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Novel approaches on melatonin role: Presence of clock-hormone in fish seminal plasma

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ABSTRACT

The study of melatonin is of great importance for the fundamental knowledge of any living system since it displays many different physiological roles, including being a potent natural antioxidant. To the best of our knowledge, there is no information regarding melatonin in fish seminal plasma. This study aimed to determine this clock-hormone levels in the seminal plasma of three aquaculture fish species: European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), and Senegalese sole (*Solea senegalensis*) (both wild and F1 breeders), and to explore melatonin potential role in fish reproduction and spermatozoa antioxidant status. Blood and seminal plasma were collected from fish during their reproductive season, at two different times of the day [mid-light (ML) and mid-dark (MD)], and melatonin concentration was determined by radioimmunoassay (RIA). Testosterone (T), 11-ketotestosterone (11KT), and total antioxidant status (TAS) were also determined, to investigate the putative role of seminal melatonin in fish reproduction, both at endocrine and antioxidant levels. For each species, Pearson's correlation analysis was performed between all possible factors. Blood plasma melatonin showed higher average values at night in the three species: gilthead seabream (808 ± 139 pg/mL), European seabass (364 ± 85 pg/mL), and Senegalese sole (248 ± 40 and 88 ± 11 pg/mL in F1 and wild males, respectively). However, melatonin levels in seminal plasma were species-specific: in European seabass, melatonin levels were not detectable at any time-point, whereas in gilthead seabream it was only found at MD (average of 21 pg/mL), and in Senegalese sole, different melatonin patterns were observed between F1 and wild males, but both had higher melatonin at MD (6.84 and 14.26 pg/mL, respectively). In gilthead seabream, at MD seminal melatonin levels correlated with the antioxidant status of seminal plasma. A relationship between blood melatonin and seminal TAS levels was observed in European seabass at ML: in this species, seminal melatonin could not be detected and the lowest seminal TAS levels were found. Regarding steroid analysis, opposite patterns in the seminal plasma of F1 and wild Senegalese sole were observed: at MD, wild Senegalese sole had substantially greater 11KT levels (2.53 ng/mL), whereas F1 males had higher T levels (1.92 ng/mL). In gilthead seabream, a positive correlation between T and ML blood melatonin and seminal TAS was observed. This study unraveled the species-specificity and daily changes of melatonin in fish seminal plasma.

1. Introduction

Since melatonin discovery, the attention of research studies has fluctuated between its origins, biosynthesis, and metabolism. However, more recent melatonin experiments have focused on its different physiological roles and extra-pineal production sites in a great variety of species (Yu et al., 2020). Known as the time-keeper hormone, this lipophilic and small structure molecule has tremendous importance in vertebrates by transducing the photoperiodic information to the animal body (Reiter, 1993). Produced during the night, and known to control circadian rhythms, melatonin is also an immunostimulant, an oncostatic agent, and can be an efficacious antioxidant (Acuna-Castroviejo et al., 2007), counterbalancing mitochondrial oxidative stress and apoptotic events (Fang et al., 2019). Melatonin regulates many biochemical,

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physiological, and behavioral processes associated with circadian, seasonal, and annual rhythms (Nisembaum et al., 2021), including reproduction (Ekstrzm and Meissl, 1997; Reiter et al., 2009). In photoperiodic animals, melatonin is important for gonadal maturation, in which the highest nocturnal melatonin levels regulate the gonadotropin-releasing hormone (GnRH), exerting positive feedback on the release of the luteinizing hormone (LH), responsible for male sperm production and female ovarian activity (Cebrian-Perez et al., 2014; Chaube et al., 2020). Literature also describes similar effects in fish species, suggesting an important role of melatonin in oocyte maturation and showing some antioxidant protection role (Maitra and Hasan, 2016). Moreover, in some species, like major carp (*Catla catla*) it influences the annual testicular maturation through the regulation of testosterone (T) production (Bhattacharya et al., 2007), and in Atlantic salmon (*Salmo salar*) it has an important role in the expression of pituitary melatonin receptors at the beginning of the reproductive season (Ciani et al., 2019). However, in temperate regions, where photoperiod defines most of the species' reproductive strategy, melatonin does not influence all animals in the same way. In summer breeders, known as long-day spawners, reproduction is triggered by increasing photoperiod, whereas in short-day breeders, longer nights stimulate the reproductive process (Falcón et al., 2007). Nonetheless, contradictory data have been reported in studies focusing on the effect of melatonin in fish reproduction, mainly due to differences in the species, age of animals, sex, light regime, time of the year, reproductive stage and experimental conditions (Falcón et al., 2010).

