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Motility of sea urchin *Paracentrotus lividus* spermatozoa in the post-activation phase

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Abstract

The characterization of sperm motility patterns, particularly post-activation changes, is the first step in setting up species-specific protocols involving gamete management and embryo production, for both aquaculture and laboratory research purposes. This study is aimed at the characterization of the sperm motility pattern of the purple sea urchin *Paracentrotus lividus*. Semen samples were individually diluted in artificial sea water for sperm motility activation. They were then incubated at 18°C for up to 24 hr. Motility was evaluated on dilution, and 1 hr, 3 hr and 24 hr after activation, by computerized analyser. The semen fertilization capacity was also evaluated. Under our experimental conditions (dilution 1:1,000 in artificial sea water plus 0.05% BSA, 18°C, in the dark), *P. lividus* semen remained viable for up to 24 hr, as the total motile sperm and the fertilization percentages did not change significantly during the incubation time. In contrast, the mean curvilinear velocity and the subpopulation of rapid sperm (those having a curvilinear velocity > 100 μm/s) slightly but significantly decreased after 3 hr, thereafter remaining unchanged for up to 24 hr after activation. In conclusion, our results show that diluted *P. lividus* semen can be used for a longer period than that of most fish species, with no need for motility inhibition procedures, supporting its wider use in laboratory research. In addition, the development of artificial fertilization protocols for aquaculture production is simplified by long-lasting sperm motility.

KEYWORDS
computer-assisted sperm motility analysis, fertilization, motility duration, *Paracentrotus lividus*, sea urchin, spermatozoa

1 | INTRODUCTION

Sperm motility assessment is the most common way of evaluating sperm quality. Sperm motility is indeed a key factor in fertilization success, as sperm cells generally have to swim to reach the egg surface (Cosson et al., 2008).

Traditionally, sperm motility in aquatic species is visually assessed by microscope, evaluating parameters such as the percentage of motile cells, the first significant decrease in motility and the total duration of motility. Developments in the application of computerized sperm motility analyses have made it possible to provide information on parameters not detectable by visual analysis, such as velocity or trajectory, that closely depend on the structural integrity of the sperm cell and functionality of the metabolic pathways involved in sperm movement (Fabbrocini et al., 2016; Fauvel, Suquet & Cosson, 2010; Liu, Innes & Thompson, 2011).

Sperm movement has been widely investigated, in the past and in recent years, in a wide range of model species, from mammals to invertebrates, both terrestrial and aquatic (Cosson et al., 2008; Miller, Mansell, Meyers & Lishko, 2015). Understanding the mechanisms of sperm movement in aquatic species, particularly the changes occurring during the motile phase, is of great concern.

Species-specific characterization of sperm motility patterns and the development of routine tools for their rapid and easy assessment are key steps in setting up species-specific protocols involving gamete management and embryo production, for both aquaculture and laboratory research purposes (Cabrita et al., 2014; Lahnsteiner & Caberlotto, 2012; Suquet et al., 2012; Yang & Tiersch, 2009).

Sperm motility features may vary widely from one species to another, as a result of the development of different reproductive traits and fertilization strategies (Hassan, Qin & Li, 2016), reflected in differences in sperm morphology and physiology (Gallego, Pérez, Asturiano & Yoshida, 2014; Suquet et al., 2010, 2012). Sea urchin spermatozoa have relatively long-lasting motility (Au, Lee, Chan & Wu, 2001; Ohtake, Mita, Fujiwara, Tazawa & Yasumasu, 1996), and thanks to this feature, they are widely used in laboratory research, from ecotoxicology to molecular biology, being among the best models for developmental and evolutionary studies (D'Adamo et al., 2014; Kazama & Hino, 2012; McClay, 2011). Future research in these fields requires further technological advances, especially in reproductive biotechnologies such as controlled crosses and the creation of specific cell lines and gamete cryobanks (McClay, 2011; Suquet et al., 2012). Moreover, most sea urchin species are considered a food delicacy and have a great economic value and are therefore of great interest for aquaculture (Fabbrocini et al., 2012; Parisi et al., 2012). Thus, echiniculture, both large scale for commercial production and small scale for laboratory research purposes, also stands to benefit from the wider application of reproductive biotechnologies (Fabbrocini et al., 2014; Paredes, Bellas & Costas, 2015), and the characterization of the sperm motility patterns of the most important sea urchin species is the first step in their development.

