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The protective effect of endogenous melatonin on gilthead seabream sperm during cryopreservation

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ABSTRACT

Cryopreservation is widely used for artificial reproduction techniques (ART) but requires good gamete quality to succeed. Considering the endogenously produced melatonin by the fish and its protective role in the organism, the objective of this experiment was to search for the best moment of the day to collect gilthead seabream (*Sparus aurata*) sperm with better quality and, at the same time, to test the potential protective effect of the exogenous melatonin during sperm freezing and thawing processes. Fish were first sampled every 6 h, corresponding to the beginning of the light period (6 h), mid-light (12*h*), beginning of the dark period (18 h), and mid-dark (24 h) to determine melatonin concentration in blood plasma by radioimmunoassay (RIA). The analysis showed higher values at 24 h (244 pg/mL) compared with 12 h (99 pg/mL), which allowed the selection of those moments for the next experiment. After, fish were sampled for sperm collection at mid-light (ML, 12 h) and mid-dark (MD, 24 h), and fresh sperm was used to assess gamete quality and for the cryopreservation assay. Sperm samples were cryopreserved to test supplementation of different melatonin concentrations (0.001 mM, 0.01 mM and 0.1 mM) together with a control group without added melatonin. Gamete quality was assessed through spermatozoa concentration and motility (CASA system), cell viability (PI/SYBR-green) and DNA fragmentation (Comet assay). Despite cell viability that was higher at ML, most of the fresh sperm motility parameters did not differ between ML and MD, only linearity (LIN) was enhanced at MD. Nevertheless, in cryopreserved samples, total motility (TM) was significantly higher at MD in all melatonin treatments, control, and fresh samples, revealing an endogenous night-effect. Moreover, spermatozoa concentration was also higher at MD (28.9×10^9/mL) than at ML (20.7×10^9/mL). Supplemented melatonin did not confer extra protection to gilthead seabream sperm during cryopreservation since the tested concentrations did not differ between the control in any sperm quality test. It is here suggested that endogenously produced melatonin may contribute to the improvement of some gamete quality parameters at mid-dark, allowing the aquaculture sector to select better sperm quality in a non-invasive way by choosing it as the best moment of the day for sperm collection.

1. Introduction

Through the development of techniques for sperm management, cryopreservation became a very useful tool in the aquaculture environment. This technique, widely used for artificial reproduction, allows accessibility to sperm samples all year round, permits synchronization on the availability of female and male biological material, facilitates the transport of samples to other research centers or production sites, and decreases the need and costs of keeping broodstock in captivity (Martínez-Páramo et al., 2017). Despite the convenience of this procedure, researchers keep trying to find new methodologies to avoid and counteract the cryodamage inflicted on cells, which is associated with membrane impairments, DNA fragmentation, alterations in mitochondrial membrane potential, loss of spermatozoa motility and viability, which ultimately compromise the fertilization success, further larval survival and correct development (Cabrita et al., 2010; Figueroa et al., 2019).

Recently, antioxidants have appeared as a viable supplement to the cryoprotectant medium, since they are able to protect spermatozoa against the oxidative stress induced by the freezing and thawing processes (Sandoval-Vargas et al., 2020). In fact, there are several papers describing those positive effects in thawed sperm from commonly produced fish species, like salmonids (Kutluyer et al., 2014; Figueroa et al.,

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2018; Merino et al., 2020), different sturgeon species (Li et al., 2018), common carp (*Cyprinus carpio*) (Ogretmen et al., 2015), European seabass (*Dicentrarchus labrax*) (Martínez-Páramo et al., 2012), gilthead seabream (*Sparus aurata*) (Cabrita et al., 2011), spotted halibut (*Verasfer variegatus*) (Zidni et al., 2022), among others. Noteworthy, the usage of natural antioxidants has been increasing, as is the case of polyphenols, compounds that are only synthesized by plants (Dhakal et al., 2019). Recently, our group reviewed the studies testing the effects of diets supplemented with plant extracts and spices (*e.g.* curcumin, black pepper, paprika, ginger, blueberries, or sesame seed) in fish sperm (Félix et al., 2021), and even extracts from different algae species are now being tested (Félix et al., 2022). However, the incorporation of natural antioxidants as cryoprotectant agents in fish sperm freezing trials is still neglected. As examples, the non-flavonoid polyphenol methylenediphosphonicacid (MDPA) was supplemented in the cryoprotectant medium of beluga (*Huso huso*) sperm, and it increased the fertilization capacity of sperm after thawing (Osipova et al., 2016), and the usage of propolis increased the spermatozoa integrity, motility and hatchability of common carp (Ogretmen et al., 2014). Also, the indolamine melatonin had promising results on fish sperm short-term storage. Different melatonin concentrations were added to paddlefish (*Polyodon spathula*) sperm stored at 4 °C, showing to be effective in protecting several spermatozoa traits [motility, ATP content, reactive oxygen species (ROS) levels, membrane integrity, mitochondria membrane potential] (Gao et al., 2019).