In teleosts, melatonin is mainly produced during the night by the pineal organ, but other extra-pineal tissues have been identified as melatonin production sites, such as the retina, brain, liver, heart and gut (Cahill, 1996; Falcón et al., 2010). However, there is no clear information regarding fish gonadal melatonin production. In mammals, some studies showed the important role exerted by melatonin on seminal plasma (Awad et al., 2006; Casao et al., 2010; Cebrian-Perez et al., 2014), but this topic remained unexplored in fish. Fish seminal plasma has a specific composition that confers the main antioxidant defense for spermatozoa (Cabrita et al., 2011a), through the presence of some antioxidant components responsible for free radicals scavenging (Jacobsen, 2019) but it is still unknown if melatonin is one of those components. Nonetheless, melatonin has been tested as an antioxidant supplement in some fish sperm cryopreservation procedures, such in paddlefish (*Polyodon spathula*), curimba (*Prochilodus lineatus*), and piracanjuba (*Brycon orbignyanus*), as previously reviewed by our group (Félix et al., 2021), but results are very divergent, in part due to a lack of standardized protocols, but also due to a species-specific melatonin effect.

In aquaculture, counteracting the oxidative stress induced by procedures and protocols applied in breeding management is important for the overall gamete quality, which ultimately impacts the reproductive performance of a species (Félix et al., 2021). In fact, some cultured species, such as the long-day spawner Senegalese sole (*Solea senegalensis*), still present reproductive constraints. In this species, the males born and raised in captivity produce low sperm quantity and quality when compared with wild breeders (Cabrita et al., 2006). Contrarily, the short-day spawners European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) do have optimized conditions for reproduction in captivity, being two of the main cultured finfish species in Europe (FAO, 2022). Based on the above-mentioned lack of knowledge, and on the fact that melatonin is a potent antioxidant, this work aimed to determine, for the first time, the presence of melatonin in fish seminal plasma and, at the same time, understand its putative role in fish reproduction and spermatozoa antioxidant capacity. Furthermore, through the comparative and integrative data analysis from three important aquaculture species (European seabass, gilthead seabream, and Senegalese sole), this study contributes to a better comprehension of melatonin role in each species reproductive strategy which, ultimately, can be used to overcome some reproductive bottlenecks in captivity and contribute to the sustainability of aquaculture.

2. Materials and methods

2.1. Ethic statement

All experimental procedures were conducted in accordance with ARRIVE guidelines, with directives 86/609/EU and 2010/63/EU of the European Parliament and Council, and Portuguese legislation for the use of laboratory animals (PORT 1005/92) of the Portuguese direction for veterinary and food services (Direção Geral de Alimentação e Veterinária, DGAV). Moreover, both infrastructures of CCMAR and IPMA are certified to house and conduct experiments with live animals, as well as all technicians and researchers involved in animal handling and sampling hold a FELASA B and C category certification, approved by DGAV. Finally, the authorization for experimental procedures with germ cells were previously approved by DGAV (ref. 003289). It is hereby certified that all animals involved in the present study were reared according to the best practices.

2.2. Broodstock management

For this experiment, different broodstocks of European seabass, gilthead seabream, and Senegalese sole were used. A broodstock of 18 European seabass males, with 2.5 years and a mean body weight (BW) of 512 ± 94 g, were sampled *in loco* at the semi-intensive aquaculture farm Aqualvor (37.13985, −8.62403 - Lagos, Portugal). These fish were maintained outdoors in an earthen pond of 12.000 m^3 (100 m length, 40 m width, 3 m height), under natural conditions of photoperiod and temperature. The gilthead seabream broodstock used in this study was acquired from Aqualvor fish farm and maintained at Ramalhete experimental station at the University of Algarve (37.00658, −7.96731 - Faro, Portugal). After quarantine and acclimatization, the 2.5 years old broodstock (547 ± 59 g BW) of 22 fish was tagged, for individual identification, and kept outdoors in two fiber-glass tanks of 3.27 m^3 (Ø2.04 m, 1.0 m height) on an open system under natural environmental conditions. At the time of the experiment (January), the photoperiod was of 10 h: 14 h light:dark (LD), and water temperature 13 ± 0.6 °C for both species. Regarding Senegalese sole, two different groups of breeders were used. The first was an established stock of 6 years old F1 breeders (1014 ± 221 g BW), divided in 6 fiber-glass tanks of 5.89 m^3 (Ø2.5 m, 1.2 height), each one with 18 animals, at Ramalhete station; while the second, established at IPMA facilities (37.03357, −7.82012 - Olhão, Portugal), consisted of 6–8 years old wild individuals (1815 ± 256 g BW) divided in 2 tanks of 18 m^3 (4.30 m length, 3.63 m width, 1.20 m height), each one with 12 animals. Both research facilities maintained the fish on a semi-closed system with a 2:1 sex ratio (male: female), necessary for reproduction in this species, and under the same photoperiod and temperature oscillations. During the experiment, photoperiod naturally oscillated between 12 L:12D (March) to 14 L:10D (June), and water temperature ranged from 14.6 to 20.4 °C. In all species, males and females were maintained together but only males were sampled for blood and sperm collection.