In the last few decades, the interest of aquaculturists in the Mediterranean sea urchin *Paracentrotus lividus* has greatly increased (Parisi et al., 2012). The purple sea urchin *P. lividus* (Lamarck, 1816) is widely distributed in the Mediterranean area and along the North-Eastern Atlantic coasts (Bouduresque & Verlaque, 2013). It is a species of high ecological importance, commonly used in laboratory research. Due to its high value as luxury food, it is intensely harvested, with a consequent decline in wild stocks (Pais et al., 2007).

As a part of a wider study aimed at the characterization of the sperm motility pattern of *P. lividus*, the data presented here describe sperm motility parameters, assessed by means of computerized analysis, in the period after semen activation. Sperm fertilization ability was also evaluated in the same period.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Thirty semen samples of *P. lividus* were individually diluted in artificial sea water to induce sperm motility activation. They were then incubated at 18°C for up to 24 hr. Motility was evaluated on dilution (t0), and 1 h, 3 h and 24 h after activation, by computerized analyser (n = 30). In addition, in six randomly selected activated semen samples, the fertilization capacity of spermatozoa was evaluated at the same post-activation times (n = 6).

2.2 | Sea urchin collection

Adult sea urchins of the species *P.lividus* (test diameter without spines 35-45 mm) were hand-collected with the aid of scuba-diving equipment along the southern Adriatic coast of Italy (Termoli, CB; 41°54' N, 16°10' E) from October to December, that is in the middle of the reproductive season for the Mediterranean area (Fabbrocini, Maurizio & D'Adamo, 2016; Sánchez-España, Martinez-Pita & Garcìa, 2004). The collected specimens were placed in a cooler and carried to the laboratory under moist conditions within two hours.

2.3 | Gamete collection

The sea urchins were induced to spawn by injection of 1 ml of KCl 0.5 M. An aliquot of gametes from each sea urchin was immediately diluted in a drop of ASW (artificial sea water 3.5 g/L; ASTM (American Society for Testing and Materials), 2004) for the preliminary evaluation of sperm motility activation and egg morphology by direct observation under a microscope.

Gametes from each animal were then separately collected and stored under appropriate conditions until use: undiluted at 4°C for sperm and diluted in ASW at 18°C for eggs. Only egg samples showing good morphology were pooled (three females for each pool) and used to evaluate the fertilization capacity of the semen samples.

2.4 | Assessment of sperm motility parameters

Each semen sample was diluted at a rate of 1:1,000 in ASW containing BSA (bovine serum albumin, fraction V, Sigma, Milan, Italy) at a final concentration of 0.05% as an antisticking agent and incubated at 18°C in the dark for up to 24 hr. Motility was evaluated on dilution (t0) and 1 hr, 3 hr and 24 hr after activation by computerized analyser, as described in Fabbrocini, Di Stasio and D'Adamo (2010).

Sperm movement was recorded using a 100 frame/s camera (Basler, 782 × 582 resolution) attached to a Nikon Eclipse E600 microscope (Nikon Instruments, Florence, Italy) with a phase-contrast objective (10 × 10 magnification) connected to a computerized motion analysis system, the Sperm Class Analyzer® (SCA®, Microptic, s.l., Barcelona, Spain). The SCA acquisition parameters were set as follows: max area = 400 μm^2, min area = 50 μm^2; frame rate = 100/s; total captured images = 100. Each recorded field consisted of a mean of three replicates, each one analysing from 250 to 500 sperm tracks. Records were carefully checked for sample drifting.

The following motility parameters were assessed: (i)Total motile sperm (TM), as the percentage of sperm with a curvilinear velocity > 10 μm/s; (ii) rapid sperm (RAP), as the percentage of sperm with a curvilinear velocity > 100 μm/s; (iii) curvilinear velocity (VCLtm, μm/s); (iv) straight-line velocity (VSLtm, μm/s); (v) average path velocity (VAPtm, μm/s); (vi) beat-cross frequency (BCFtm, hz); (vii) lateral head displacement (ALHtm, μm). For the subpopulation of

rapid sperm, the following parameters were also evaluated: (viii) VCLrap (μm/s); i) VSLrap (μm/s); l) VAPrap (μm/s).