Melatonin is known to be produced by the organism during the night, having an important role in sleep regulation (Reiter, 1993), but is also a powerful antioxidant, immunostimulant, anti-inflammatory and oncostatic agent, and it is involved in the regulation of other physiological processes, like reproduction (Zhao et al., 2019). Recent findings from our group demonstrated that melatonin is present in fish seminal plasma, and it may have a protective role for spermatozoa by being correlated with the antioxidant status of this fluid (Félix et al., 2023). Moreover, similarly to what happens with melatonin circulating in the bloodstream, it was shown that melatonin profile in seminal plasma also changed between mid-light (ML) and mid-dark (MD) moments of the day. The moment of highest melatonin concentration in blood varies within species, but normally it raises during the dark period and falls to basal levels during the day (Bromage, 2001). Despite photoperiod, melatonin production is also influenced by other environmental factors, that are normally under control in aquaculture, like temperature, light intensity, and salinity (Porter, 2001; Lopez-Olmeda et al., 2009b).

Gilthead seabream is one of the most produced species in Europe, and a good model for reproductive studies due to the amount of sperm obtained by stripping. It is a winter spawner, meaning its reproductive season is triggered by the shortening of photoperiod (Meseguer et al., 2008), and males are normally fluent from December to February. The circulating melatonin in the bloodstream of this species has basal levels during the day and peaks at night (Molina-Borja et al., 1996), and was only found in seminal plasma during the night (Félix et al., 2023). Considering all those recent findings, and the fact that gilthead seabream has a well-established cryopreservation protocol (Cabrita et al., 2005), this study aimed at exploring the potential use of melatonin as an antioxidant and natural protecting agent of spermatozoa during cryopreservation. This was attained under two different approaches: 1) find the most suitable moment for sperm collection, considering that fish has endogenous melatonin in circulation at night, and 2) study the effects of exogenous melatonin supplemented as an antioxidant in the cryopreservation media.

2. Material and methods

2.1. Animal rearing

For this experiment, gilthead seabream broodstock was acquired from a semi-intensive aquaculture farm, Aqualvor (Portugal), and maintained at *Ramalhete* experimental station, Faro (Portugal). Fish had a mean body weight of 520 ± 90 g and were kept in two fiberglass tanks in a semi-open water system at 13 ± 0.6 °C, and under a controlled short photoperiod of 8 h:16 h light:dark (LD) in order to simulate the environmental conditions of the reproductive season. All experimental conditions were established and performed in accordance with ARRIVE guidelines, with directives 86/609/EU and 2010/63/EU of the European Parliament and Council, and Portuguese legislation from Direção Geral de Alimentação e Veterinária (DGAV) for the use of laboratory animals (PORT 1005/92). CCMAR facilities (licence number 009238) and their staff are certified to house and conduct experiments with live animals (Group-C licenses by DGAV). The experimental procedures with germ cells were previously approved by DGAV with reference 003289.

2.2. Experimental design and sampling procedures

To explore the potential role of circulating melatonin in spermatozoa protection during cryopreservation, the moments of the day with the highest and lowest melatonin concentration in the bloodstream were firstly determined. A first sampling set was performed in the middle of the breeding season (January), to characterize the broodstock daily melatonin rhythm, randomly collecting blood samples every 6 h (6 h – beginning of light period, 12 h – mid-light, 18 h – beginning of dark period, and 24 h – mid-dark) for melatonin determination. After anesthesia with 200 ppm phenoxyethanol, blood was collected from the caudal vein with a heparinized syringe and immediately centrifuged at 3000*g*, 4 °C, for 15 min. The supernatant plasma was kept at −80 °C until analysis. Afterwards, the most suitable moments of the day to perform the cryopreservation assays were decided as the one with the lowest melatonin concentration in the bloodstream, corresponding to the mid-light (ML, 12 h), and the one with the highest registered melatonin values, at mid-dark (MD, 24 h). In a second sampling event, sperm samples were randomly collected from fluent males at both time points, ML and MD (6 males at each daytime), by an abdominal massage using a sterilized 1 mL syringe and cleaning the urogenital pore with PBS solution to avoid contamination with seawater, mucus or feces. Samples were kept in 5 mL Eppendorf tubes at 4 °C in a styrofoam box until analysis. Once in the laboratory, an aliquot from each male (approximately 200 μL) was kept aside for fresh gamete quality assessment (cell concentration, viability and motility parameters) to discard contaminated samples, and another part of the sperm sample was prepared for the cryopreservation assay. All nocturnal samplings were performed under dim red light and using aluminum foil to protect the fish's head and eye from scattering light, thus not interfering with the natural melatonin production during the night.

2.3. Blood melatonin concentration

The gilthead seabream blood melatonin concentration was assessed for the four different daytimes by radioimmunoassay (RIA), following the manufacturer protocol for the Melatonin direct Serum/Plasma/Saliva RIA kit (RE 29301, IBL International, Germany), which was previously validated for this species (Lopez-Olmeda et al., 2009a). The final quantification of radioactivity was measured in a ray-gamma counter (WALLAC 1470 Automatic Gamma Counter, Perkin Elmer, Waltham, Massachusetts, USA) for 1 min.