2.3. Experimental design

The main objective of this study was to determine the presence of melatonin in seminal plasma from different teleost species, and further determine its putative effect in reproduction and spermatozoa antioxidant capacity. For that purpose, all animals were sampled during their reproductive season: European seabass and gilthead seabream in winter (January), and both F1 and wild Senegalese sole in spring (from March to June). All species were sampled for blood and sperm at two different times of the day, mid-light (ML) and mid-dark (MD), to determine the daily fluctuations in the parameters analyzed. To avoid light contamination, MD samplings were performed on new-moon nights, using a red

head-torch and covering the fish head with aluminum foil, so the retina and pineal organ could not detect any scattering light. European seabass and gilthead seabream males were previously anesthetized with 200 ppm phenoxyethanol, and Senegalese sole males with 300 ppm phenoxyethanol. After anesthesia, the collection of biological samples was performed similarly for the three species, with small adjustments. The genital pore was cleaned with PBS to avoid any contamination from mucus, anesthesia or urine, and sperm samples were collected by abdominal massage using a sterile syringe for European seabass and gilthead seabream, and a 10 μL pipette for Senegalese sole. Blood was collected from the caudal vein using heparinized syringes, and both biological samples (blood and sperm) were kept at 4 °C in a styrofoam box until centrifugation. Blood samples were centrifuged at 3000*g* for 15 min at 4 °C, and supernatant plasma was collected and kept individually. The number of blood samples collected at each sampling point (ML and MD) were: $n = 11$ (European seabass); $n = 7$ (gilthead seabream); $n = 14$ (Senegalese sole F1 males) and $n = 9$ at ML and $n = 10$ at MD (Senegalese sole wild males). Sperm samples were centrifuged as follows: 11000 *g*, 20 min, 10 °C (European seabass); 6000 g, 15 min, 10 °C (gilthead seabream) and 1200 g, 15 min, 10 °C (Senegalese sole). The supernatant plasmas were kept individually for European seabass (ML: $n = 18$, MD: n = 14) and gilthead seabream (n = 9 each point), and 12 μL of each sample were aliquoted separately for TAS and sex steroid analysis. Due to the low volume of sperm obtained in Senegalese sole, plasma samples were pooled using 4–6 males, in order to obtain the 100 μL volume necessary for the RIA analysis (F1 males had n = 9 pools at each point; and wild males had $n = 6$ pools at ML, and n = 7 at MD). In this last species, a second sampling was performed to collect seminal plasma for TAS and sex steroids analysis. All samples were stored at −80 °C until further analysis. Melatonin concentration was determined by RIA in seminal and blood plasma and compared between day (ML) and night (MD) periods. In order to understand the role of seminal plasma melatonin in fish reproductive physiology, total antioxidant status (TAS), testosterone (T) and 11-ketotestosterone (11KT) were determined in seminal plasma samples.

2.4. Melatonin determination

To evaluate and quantify the presence of melatonin in both blood and seminal plasma samples, a RIA assay was performed according to the manufacturer protocol for the Melatonin direct Serum/Plasma/Saliva RIA kit (RE 29301, IBL International, Germany). The melatonin detection on fish blood plasma by RIA was previously optimized by our team (Vera et al., 2007; Lopez-Olmeda et al., 2009a, 2009b) but minor adjustments had to be done to adapt the protocol for seminal plasma: the technique was performed using half of volumes and, on the second day, the supernatants were aspirated manually with a Pasteur pipette to avoid losses of the precipitated pellet. In order to validate the method for this type of sample, a known amount of melatonin was added to pooled seminal plasma samples and serial dilutions were made. Then, after measurement of radioactivity in a ray-gamma counter (WALLAC 1470 Automatic Gamma Counter, Perkin Elmer, Waltham, Massachusetts, USA) for 1 min, the parallelism between the seminal plasma serial dilutions and the standard curve was checked. The lower limit of quantification (LLOQ) of the kit was 0.9 pg/mL, the intra-assay coefficient of variation (CV) was 3.9–6.9% and inter-assay CV was 6.2–16.0%.

2.5. Total Antioxidant Status (TAS)

The seminal plasma antioxidant status was determined using the commercial kit TAS (REF 2332) from Randox Laboratories Limited (United Kingdom), following the protocol indicated by the manufacturer and adapted by Martínez-Páramo et al. (2013) for fish seminal plasma. For the analysis, 2 μL of each sample reacted with 100 μL of chromogen (R2) from the kit, and the absorbance was immediately read at 600 nm at 37 °C. Then, 20 μL of substrate (R3) was added and incubated for 3 min, after which the absorbance was read at the same wavelength. A control sample (TAS control, REF 2331, Randox Laboratories Ltd., United Kingdom) was added to ensure the functionality of the kit. After calculations, results were expressed as mmol/L. A total number of 7–13 individual samples were used per species at each time of the day (ML/MD).

2.6. Sex steroids determination

In this study, the circulating levels of T and 11KT were determined in seminal plasma samples, for each species, by an enzyme-linked immunosorbent assay (ELISA). Following the manufacturer instructions, the assay was performed using the respective commercial ELISA kits from Cayman Chemicals (Ann Arbor, Michigan, United Stated), previously applied by Oliveira et al. (2020) to Senegalese sole blood plasma samples. For each hormone measured, a total of 6–10 individual samples were analyzed per time point and species.