2.5 | Assessment of sperm fertilization capacity

The fertilization capacity of spermatozoa on activation (t0) and 1 hr, 3 hr and 24 hr post-activation was evaluated in accordance with the protocol standardized for the *P. lividus* sea urchin spermiotoxicity tests (Volpi Ghirardini, Arizzi Novelli, Losso & Ghetti, 2005) and routinely used in ecotoxicology (Fabbrocini et al., 2010, and references herein). Briefly, the concentration of sperm samples and egg suspensions was determined by counting under a microscope. A sperm aliquot from each sample was then added to 10 ml of the egg solution (adjusted to 200 eggs/ml in ASW) to obtain a sperm–egg ratio of 15,000:1 and incubated in polystyrene multiwell dishes (18°C, in the dark). After 30-minute incubation, samples were fixed in concentrated buffered formalin and the percentages of fertilized eggs were scored by observing 200 eggs from each sample. Three replicates for each semen sample were run. For the fertility evaluations carried out 24 hr post activation, a pool of freshly spawned eggs was used.

2.6 | Statistical analysis

Differences in the assessed sperm parameters as a function of the time post activation were statistically evaluated by one-way ANOVA, and, where appropriate, significant differences were explored by Tukey's post hoc test. Prior to analysis, data were tested for normality using Kolmogorov–Smirnov's test and for homogeneity of variance using Cochran's C test. Data that did not meet the ANOVA assumptions were log-transformed before analysis. Percentage data were arcsine square-root-transformed before analysis. Only ALH transformed data did not meet the ANOVA assumptions and were therefore analysed by nonparametric Kruskal–Wallis ANOVA on ranks. $p < .01$ was considered as significant.

3 | RESULTS

3.1 | Sperm motility

The values of the sperm motility parameters recorded on dilution (t0) and at the other times following activation are shown in Figure 1a-f. The total motile sperm (TM; Figure 1a) was always around 90%, with no significant differences linked to activation time. In contrast, the subpopulation of rapid sperm (RAP; Figure 1a) was found to vary significantly as a function of the time from activation. It was unchanged after 1 hr (about 80%) and slightly but significantly lower after 3 hr, remaining thereafter at around 70% for up to 24 hr after activation. The sperm velocity parameters were also observed to decrease in the period after activation, in terms of both the total motile spermatozoa and the subpopulation of rapid ones: all the analysed velocity parameters were unchanged 1 hr after dilution, significantly lower at the 3-h evaluation and unchanged after 24 h. Briefly, the curvilinear velocity of the TM spermatozoa ranged from 200 to 350 μm/s at t0 and 1 h after activation and around 150 μm/s at the other times (VCLtm; Figure 1b). The straight-line velocity initially ranged from 150 to 200, decreasing to about 75 μm/s at the later times (VSLtm; Figure 1c). Similarly, the average path velocity (VAPtm; Figure 1d) showed values from 150 to 250 μm/s at t0 and 1 hr after activation, and lower levels (around 100 μm/s) at the 3-hr and 24-hr evaluations. The velocity parameter values recorded in the RAP subpopulation (VCLrap, VSLrap, VAPrap; Figure 1b-d, respectively) were quite similar to those of the TM sperm. Despite the different trend in function of the time post activation, most of the motile sperm were still rapid even 24 h after dilution. The beat-cross frequency (BCFtm; Figure 1e) ranged between 36 and 40 hz, with no significant difference in relation to the time post activation. The amplitude of lateral head displacement (ALHtm; Figure 1f) did not vary significantly with time after activation either, remaining about 2 μm.

3.2 | Sperm fertilization capacity

The fertilization capacity of semen samples on dilution (t0) and 1 hr, 3 hr and 24 hr after activation is shown in Figure 2. The fertilization percentages were always around 80%, with no significant difference in relation to the time post activation.

4 | DISCUSSION

In the present study, we evaluated the sperm quality of the sea urchin *P. lividus* at successive times after activation. On activation, the velocity values, relative size of total motile and rapid sperm subpopulations, and fertilization rate all lay within the range previously reported for mature and in-spawning specimens (Fabbrocini et al., 2016). The total motile sperm and fertilization rates did not significantly change in the 24 h after activation, while the velocity values and the relative size of the rapid sperm subpopulation slightly decreased but significantly.