2.4. Cryopreservation assay

During the second sampling event, sperm was cryopreserved shortly after collection, both at 12 h (ML) and 24 h (MD), to evaluate the protective role of endogenous and exogenous melatonin, following a protocol previously optimized by our group for gilthead seabream sperm (Cabrita et al., 2005). For the cryopreservation procedure, 400 μL of sperm were diluted 1:6 (*v*/v) in the cryoprotectant medium [5% dimethyl-sulfoxide (DMSO) in 1% sodium chloride (NaCl)],

supplemented with different melatonin concentrations: 0.001 mM, 0.01 mM and 0.1 mM, together with a control group without added melatonin. Those concentrations were chosen according to the available literature and to preliminary results obtained by our group in Senegalese sole sperm (Ferrão, 2020). All the cryoprotectant solutions were prepared freshly before use and covered with aluminum foil, due to melatonin light sensitivity and quick degradation. The 500 μL French straws (3–5 per treatment) were filled and placed 2 cm above the liquid nitrogen in a floating rack for 10 min. After that, straws were immediately submerged and transferred to the storage tank until further analysis. For the post-thaw quality analysis, straws were placed in a water bath at 25 °C, for 30 s, according to the protocol described by (Cabrita et al., 2011) for gilthead seabream.

2.4.1. Sperm quality analysis using CASA system

Spermatozoa concentration and motility parameters were assessed using a Computer Assisted Sperm Analysis (ISAS software, Proiser, Valencia, Spain), coupled to a phase-contrast microscope (Nikon E-200; Nikon, Tokyo, Japan) with an ISAS camera (25 fps), to check for potential gamete quality differences among fresh sperm ($n = 8$) and all cryopreserved treatments ($n = 6$ for each), and between ML and MD moments. For cell concentration, sperm samples were first diluted 1:10 in a non-activating medium (1% NaCl) and 10 μL of cell suspension were placed in a Makler counting chamber under a 10× negative phase contrast objective. For each sample, 3 photos of different fields were taken, and the software calculated the cell concentration in millions of spermatozoa/mL ($n = 8$), as previously described for this species (Cabrita et al., 2011). For the spermatozoa motility assessment, sperm was first activated using 1 μL of sperm sample and 10 μL of artificial seawater. Immediately after, 15 s post activation, different parameters were registered: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s) and linearity (LIN, %). TM refers to any spermatozoa showing movement; PM refers to spermatozoa moving in a progressive manner; VCL is defined as the spermatozoa velocity along the real trajectory; VSL refers to the straight-line velocity from the starting to the finishing point of the movement; and LIN is calculated according to VSL and VCL (LIN = VSL/VCL*100) (Gallego and Asturiano, 2018).

2.4.2. Cell viability

Cell viability was determined in fresh (n = 8) and post-thaw ($n = 6$) sperm samples using fluorescence microscopy, and following the protocols described by Martínez-Páramo et al. (2013) and Cabrita et al. (2011). For this analysis, 1 μL of sperm was diluted 1:100 in 1% NaCl with a mix of 0.5 μL propidium iodide (PI) (0.6 M) and 1 μL SYBR-green (final concentration of 100 nM) dyes to stain non-viable and viable cells, respectively. Three photos of different fields were taken (magnified 20×) per sample under a fluorescent microscope (Nikon E200, Tokyo, Japan), and at least 100 cells per field were counted using the "cell counter" feature from ImageJ (Java) software. Results were expressed as a percentage of viable cells.

2.4.3. Evaluation of DNA fragmentation

The fragmentation of spermatozoa DNA was assessed by Comet assay, as described by Cabrita et al. (2005) for gilthead seabream sperm (n = 6–8). Briefly, sperm was first diluted 1:75 in 1% NaCl, cells embedded in 0.5% low melting agarose (Invitrogen) and introduced in pre-treated agarose slides with a coverslip (in duplicates). After drying, the slides were immersed in a lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM tris pH 10, 1% triton X-100, and 1% lauril sarcosin) for 1 h, after which, 10 mM dithiothreitol (DTT) was added and, after 30 min, 4 mM lithium diodosalicilate was introduced to help DNA unwind. The slides were kept in this solution for 1.30 h at room temperature. Once finished the lysis process, slides were immersed in an alkaline electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) for 20 min before submitted to electrophoresis for 10 min, at 25 V and 300 mA. After electrophoresis, the slides were neutralized in two baths with 0.4 M Tris HCl for 5 min each. Afterwards, samples were fixed in 96% ethanol for 3 min and stored at 4 °C until analysis. To visualize the DNA, 10 μL of PI (2.5 μg/mL) were added to each slide and placed in a fluorescence microscope. An average of 50 photos per sample (magnified 40×) were taken with a digital camera (VisiCam 5 Plus V1900119), in order to have a minimum of 100 cells per sample to analyze. The final analysis of the DNA fragmentation was performed using the KOMET software, version 6.0 (Andor Technology, Belfast, Ireland), as described by Cabrita et al. (2011).

2.5. Statistical analysis

Statistical analysis was performed using SPSS software (IBM). In blood melatonin data, outliers were first identified and removed and afterwards a one-way ANOVA, followed by the *post-hoc* Tukey test, was applied. A student-*t*-test was applied to spermatozoa concentration and, additionally, to VCL, to identify differences between the two time points. In the case of motility parameters, cell viability and DNA fragmentation, data was previously logarithmic transformed when necessary and checked for normality and homogeneity of variances, using Shapiro-Wilk and Levene tests, respectively. To the data that assumed those principles was applied a two-way ANOVA followed by a Tukey *post-hoc* test. The respective non-parametric Kruskal-Wallis test was applied to data that did not assume the above-mentioned principles. Significant differences were assumed when $p < 0.05$.