2.7. Statistical analysis

Statistical analyses were performed using SPSS (IBM) software, and graphical plotting was done with GraphPad prism 9. After removing the statistical identified outliers (extreme outlier was considered when sample was on 1st or 3rd quartile ±3 x interquartile range), data that assumed the principles of normality and homogeneity of variance (Shapiro-Wilk and Levene tests, respectively) were analyzed by General Linear Model (GLM) univariate with two factors with Bonferroni correction, or student *t*-test to identify statistical differences between groups. For data that did not assume the above-mentioned principles, the Mann-Whitney or Wilcoxon nonparametric tests were applied for independent and dependent samples, respectively. Significant differences were assumed when $p < 0.05$. Finally, in order to ascertain possible correlations between the different parameters under study, the Pearson's analysis was applied. In the case of gilthead seabream, since day/night samples were dependent, it was possible to correlate more data than in other species.

3. Results

3.1. Melatonin levels in blood and seminal plasma

3.1.1. Assay validation for seminal plasma

Seminal plasma samples from European seabass and gilthead seabream were used to validate the RIA assay for this type of sample. The high amount of volume necessary for the analysis, prevented us from using Senegalese sole samples too, due to the low sperm volume produced by this species. Parallelism between seminal plasma samples and standard curve was observed in both recovery tests (supplementary Fig. S1), validating the usage of RIA to quantify melatonin in fish seminal plasma samples.

3.1.2. Melatonin levels in different fish species

In European seabass, blood plasma melatonin levels were 4 times higher at MD (364 pg/mL) than at ML (91 pg/mL) (Student *t*-test, $p < 0.05$) (Fig. 1A), whereas in seminal plasma no significant differences were found between day and night (Fig. 1B) since melatonin was not detected at all at MD and was only detected in one sample at ML (concentration ranged from 0 to 10 pg/mL).

Regarding gilthead seabream, RIA analysis revealed that blood plasma had significantly higher levels of melatonin at MD (808 pg/mL) than at ML (198 pg/mL) (paired Student *t*-test, $p < 0.05$) (Fig. 1C). Melatonin was not detected in any seminal sample collected at ML (Fig. 1D), and the average of melatonin detected at MD was approximately 21 pg/mL (supplementary Table S1). Since the analysis was performed at both sampling times (ML and MD) using the same individuals, it was possible to determine the day/night differences in