Computer-assisted sperm motility analysis makes it possible to evaluate not only the number of motile spermatozoa, but also how they move (Gallego et al., 2014). A wide number of parameters can be assessed to characterize sperm motility. Among these, velocity parameters are the most widely used in aquatic species (Liu et al., 2011). In the last few years, it has been demonstrated that grouping spermatozoa in subpopulations on the basis of their velocity makes it possible to identify changes occurring in the semen with reference to the coexistence of different subpopulations with distinct motility characteristics within the same sample (Beirão, Cabrita, Pérez-Cerezales, Martinez-Pánaramo & Herráez, 2011; Gallego et al., 2014). Indeed, in the sea urchin *A. crassispina*, velocity parameters showed significant reduction after exposure to cadmium (Au et al., 2001), while in the sea bream *S. aurata*, cryopreservation proved to affect different sperm subpopulations in different ways (Beirão et al., 2011), while the relative size of the RAP sperm subpopulation was more strongly impaired by toxicants than the TM population

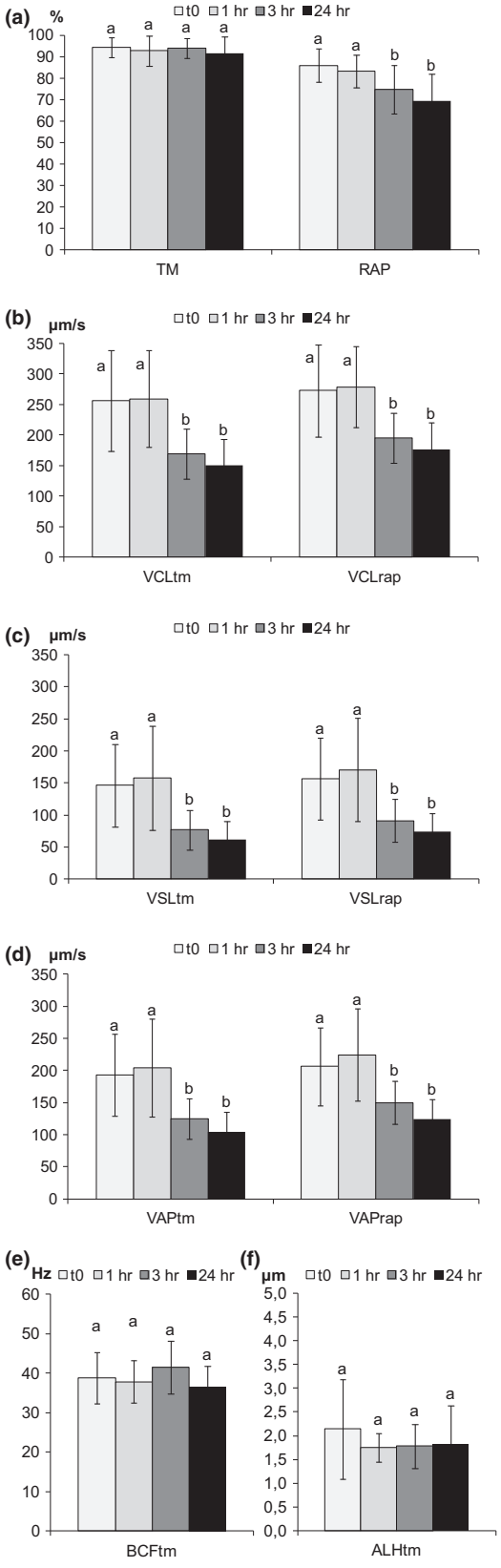

FIGURE 1 Motility parameters of *Paracentrotus lividus* spermatozoa recorded at different post-activation times. (a) TM = total motile sperm (VCL > 10 μm/s); RAP = rapid sperm (VCL > 100 μm/s). (b) VCL = curvilinear velocity (μm/s) of TM and RAP sperm subpopulations. (c) VSL = straight-line velocity (μm/s) of TM and RAP sperm subpopulations. (d) VAP = average path velocity (μm/s) of TM and RAP sperm subpopulations. (e) BCFtm = beat-cross frequency (hz) of TM sperm. (f) ALHtm = lateral head displacement (μm) of TM sperm. Data represent mean values ± standard deviation (n = 30). For each evaluated parameter, different superscripts indicate significant differences ($p < .01$)