3. Results

3.1. Blood melatonin concentration

The characterization of gilthead seabream blood melatonin daily rhythm confirmed that concentrations fluctuated along the 24 h of the day. The maximum melatonin concentration in blood (244 pg/mL) was found at 24 h, coinciding with the MD time point, and the lowest (99 pg/mL) at ML, which occurred at 12 h (Fig. 1). The analysis of data revealed significant differences (one-way ANOVA, Tukey, $p < 0.05$) between different sampling points (12 h and 24 h).

3.2. Sperm quality analysis using CASA system

Day and night sperm collection revealed that cell concentration at MD (28.9×10^9/mL) was significantly (student-*t*, $p < 0.05$) higher than

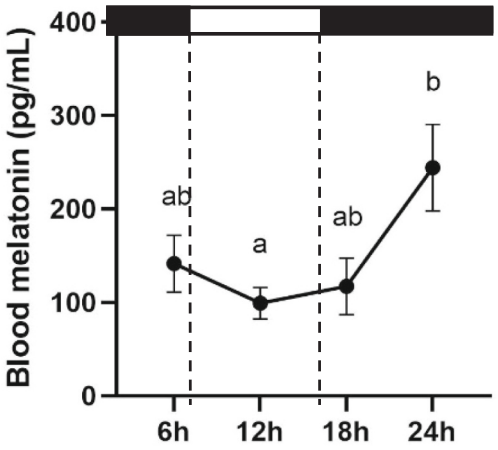

Fig. 1. Blood melatonin concentration in gilthead seabream kept under a photoperiod of 8 h light: 16 h dark. Blood plasma samples were collected during breeding season at 6 h ($n = 5$), 12 h ($n = 6$), 18 h ($n = 7$) and 24 h (n = 6). Results are expressed as mean ± SE. Significant differences (one-way ANOVA, Tukey test, $p < 0.05$) are identified with different letters.

at ML (20.7×10^9/mL) (Fig. 2).

The statistical analysis (two-way ANOVA, Tukey, $p < 0.05$) of motility parameters determined in fresh and cryopreserved sperm, revealed that the time of the day when the samples were collected (ML, MD) had a significant effect on TM, VCL and LIN. The treatment of samples (fresh, cryopreserved with or without supplemented melatonin) showed a significant effect in TM, VCL and VSL; and the interaction between Time and Treatment also had a significant effect on TM, PM, VSL and LIN (Table 1). There were no significant differences in TM in fresh samples between ML and MD moments of the day (87.6 and 82.1%), however, this parameter decreased significantly in all the cryopreserved treatments at ML (Fig. 3A). In addition, all cryopreserved treatments, including the control group (only DMSO), had higher TM at night-time (CTR: 82.4%; 0.001 mM: 82.8%; 0.01 mM: 84.0%; 0.1 mM: 85.6%), reaching values similar to the fresh samples (82.1%). Similar results were observed in VCL, in which MD values were significantly higher than those at ML (irrespective of the treatment) and also the treatment factor was significant (irrespective of the time of day) (two-way ANOVA, Tukey, $p < 0.05$), revealing differences (student-*t*-test, $p < 0.05$) in CTR, 0.01 and 0.1 mM melatonin treatments in terms of daytime (Fig. 3C). Regarding the percentage of progressive motile spermatozoa (Fig. 3B), and VSL (Fig. 3D), fresh samples registered significantly higher values at MD, than all cryopreserved treatments during the same sampling point, whereas at ML no differences were observed. In addition, regarding LIN, all cryopreserved treatments had significantly higher results at ML (average: 61.7%) than at MD (average: 43.3%) (Fig. 3E). Overall, comparing ML with MD moments, sperm collected and cryopreserved at MD showed a high percentage of motile cells, similar to fresh sperm, but with lower progressiveness, lower straight-line velocity and consequently lower linearity. Contrarily, sperm collected and cryopreserved during the day-time period (ML) showed a decrease in the percentage of motile cells compared with fresh samples. However, it seems that sperm motility pattern was not affected by cryopreservation since PM and VSL were similar to fresh. Moreover, at each daytime, there were no significant differences between the different concentrations of melatonin used in the cryopreservation treatments.

3.3. Cell viability

According to the data analysis (two-way ANOVA, Tukey, $p < 0.05$), the treatments (fresh samples, cryopreserved with or without melatonin) and the interaction between treatment and sperm collection time had a significant impact on cell viability (Table 1). In fresh spermatozoa, cell viability was significantly higher at ML (79.5%) than at MD (52.3%), and it was significantly higher than all cryopreserved treatments (Fig. 4). There were no significant differences between cryopreserved treatments and fresh samples (52.3%) at MD.

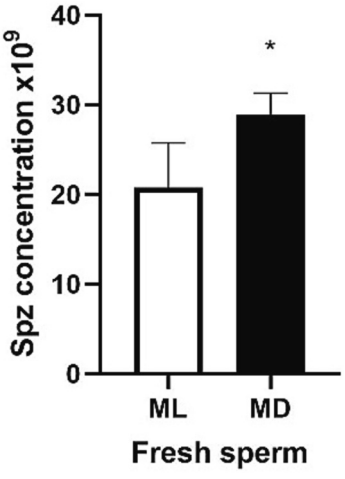

Fig. 2. Gilthead seabream spermatozoa concentration at two different moments of the day (ML and MD). Results are expressed as mean ± SD (n = 7). Significant differences (student-*t*, $p < 0.05$) are identified with an asterisk (*).