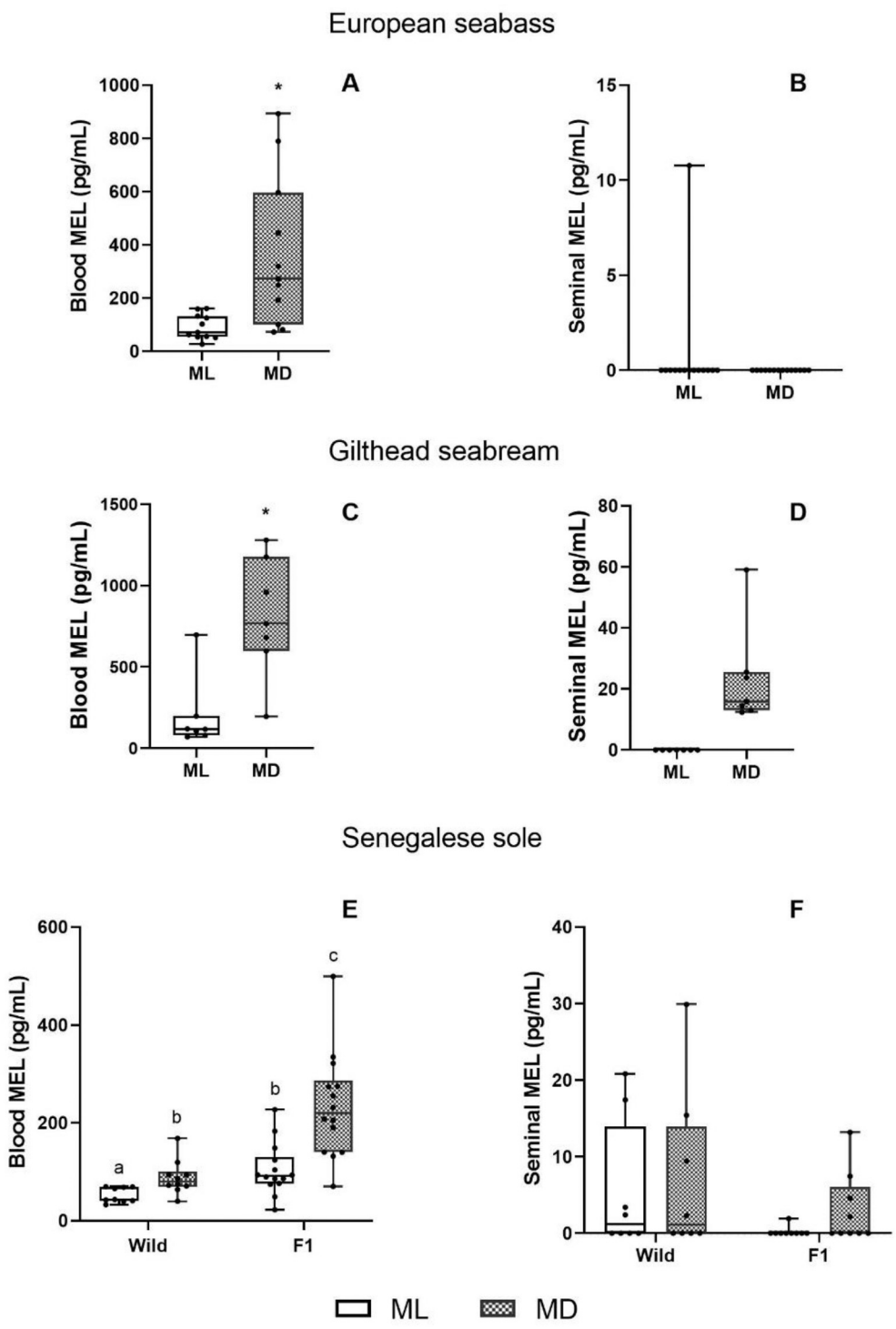

Fig. 1. Melatonin (MEL) concentration (pg/mL) in blood and seminal samples of European seabass (A: blood n = 11, B: seminal n = 14), gilthead seabream (C: blood n = 7, D: seminal n = 7), and wild (blood n = 9 ML, n = 10 MD; seminal n = 8) and F1 (blood n = 14, seminal n = 9) Senegalese sole (E, F) collected during breeding season at mid-light (ML) and mid-dark (MD) moments. Data are expressed as mean ± SE and the results are represented by interleaved boxes and whiskers with individual points representing the variance of the data. Student t-test (A) and paired t-test (C) significant differences between ML and MD are identified with an asterisk (*), GLM univariate with two fixed factors, with Bonferroni correction (E, F) significant differences are identified with different letters. Statistical differences were considered when $p < 0.05$.

melatonin levels for each male. In blood plasma samples, high inter-individual variability was observed for melatonin levels at both ML (concentrations ranged from 54 pg/mL to 697 pg/mL) and MD (concentrations between 196 and 1279 pg/mL).

In Senegalese sole blood samples, a significant interaction (p = 0.048) between the variables broodstock and time-of-day (GLM univariate with Bonferroni correction, $p < 0.05$) was found. In addition, the main effect time-of-day was found to be significant for melatonin concentrations in blood plasma (GLM univariate with Bonferroni correction, $p < 0.05$), which were significantly higher at MD than at ML for both wild (88 and 52 pg/mL) and F1 (248 and 104 pg/mL) males. Also, a significant main effect of broodstock was observed: at both ML and MD melatonin levels were significantly higher in F1 males than in wild ones (Fig. 1E). Contrarily, in seminal plasma, no significant differences (Kruskal Wallis, $p < 0.05$) were observed neither between time points (p = 0.279), nor between wild and F1 males (p = 0.097), despite the observed tendency for higher concentration of melatonin at night in seminal plasma, in both groups of males (Fig. 1F). It is worth to mention that melatonin was not detected in a considerable number of samples. Specifically, it was only possible to find it in 50% (ML) and 56% (MD) of wild seminal plasma samples, and less results were obtained with F1 males, with only 11% (ML) and 44% (MD) of samples revealing

melatonin content. Moreover, variability was high, as it is represented in Fig. 1F. The overall results of melatonin determination in fish seminal plasma are summarized in supplementary Table S1 (supplementary material), for better comparison among species.

3.2. Total Antioxidant Status (TAS)

TAS analysis in seminal plasma revealed significant differences between fish species ($p = 0.000$) (GLM univariate, followed by Tukey test $p < 0.05$). According to the results, the antioxidant status of the European seabass seminal plasma is significantly lower than the antioxidant status of the other species, independently of the time of the day (Fig. 2). Moreover, significant differences (Student *t*-test, $p < 0.05$) were observed between sampling times (ML and MD) in Senegalese sole. Both wild and F1 males presented higher TAS at MD (1.23 and 1.21 mmol/mL) than at ML (1.07 and 1.01 mmol/mL).

3.3. Sex steroids analysis

The determination of T and 11KT in seminal plasma from the different fish species revealed significant day/night differences only in Senegalese sole samples (Fig. 3). At MD, 11KT levels were significantly higher in wild Senegalese sole (2.53 ng/mL) whereas T was higher in F1 males (1.92 ng/mL) (*t*-Student, $p < 0.05$). European seabass and gilthead seabream presented higher levels of both T and 11KT at ML, although differences were not statistically significant.

3.4. Correlation between factors in blood and seminal plasma

The results from Pearson's correlation in European seabass samples only had relevance at ML, since no correlations were found between parameters at MD. The statistical analysis revealed that during the day the melatonin concentration on blood plasma was positively correlated with the antioxidant status of seminal plasma (Table 1). Moreover, the levels of T and 11KT were also correlated between them.

In gilthead seabream dependent samples, Pearson's analysis revealed that melatonin concentration found in blood plasma was not correlated with seminal plasma levels at MD, which was easily visualized on the different black bars showed on Fig. 3. However, the MD melatonin concentration in seminal plasma was positively correlated with the antioxidant status of these samples (Table 2). Also, T levels at MD were positively correlated with antioxidant status of seminal plasma at ML.

