejko, B.I., Sarosiek, B., Kowalski, R.K., Krejszeff, S. and Kucharczyk, D. 2013. Application of Computer-assisted Sperm Analysis in Selecting the Suitable Solution for Common Carp, *Cyprinus carpio* L., Sperm Motility. Journal of the World Aquaculture Society, 44: 466–472. doi:10.1111/jwas.12043

Cosson, J. 2010. Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. Journal of fish biology, 76: 240–279. doi:10.1111/j.1095-8649.2009.02504.x

Dietrich, G.J., Kowalski, R., Wojtczak, M., Dobosz, S., Goryczko, K. and Ciereszko, A. 2006. Motility Parameters of Rainbow Trout (*Oncorhynchus mykiss*) Spermatozoa in Relation to Sequential Collection of Milt, Time of Post-mortem Storage and Anesthesia. Fish Physiology and Biochemistry, 31: 1–9. doi:10.1007/s10695-005-3527-4

Donoghue, A.M., Kirby, J.D., Froman, D.P., Lerner, S.P., Crouch, A.N., King, L.M., Donoghue, D.J. and Sonstegard, T.S. 2010. Field testing the influence of sperm competition based on sperm mobility in breeder turkey toms. British Poultry Science, 44: 498-504. doi:10.1080/00071660310000855l7

Dziewulska, K. and Domagała, J. 2013. Effect of pH and cation concentrations on spermatozoan motility of sea trout (*Salmo trutta m. trutta* L.) Theriogenology, 79: 48–58. doi:10.1016/j.theriogenology.2012.09.008

Hagedorn, M. and Carter, V.L. 2011. Zebrafish reproduction: revisiting in vitro fertilization to

increase sperm cryopreservation success. PloS one, 6: e21059.doi:10.1371/journal.pone.0021059

Hoo, J.Y, Kumari, y., Shaikh M.F., Hue, M.S,. Hing, G.B. 2016. "Zebrafish: A Versatile Animal Model for Fertility Research." Biomed Research International.

Ingermann, R.L., Schultz, C.L.F., Kanuga, M.K. and Wilson-Leedy, J.G. 2011. Metabolism of motile zebrafish sperm. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology, 158: 461–467. doi:10.1016/j.cbpa.2010.12.008

Islam, M.S. and Akhter, T. 2011. Tale of Fish Sperm and Factors Affecting Sperm Motility: A Review. Advances in Life Sciences, 1: 11-19. doi:10.5923/j.als.20110101.03

Jing, R., Huang, C., Bai, C., Tanguay, R. and Dong, Q. 2009. Optimization of activation, collection, dilution, and storage methods for zebrafish sperm. Aquaculture, 290: 165–171. doi:10.1016/j.aquaculture.2009.02.027

Lammers, J., Splingart, C., Barrière, P., Jean, M. and Fréour, T. 2014. Double-blind prospective study comparing two automated sperm analyzers versus manual semen assessment. Journal of assisted reproduction and genetics, 31: 35–43. doi:10.1007/s10815-013-0139-2

Li, P., Li, Z.-H., Hulak, M., Rodina, M. and Linhart, O. 2012. Regulation of spermatozoa motility in response to cations in Russian sturgeon Acipenser gueldenstaedtii. Theriogenology, 78: 102–109. doi:10.1016/j.theriogenology.2012.01.025

Morisawa, M. and Suzuki, K. 1980. Osmolality and potassium ion: their roles in initiation of sperm motility in teleosts. Science (New York, N.Y.), 210: 1145–7.

Perchec, G., Jeulin, C., Cosson, J., André, F. and Billard, R. 1995. Relationship between sperm ATP content and motility of carp spermatozoa. Journal of cell science, 108: Pt 2, 747–753.

Pérez-Cerezales, S., Martínez-Páramo, S., Cabrita, E., Martínez-Pastor, F., de Paz, P. and Herráez, M.P. 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology, 71: 605–613. doi:10.1016/j.theriogenology.2008.09.057

Reinardy, H.C., Skippins, E., Henry, T.B. and Jha, A.N. 2013. Assessment of DNA damage in sperm after repeated non-invasive sampling in zebrafish Danio rerio. Journal of Fish Biology, 82: 1074–81. doi:10.1111/jfb.12042

Rurangwa, E, D.E Kime, F Ollevier, and J.P Nash. 2004. "The Measurement of Sperm Motility and Factors Affecting Sperm Quality in Cultured Fish." Aquaculture, 234 (1): 1–28. doi:10.1016/j.aquaculture.2003.12.006.

Sahin, T., Kurtoğlu, İ.Z. and Balta, B 2013. Effect of Different Extenders and Storage Periods on Motility and Fertilization Rate of Rainbow Trout (Oncorhynchus Mykiss) Semen. Universal Journal of Agricultural Research, 1: 65–69.

Schleh, C. and Leoni, A.L. 2013. How to optimize the benefits of computer assisted sperm analysis in experimental toxicology. Journal of occupational medicine and toxicology (London, England), 8: 6. doi:10.1186/1745-6673-8-6

Targońska, K., Kucharczyk, D., Mamcarz, A., Glogowski, J., Krejszeff, S., Prusińska, M. and Kupren, K. 2008. Influence of Individual Variability in the Percentage of Motile Spermatozoa and Motility Time on the Survival of Embryos of Chosen Fish Species. Polish Journal of Natural Science, 23: 178–187. doi:10.2478/v10020-008-0013-3

Seok, S.H., Baek, M.W., Lee, H.Y., Kim, D.J., Na, Y.R., Noh, K.J., Park, S.H., Lee, H.K., Lee, B.H., Park, J.H. 2008. In vivo alternative testing with zebrafish in ecotoxicology. Journal of veterinary science, 9: 351–7

Tejerina, F., Buranaamnuay, K., Saravia, F., Wallgren, M. and Rodriguez-Martinez, H. 2008. Assessment of motility of ejaculated, liquid-stored boar spermatozoa using computerized instruments. Theriogenology, 69: 1129–1138. doi:10.1016/j.theriogenology.2008.01.027

Trigo, P., Merino, O., Figueroa, E., Valdebenito, I., Sánchez, R. and Risopatrón, J. 2015. Effect of short-term semen storage in salmon (*Oncorhynchus mykiss*) on sperm functional parameters evaluated by flow cytometry. Andrologia, 47: 407–411. doi:10.1111/and.12276

Wilson-Leedy, J.G., Kanuga, M.K. and Ingermann, R.L. 2009. Influence of osmolality and ions on the activation and characteristics of zebrafish sperm motility. Theriogenology, 71: 1054–62. doi:10.1016/j.theriogenology.2008.11.006

Yang, H. and Tiersch, T.R. 2009. Sperm motility initiation and duration in a euryhaline fish, medaka (*Oryzias latipes*). Theriogenology, 72: 386–392. doi:10.1016/j.theriogenology.2009.03.007