3.4. Evaluation of DNA fragmentation

Regarding DNA fragmentation, the results did not show any significant differences (two-way ANOVA, Bonferroni, $p < 0.05$) between treatments in tail DNA (%) (Fig. 5).

4. Discussion

The emerging of potential cryoprotecting agents, like melatonin, led the scientific community to constantly challenge the already established cryopreservation protocols, to decrease even more the possibility of cell damage during freezing and thawing processes. In this experiment, it was demonstrated that rather than the supplemented melatonin on the cryopreservation assay, the moment of the day for sperm collection could be an effective way to obtain better sperm quality which further influences spermatozoa performance after cryopreservation.

For this study, fish were maintained under a controlled short photoperiod of 8 L:16D, coinciding with the mid-light (ML) moment with the 12 h and the mid-dark (MD) moment with the 24 h. As photoperiod changes along the year, mid-light and mid-dark times do change too, the reason why in chronobiology studies the usage of ML and MD has been standardized and allows to compare all species, irrespective of the photoperiod. The first experiment, the daily melatonin rhythm characterization of our broodstock, aimed to find the moment of the day with the highest and lowest melatonin concentrations in the gilthead seabream bloodstream and revealed that MD and ML points corresponded, respectively, to those moments of the day. Melatonin can be produced by the pineal organ and many other extra pineal sites that, altogether, contribute to the overall melatonin concentration in the organism (Cebrian-Perez et al., 2014). In species like European seabass (Bayarri et al., 2002), rainbow trout (Besseau et al., 2006), and brook trout (*Salvelinus fontinalis*) (Zachmann et al., 1992), ocular melatonin production occurs during the day, which is thought to have a local effect and not contribute to circulating levels, that normally rise during the dark period (Falcón et al., 2010). In addition, the timing and amplitude of melatonin peak also depend on the species and, as reviewed by Bromage (2001), within the same photoperiod, there are species with a prolonged nocturnal plasma melatonin peak, reflecting the duration of the dark period, such as Atlantic salmon and rainbow trout, and others that have only a single melatonin peak that can occur in the beginning or at the end of the dark phase, like the Atlantic cod (*Gadus morhua*) (Porter et al., 2000). In gilthead seabream, plasma melatonin increased during the night, displaying a profile similar to European seabass and Atlantic cod (Bromage, 2001) and, although there were no significant differences between the three measurements of melatonin during the dark phase, there was a clear single peak of melatonin concentration at 24 h (MD) that was significantly different from the levels of the sampling point at 12 h (ML). This difference allowed the selection of those moments as the most suitable day-timings for sperm collection in the further assays.

The first suggestive data of the beneficial night effect on gilthead seabream spermatozoa quality was the result of sperm concentration analysis. Measured with the CASA system, the concentration of fresh samples collected at ML and MD proved a daily oscillation in this parameter, being higher at MD and coinciding with the moment of higher melatonin concentration in the bloodstream. In most fish species, sperm production depends on seasonal changes that occur in the reproductive tract (Butts et al., 2010), and gilthead seabream reproductive behaviour and spawning are triggered by the shorter (winter) photoperiods (Meseguer et al., 2008). Since melatonin has a neuroendocrine effect on fish reproduction (Falcón et al., 2007) and is also a key factor that transduces photoperiodic information to fish, it may have an

Table 1
Effects of factors Time (ML and MD) and Treatment (fresh, control, 0.001, 0.01 and 0.1 mM melatonin) and interaction between Time*Treatment for all the motility parameters [total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight-line velocity (VSL) and linearity (LIN)] and cell viability. Significant differences (two-way ANOVA, Tukey test, $p < 0.05$) are identified with asterisks ($p < 0.01$* and $p < 0.001$**).

	TM	PM	VCL	VSL	LIN	Viability
Time	< 0.001**	0.0595	< 0.001**	0.1132	< 0.001**	0.838
Treatment	0.0014*	0.0837	< 0.001**	0.0011*	0.3720	< 0.001**
Time*Treatment	< 0.001**	< 0.001**	0.4380	0.0012*	< 0.001**	< 0.001**