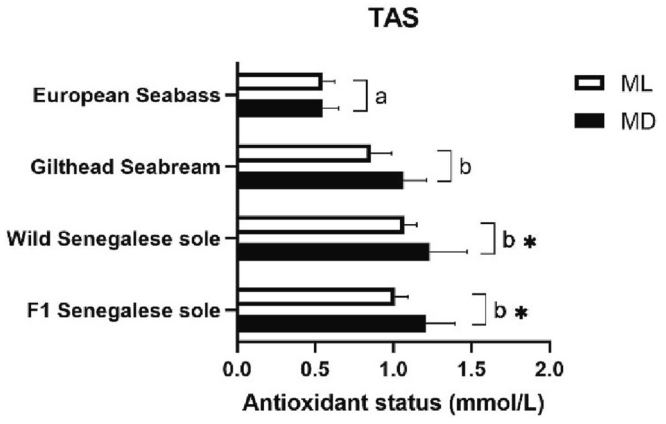

Fig. 2. Total Antioxidant Status of seminal plasma from European seabass (n = 9–13), gilthead seabream (n = 6), and Senegalese sole F1 ($n = 7–8$) and wild ($n = 8–9$) males. White bars refer to the mid-light (ML) sampling point and black bars to the mid-dark (MD). GLM univariate, followed by *post-hoc* Tukey test, significant differences between species are identified with different letters. Student *t*-test differences within the same species between ML and MD are identified with an asterisk (*) ($p < 0.05$).

Finally, at ML, the levels of both T and 11-KT were correlated, whereas T and melatonin values were positively correlated in blood plasma.

In Senegalese sole F1 males, seminal plasma T and antioxidant status were positively correlated at MD (Table 3), whereas no correlations were found at ML. In wild males, both androgens (T and 11KT) were strongly correlated at both times of the day (ML and MD) (Table 4). Moreover, at MD, seminal T was correlated with seminal antioxidant status.

It was not possible to perform the correlation analysis between blood and seminal plasma samples used to determine melatonin concentration in sole, since the latest samples were pooled at each sampling time.

4. Discussion

Fish chronobiology studies have been focusing on the comprehension of melatonin role in diverse fish systems, aiming to discover new production sources. In this study we aimed to understand the importance of melatonin in fish reproductive system, and more specifically to determine its presence in fish seminal plasma, its putative roles, and ultimately if it impacts the antioxidant status of spermatozoa. Our comparative study in gilthead seabream, European seabass, and Senegalese sole enlightened important aspects regarding the role of melatonin in marine fish species with different reproductive strategies. The three aquaculture-relevant species analyzed showed higher levels of circulating melatonin in blood plasma at night rather than during the day, which is in accordance with previous results obtained in European seabass (Sánchez-Vázquez et al., 1997), gilthead seabream (Molina-Borja et al., 1996), and Senegalese sole (Vera et al., 2007). Interestingly, melatonin measurement in fish seminal plasma revealed that melatonin concentration in this fluid not only varies between day and night, but also its presence in seminal plasma can be species-specific.

Melatonin has been reported to have an inhibitory effect on the expression of different GnRH receptor subtypes, which ultimately impacts seasonal biological functions like reproduction (Servili et al., 2013). Alvarado et al. (2015) suggested that the impact of melatonin in brain and gonadal development in fish could be species-specific, since European seabass spermatogenesis was negatively affected by melatonin supplementation. In their study, melatonin was administered to European seabass *via* abdominal implants, which inhibited the expression of kisspeptin and GnRH, reduced the levels of circulating androgens (T and 11KT), decreased gonadal growth and, consequently, affected spermatogenesis. In our research, the observed levels of T and 11KT in European seabass seminal plasma correlated with each other and were similar to those reported in blood plasma during the reproductive season (Bayarri et al., 2004). However, in this species, melatonin was only detected in one seminal plasma sample taken during the day and none at night, whereas the concentration of both androgens did not differ between day and night. As described by Sanchez-Vázquez et al. (1995), European seabass is a dual species, which means that its locomotor activity levels peak at night during the spawning season (winter), but during the resting season this pattern shifts and fish become diurnal, showing higher activity levels during the day. Altogether, and since European seabass does not have gonadal maturation or spermiation problems in captivity, our results suggest that in this species melatonin production may be more related with the selected environmental conditions for reproduction (longer periods of darkness), than with gonadogenesis, as advanced by other authors (Alvarado et al., 2015), or gamete antioxidant status according to our data (absence of melatonin in seminal plasma and low TAS in the fluid). In fact, circulating melatonin seems to be more important for the antioxidant capacity of sperm since a positive correlation was found between both variables and no melatonin was detected locally. In other words, for European seabass, melatonin may be mainly produced by the pineal organ in response to the environmental cues and released into the bloodstream, reaching the fish testis, where it would exert its antioxidant properties through an already described paracrine mechanism (Tan et al., 2003), and