(Fabbrocini et al., 2016). For this reason, we evaluated the mean velocity parameters in both the total motile sperm (TM) population (VCLtm, VSLtm, VAPtm) and the subpopulation of rapid sperm (RAP), that is those having VCL > 100 μm/s (VCLrap, VSLrap, VAPrap). Moreover, the beat-cross frequency (BCFtm), which is sublinearly proportional to the beating frequency of the tail, and the amplitude of lateral head displacement (ALHtm), which is related to the level of bending in the proximal region of the tail (Su, Xue & Ozcan, 2012), were also quantified to describe how motile cells progress along their tracks.

In the present study, the subpopulations composing the assemblage of *P. lividus* semen were found to vary with time after activation. The mean curvilinear velocity fell significantly, considering both VCLtm and VCLrap. However, this decrease affected only the relative size of the RAP subpopulation, whereas that of the TM population remained practically unchanged for up to 24 h after activation. Considering that these subpopulation changes are the result of the decrease in curvilinear velocity, it can be surmised that they are indicators of sublethal alterations in sperm physiology occurring in the period after activation. However, these alterations do not affect semen viability, as demonstrated by the absence of effects on the relative size of the TM sperm population, ALH and BCF values, and the fertilization rate.

The egg-to-sperm ratio, fertilization volume, gamete contact time and temperature used here were the same as the standardized

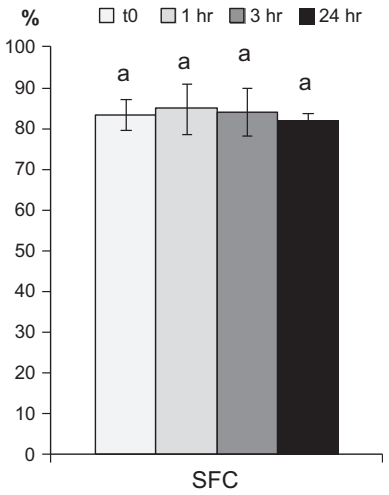

FIGURE 2 Sperm fertilization capacity (SFC, expressed as the percentage of fertilized eggs) of *Paracentrotus lividus* spermatozoa at successive post-activation times. Data represent mean values ± standard deviation (n = 6). Different superscripts indicate significant differences ($p < .01$)

fertilization conditions adopted for the spermiotoxicity test with *P. lividus* (Fabbrocini et al., 2010; Volpi Ghirardini et al., 2005), which is designed to be able to detect and quantify any impairment in the sperm's egg-fertilizing capacity. Our results show that no effect on fertilization ability was observed in the period after activation, even when semen is activated 24 h before being used. Similarly, long-lasting sperm viability, in terms of fertilization ability, has also been reported for other sea urchin species (Ohtake et al., 1996; Rahman, Tsuchiya & Uehara, 2009).

The fall in sperm motility after activation has been studied in many aquatic species, and the exhaustion of ATP reserves (with a contemporary increase in internal ionic concentrations) is considered to be the main cause (Cosson, 2010; Dadras et al., 2017; Suquet et al., 2010). Most studies of sperm flagellar movement use sea urchins as model species, and it has been established that the energy for their flagellar beating is produced by the oxidation of endogenous substrates. The respiration rate is also known to decrease in the period after activation, leading to a decrease in sperm motility (Mita, Fujiwara, De Santis & Yasumasu, 1994; Mita & Nakamura, 1998; Ohtake et al., 1996; Tombes, Brokaw & Shapiro, 1987). In addition, mitochondrial damage has been observed in *H. pulcherrimus* spermatozoa after prolonged incubation in seawater (Mita & Nakamura, 1998).

Oxidative stress is a major factor affecting semen viability, as sperm, being terminally differentiated cells, possess limited endogenous antioxidant protection (Aitken et al., 2012; Aramli, Kalbassi, Nazari & Aramli, 2013). Reactive oxygen species (ROS) are produced during mitochondrial respiration, leading to DNA/RNA damage and membrane lipid peroxidation, thus reducing sperm motility, and their amount has been found to increase post collection as a result of the high respiration rate linked to motility (Aramli et al., 2013; Kazama & Hino, 2012). In the present study, both ALH and BCF levels remained almost unchanged for up to 24 hr after activation, suggesting the absence of severe alterations in flagellar beating. Consistent with our hypothesis that only sublethal damage had occurred in the longer post-activation times, most of the motile sperm were rapid even 24 hr after dilution, maintaining their fertilization capacity despite the significant decrease in VCL.