important role in sperm production and concentration, besides the already known antioxidant proprieties. Sperm quality improvement by the action of endogenous melatonin was previously found in humans (Ortiz et al., 2011) and, recently, diurnal oscillations of total sperm count, sperm concentration, semen volume, progressive motility and total motility were also described in a specific Chinese population (Liu et al., 2022). In their study, researchers found that the optimum quality of sperm was obtained from samples collected between 11 AM and 3 PM, and they hypothesized that this is an intrinsic window that possibly may change and move to later hours due to nowadays human lifestyle. As far as we know, there are no such studies on fish. However, there is some recent literature regarding the effects of exogenous melatonin on fish reproduction. In a dietary study, a combination of melatonin and zinc were supplemented in male walking catfish (*Clarias macrocephalus*) feeds and the effects on gonadal maturation and reproductive performance were evaluated after 8 weeks of trial (Aripin et al., 2018). In the melatonin combined treatment authors obtained higher gonadosomatic index, sperm motility and spermatozoa concentration, and it also decreased the percentage of abnormal cells. Gilthead seabream sperm concentration is normally high (Fauvel et al., 2010) and an important sperm quality parameter for this species, since it has external fertilization and an elevated number of spermatozoa is necessary to obtain success upon spawning, as it happens in other fish species (Browne et al., 2015). Also, the spermatozoa concentration and sperm volume are two important characteristics that express the reproductive performance of a species (Kowalski and Cejko, 2019) and, once it has daily oscillations, possibly influenced by endogenous melatonin and other hormonal secretions, the extraction of sperm samples in the proper moment of the day may enable efficient artificial reproductive techniques (ART) in aquaculture, like fertilizations and cryopreservation procedures.

Regarding motility analysis in fresh samples, there were no differences between samples collected at ML and MD in the majority of the parameters analyzed, but LIN was significantly enhanced at night (MD), in line with the same tendency for PM and VSL to be increased, which may reveal a possible effect from the endogenously produced melatonin on spermatozoa motility pattern. It was not possible to find on literature a similar study to discuss the obtained results regarding the influence of endogenous melatonin as an antioxidant and protective agent of fish spermatozoa, since this is a pioneering experiment on this subject. Though, in humans, Ortiz et al. (2011) reported that both melatonin and antioxidant endogenous levels influenced positively sperm motility. Furthermore, other *in vivo* studies with melatonin permit some considerations regarding the effect of melatonin itself. A study in killifish (*Fundulus heteroclitus*), in which the animals were exposed to water containing 1 μM melatonin for 8 days, revealed that the subpopulation of rapid spermatozoa had increased in the treated group, and showed higher VSL and average path velocity (VAP) (Lombardo et al., 2014). Although the origin of melatonin differs, these results are in accordance with the ones obtained in our study.

In what cryopreservation assay is concerned, comparing cryopreserved results with the ones obtained with fresh samples, different patterns were displayed depending on the time sperm was collected. Samples collected and cryopreserved at MD showed a high percentage of motile cells, similar to fresh sperm, whereas at ML a slight decrease in sperm motility and curvilinear velocity was observed, although, the motility pattern in terms of straight-line velocity was maintained as in fresh samples. In our data, linearity was the only motility parameter with an opposite tendency between fresh and cryopreserved samples: in fresh samples was higher at MD, in cryopreserved samples was higher at ML, which suggest that freezing and thawing process considerably affected this motility parameter. Since spermatozoa tracks are typically curved rather than linear, the LIN index should be considered, as it is an important parameter for fertilization success (Cosson et al., 2008). Altogether, the obtained results suggests that endogenous melatonin could be exerting a protective effect on gilthead seabream spermatozoa. An *in vitro* study performed in paddlefish, demonstrated that 0.5 μM melatonin supplemented to the extender media improved the motility duration, TM, PM and beat cross frequency (BCF) of the spermatozoa stored at 4 °C for 24 h (Gao et al., 2019). Moreover, in a cryopreservation assay with piracanjuba (*Brycon orbignyanus*) sperm, 2 mM melatonin added to cryoprotectant media improved the post-thaw spermatozoa viability, motility duration, TM and had the highest fertilization rate (Palhares et al., 2020). Although there is no literature available to discuss about protection of endogenous melatonin, the above mentioned studies demonstrated that melatonin itself can improve some fish sperm motility parameters. Regarding supplemented melatonin, the results did not differ between melatonin concentrations, inclusive from the control, in any of the motility parameters analyzed (TM, PM, VCL, VSL and LIN). The same effect has been previously seen in a different species, such as curimba (*Prochilodus lineatus*), in which spermatozoa cryopreserved with three different melatonin concentrations (1, 2 and 3 mM melatonin) had no significant differences in any motility parameter (Assis et al., 2019). The present results suggests that supplemented melatonin did not confer extra protection to the spermatozoa, as initially hypothesized, and consequently, it did not improve the original cryopreservation protocol.

Cell viability was also assessed in fresh samples and revealed to be higher at ML, but in all cryopreserved treatments the percentage of viable cells was significantly lower in both moments of the day, showing signs of cryodamage. Despite fresh samples at MD had a lower percentage of viable cells, cryopreserved treatments did not differ from the fresh, control or between melatonin supplemented treatments at MD, suggesting some protective role exerted from endogenous melatonin, likewise motility. Similarly, in an *in vitro* experiment with the freshwater species piracanjuba, 2 mM melatonin supplemented to the cryoprotectant medium maintained the viability levels of spermatozoa post-thaw (Palhares et al., 2020). The overall DNA fragmentation obtained in this study was lower than in similar studies with gilthead seabream sperm (Cabrita et al., 2005, 2011). Moreover, it did not reveal any significant differences in tail DNA percentages, either between sperm collection and cryopreservation times, or between supplemented melatonin treatments, which also reinforce the already known higher resistance of gilthead seabream sperm to the freezing and thawing processes (Guerra et al., 2013). Although literature in rats describe that melatonin can reduce oxidative stress and DNA fragmentation in testes exposed to microwaves (Sokolovic et al., 2015), our study did not demonstrated this kind of DNA protection, and there is a lack of literature about melatonin effects on fish sperm DNA.