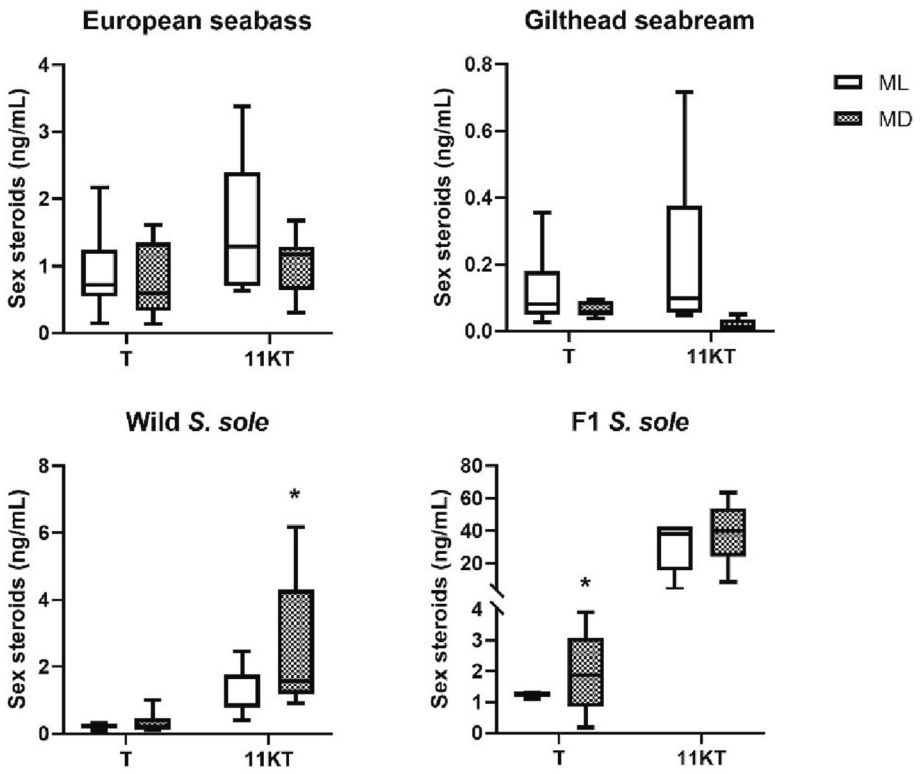

Fig. 3. Circulating levels of testosterone (T) and 11-ketotestosterone (11KT) in European seabass (n = 9–10), gilthead seabream ($n = 6$) and Senegalese sole F1 (n = 7–8) and wild (n = 6–7) males. Data were analyzed by Student *t*-test, except for gilthead seabream T data (Wilcoxon test) and European seabass 11KT data (Mann-Whitney test). Results are represented by interleaved box and whiskers, and significant differences between ML and MD are indicated with an asterisk (*) ($p < 0.05$).

Table 1
Pearson's correlations between blood and seminal melatonin concentrations, seminal antioxidant status and seminal steroids concentration in European seabass breeders at ML ($n = 10$). Melatonin (MEL), Total Antioxidant Status (TAS), testosterone (T), 11-ketotestosterone (11KT). Significant correlations are identified with asterisks (* when $p < 0.05$ and ** when $p < 0.001$).

	Blood MEL	Seminal MEL	TAS	T	11KT
Blood MEL	1				
Seminal MEL	−0.208	1			
TAS	**0.670***	−0.152	1		
T	0.090	n/a	0.111	1	
11KT	0.144	n/a	−0.111	**0.808****	1

compensating the absence of gonadal melatonin.

Both European seabass and gilthead seabream are winter spawner species. However, seabass reproduction is triggered by lower temperatures, while gilthead seabream responds to shortening photoperiod (Carrillo et al., 1993; Bromage, 2001). In addition, both species differ in their reproductive rhythms and spawn at species-specific moments of the day. Thus, while European seabass normally spawn in the middle of the dark period (Villamizar et al., 2012), gilthead seabream have a clear spawning peak just before darkness onset (Meseguer et al., 2008). The seminal plasma melatonin results of this study also demonstrate differences between both species. In the present study, melatonin in seminal

Table 3
Pearson's correlations between seminal antioxidant status and seminal steroids concentrations of F1 Senegalese sole males at MD ($n = 9$). Total Antioxidant Status (TAS), testosterone (T), 11-ketotestosterone (11KT). Significant correlations are identified with asterisks (** when $p < 0.001$).

	TAS	T	11KT
TAS	1		
T	**0.842****	1	
11KT	−0.257	0.016	1

Table 2
Pearson's correlations between blood and seminal melatonin concentrations, seminal antioxidant status and seminal steroids concentration in gilthead seabream samples collected at ML and MD ($n = 5–6$). Melatonin (MEL), Total Antioxidant Status (TAS), testosterone (T), 11-ketotestosterone (11KT). Significant correlations are identified with asterisks (* when $p < 0.05$ and ** when $p < 0.001$).