Sperm motility duration may widely vary in aquatic species from a few minutes in sea bass (Sansone et al., 2001) to several days in Persian sturgeon and medaka (Aramli et al., 2013; Yang & Tiersch, 2009). Differences in sperm motility duration have also been found among sea urchin species. In *P. lividus* spermatozoa, motility and fertilization ability are known to remain unchanged in the first hour post activation, and that is the reason for their extensive use in ecotoxicological tests (Fabbrocini et al., 2010). A similar trend has also been reported in *A. crassispina*, where motility remained constant for up to 120 minutes (Au et al., 2001). Shorter sperm swimming duration was found by Levitan (2000) in *L. variegatus* (120 min), by Rahman et al. (2009) in *E. mathaei* (85 min) and by Gallego et al. (2014) in *A. crassispina* (45 min). In contrast, Ohtake et al. (1996) found 90% of sperm to be still motile 12 h after activation in *H. pulcherrimus*, with a swimming duration of about 20 h. In the present study, more than 80% of *P. lividus* spermatozoa continued swimming 24 hr after activation, most of them still rapid.

Apart from the differences in energetic metabolism between species that have developed different fertilization strategies (Levitan, 2000; Rahman et al., 2009), it should be pointed out that the reported data refer to studies performed under different conditions of sperm activation and incubation (dilution rate, dilution buffer, temperature, etc.). In the present study, semen samples are diluted in ASW at a rate of 1:1,000 in accordance with our motility activation protocol. Interestingly, this dilution rate is 10- to 125-fold higher than that reported by other authors (Gallego et al., 2014; Mita et al., 1994; Ohtake et al., 1996), despite the fact that the rate of oxygen consumption has been found to increase with dilution and low sperm concentrations are known to reduce semen lifespan (Kazama & Hino, 2012; Levitan, Sewell & Chia, 1991). On the other hand, our dilution buffer contains BSA, one of the most common antioxidants used in culture media for mammalian sperm (Aitken et al., 2012; Yanagimachi, 1994), which has also been found to improve post-thaw motility in cryopreserved sturgeon sperm (Shaluei, Sadeghi & Zadmajid, 2017). Therefore, it could be supposed that even in very small amounts (at a 20-fold lower concentration), it offers sperm cells some protection from ROS during incubation.

In the light of these results, an in-depth investigation of the changes occurring in the metabolic pathways involved in *P. lividus* sperm motility at the longer post-activation times (≥3 hr), and of the factors that may impair sperm cells at both structural and physiological levels, would be of great interest.

5 | CONCLUSIONS

Our results allow us to conclude that under our experimental conditions (dilution 1:1000 in ASW plus 0.05% BSA, 18°C, in the dark), *P. lividus* sperm viability is retained for up to 24 hr.

Specifically, in the first hour post activation, no differences were recorded in any of the evaluated parameters. Therefore, diluted *P. lividus* semen can be used for a longer time than that of most fish species, enabling its wider use in laboratory research into sperm physiology, ecotoxicology and cryobiology, with no need for motility inhibition procedures. The development of artificial fertilization protocols for aquaculture production is also simplified by long-lasting sperm motility. Moreover, the fertilization rate and the percentage of total motile sperm did not change during the incubation time, and most of the motile sperm, despite a slight but significant decrease observed 3 hr after activation, were rapid even 24 hr after dilution.

Finally, the computer-assisted sperm motility analysis was able to detect sublethal alterations in sperm physiology at the longer post-activation times, as reflected in decreased curvilinear velocity. These alterations do not, however, affect overall sperm viability, again proving to be a very useful and sensitive "quick-response" tool for the evaluation of sperm quality.

Additional studies evaluating the relationship between incubation conditions (mainly regarding the dilution rate and the presence of

antioxidants) and sperm cell functionality (mainly regarding intracellular ATP content and the presence of oxidative damage) are needed to develop a semen management protocol able to further improve the motility quality of *P. lividus* spermatozoa at longer post-activation times.

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