It is known that melatonin effects are species-specific and its efficacious is related with the selected dose and administration method (Succu et al., 2011). Cryopreservation assays performed with melatonin supplemented solutions require a very cautious preparation and handling to

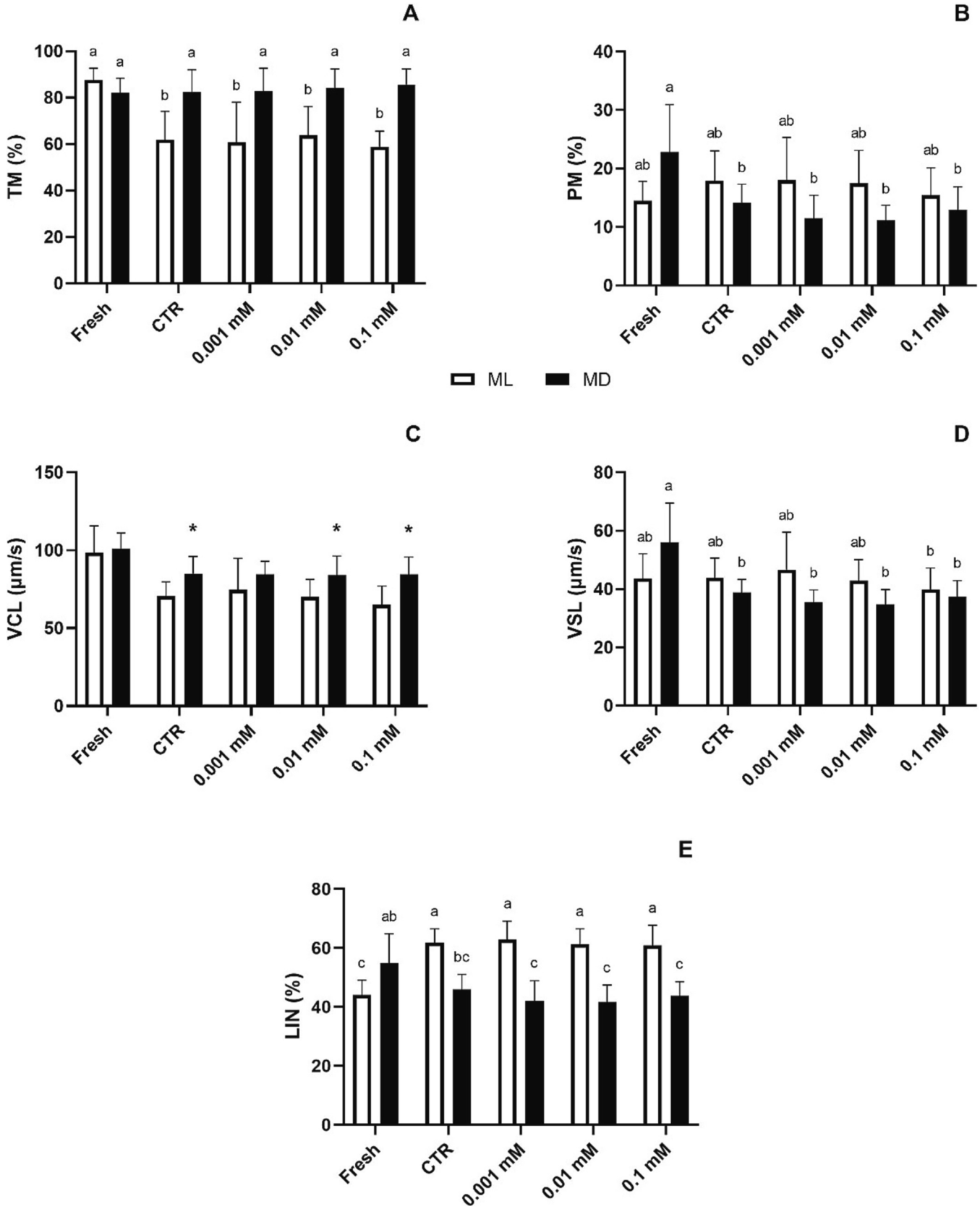

Fig. 3. Motility parameters of gilthead seabream fresh and cryopreserved sperm, at two different moments of the day, mid-light (ML) and mid-dark (MD): A) Total motility (TM), B) Progressive motility (PM), C) Curvilinear velocity (VCL), D) Straight line velocity (VSL) and E) Linearity (LIN). Cryopreservation was performed with three different melatonin supplementations (0.001, 0.01 and 0.1 mM) and a control (CTR) treatment without melatonin. Results are expressed as mean ± SD ($n = 8$). Significant differences of the two-way ANOVA are identified with different letters, and from student-*t*-test (applied to VCL to demonstrate differences between ML and MD) are identified with an asterisk (*). Significant differences were considered when $p < 0.05$.

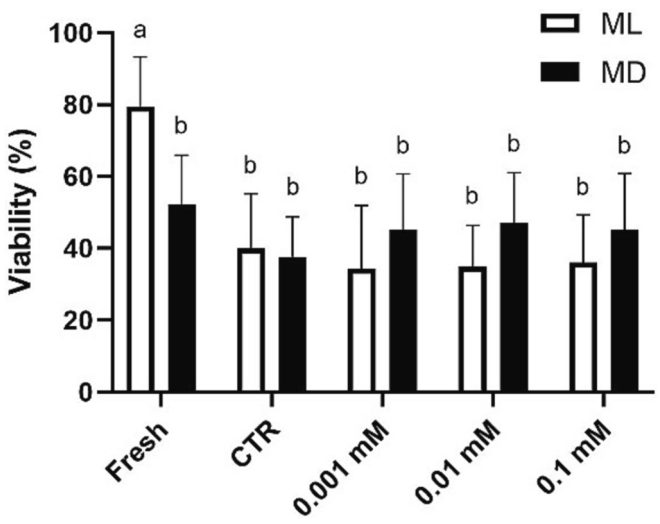

Fig. 4. Spermatozoa viability of gilthead seabream fresh (n = 8) and cryopreserved (n = 6) sperm at two different moments of the day (ML and MD). Cryopreservation was performed with three different melatonin supplementations (0.001, 0.01 and 0.1 mM) and a control (CTR) treatment without melatonin. Results are expressed as mean ± SD. Significant differences (two-way ANOVA, Tukey test, $p < 0.05$) are identified with different letters.

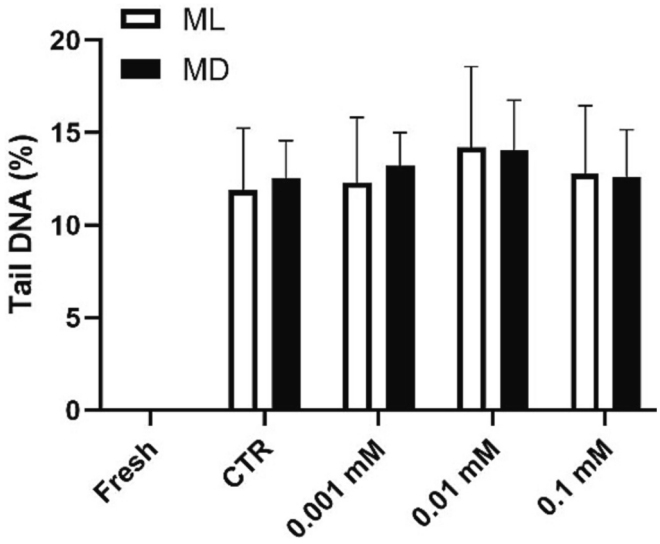

Fig. 5. DNA fragmentation of gilthead seabream sperm at mid-light (ML) (n = 6–7) and mid-dark (MD) (n = 8) moments of the day. Cryopreservation was performed with three different melatonin supplementations (0.001, 0.01 and 0.1 mM) and control (CTR) treatment without melatonin. The number of fresh samples were insufficient for the analysis. Results are expressed as mean ± SD. No significant differences (two-way ANOVA, $p < 0.05$) were found.

use them on the best conditions, due to melatonin sensitiveness to light and temperature, which can compromise the reliability of the assays. Such sensitiveness to temperature leads to the fact that the optimum of its action may have a temperature limit, however cryopreservation submits the tissues or cells to very low temperatures, until - 196 °C. In other words, more than the concentration in which it is used, the extremely low temperatures of cryopreservation may be inhibiting melatonin to act on cells with its full antioxidant proprieties. If so, the available time for melatonin to act inside the spermatozoa may take place during the equilibrium time (normally at 4 °C) and, once the samples are submitted to the freezing curve, there may be an unknown temperature below which supplemented melatonin cannot exert its protection role. This hypothesis is supported by comparing the available literature on long and short-term fish sperm storage with supplemented melatonin, that normally have better results in short-term storage assays with samples maintained at 4 °C (Ortiz et al., 2011; Gao et al., 2019; Ferrão, 2020). However, further research is needed to understand the melatonin specific entrance and mechanism of action in the spermatozoa (Zhao et al., 2019), and if there is a temperature and time limit to exert its protective functions. Besides, it would be important to try to find the optimal exposure time and conditions that enhance the protective action of melatonin without compromising cryoprotectant effects.

5. Conclusions

This study aimed to understand the impact of endogenous melatonin on fish sperm quality and, at the same time, test the antioxidant effect of melatonin as a natural supplement in the sperm cryopreservation assay. In face of the obtained results, it is possible to conclude that supplemented melatonin in the cryoprotectant medium did not confer extra protection to gilthead seabream spermatozoa. However, despite the cryopreservation outputs, this study contributed for the comprehension of melatonin role at fish reproductive level and suggested a positive impact of endogenous melatonin on different fish sperm quality biomarkers, especially, in sperm concentration, motility and velocity. The present results allow the production sector to obtain better sperm quality by choosing the best moment of the day to collect the samples, which according to our results would be at mid-dark timing. Nonetheless, further chronobiology research is needed in other fish species with commercial interest to check if melatonin naturally produced by the organism exerts its functions in the same way.

CRediT authorship contribution statement

F. Félix: Conceptualization, Methodology, Validation, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization. **R. Antunes:** Investigation. **L.M. Vera:** Writing – review & editing, Supervision. **C.C.V. Oliveira:** Writing – review & editing, Supervision. **E. Cabrita:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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