	Blood MEL ML	Blood MEL MD	Seminal MEL ML	Seminal MEL MD	TAS ML	TAS MD	T ML	T MD	11KT ML	11KT MD
Blood MEL ML	1									
Blood MEL MD	0.794	1								
Seminal MEL ML	n/a	n/a	n/a							
Seminal MEL MD	−0.103	−0.021	n/a	1						
TAS ML	−0.547	−0.711	n/a	0.629	1					
TAS MD	−0.194	0.046	n/a	**0.890***	0.604	1				
T ML	**0.893***	0.549	n/a	−0.478	−0.611	−0.585	1			
T MD	−0.494	−0.721	n/a	0.407	**0.949***	0.470	−0.476	1		
11KT ML	0.831	0.411	n/a	−0.447	−0.532	−0.642	**0.976****	−0.428	1	
11KT MD	−0.108	−0.021	n/a	−0.403	−0.543	−0.639	0.074	−0.663	0.177	1

Table 4
- Pearson's correlations between seminal antioxidant status and seminal steroids concentration of wild Senegalese sole males, at ML and MD (n = 7–9). Total Antioxidant Status (TAS), testosterone (T), 11-ketotestosterone (11KT). Significant correlations are identified with asterisks (* when $p < 0.05$ and ** when $p < 0.001$).

		TAS	T	11KT
ML	TAS	1		
	T	0.227	1	
	11KT	0.042	**0.915****	1
MD	TAS	1		
	T	**0.713***	1	
	11KT	0.571	**0.999****	1

plasma was detected only at night for gilthead seabream and was not correlated with blood levels. This result suggests that seminal plasma melatonin may not be up taken from the bloodstream but would be rather synthetized locally by the gonads. Similar results were obtained in a study by Cuesta et al. (2008), in which the highest positive effects of injected melatonin on the immune system of gilthead seabream did not correlate with melatonin levels in the bloodstream. Melatonin synthesis by gonads itself has been under study by some authors, both in mammals and fish (Hasan et al., 2014; Gonzalez-Arto et al., 2016a), and despite the most recent findings focused on melatonin receptors in these organs in rams (Gonzalez-Arto et al., 2016b) and deer (Koziol et al., 2020), it is still unclear if there is a local autocrine or paracrine mechanism of action. To date, the information on this topic is even more scarce in fish, and melatonin receptors have been detected only in ovaries of the cyprinid *Catla catla* (Chattoraj et al., 2009) and, more recently, in both female and male gonads of turbot (*Scophthalmus maximus*) (Zhao et al., 2021a). Also, some attempts were made in European seabass testis, but there was no expression of the genes coding for melatonin receptors *mt1* and *mel1c*, and the expression of the receptor *mt2* was not significant when compared to brain regions and other organs (Sauzet et al., 2008). These findings are in accordance with our results and corroborate the hypothesis that the absence of melatonin in the seminal plasma of this species could be related with a total absence of melatonin in the gonads (whether up taken from the bloodstream or produced locally). With the newest technology, the comparative study of melatonin receptors in the gonads of all the three species analyzed in the present study would be of a great interest to further explore this theory.

As mentioned previously, melatonin was only detected in gilthead seabream seminal plasma at MD, with levels strongly correlating with seminal plasma antioxidant status, which agrees with the known antioxidant properties of melatonin (Manchester et al., 2015; Mironczuk-Chodakowska et al., 2018). This protective effect of melatonin was recently described in human spermatozoa exposed to oxidative stress (Zhao et al., 2021b), and in an *in vitro* assay with bovine oocytes, in which melatonin delivered through nanocapsules decreased the ROS, not only of oocytes, but also of embryos (Remiao et al., 2016). In fish, a similar effect was observed by Hasan et al. (2014) in carp ovaries. In their study, they detected a significant negative correlation between levels of melatonin and malondialdehyde (MDA), a specific marker of intracellular oxidative stress. The results from Pearson's correlation in gilthead seabream also pointed out that testosterone may contribute to the antioxidant status of seminal plasma. However, the results indicated that seminal plasma T at MD was positively correlated to TAS at ML, which need further studies to comprehend the reasons behind such delay of action. The interaction between the endocrine and antioxidant system is complex and poorly studied in fish, and it can also be species-specific. Indeed, a study by Hoogenboom et al. (2012) indicated that female brown trout (*Salmo trutta*) with higher levels of T in the months prior to spawning, presented higher reactive oxygen metabolites at spawning, suggesting that these fish were more exposed to oxidative damage. However, in mammals, positive interactions between T and the antioxidant system have been found in humans (Mancini et al., 2008) and ram (Casao et al., 2010). Furthermore, both positive and negative correlations between melatonin and sex hormones have been reported in the literature for different fish species. Previous research in Senegalese sole described an impact of moon phases on both melatonin production and plasma levels of T and 11KT (Oliveira et al., 2010). In two different carp species, Indian major carp (*Labeo rohita*) and European major carp (*Cyprinus carpio*), Chattoraj et al. (2005) demonstrated that melatonin accelerates the action of maturing inducing hormone (MIH) on carp oocyte maturation. Nevertheless, opposite effects have been also described. For example, Sebert et al. (2008) observed that melatonin implants provoked a higher expression of tyrosine hydroxylase (TH) in various female eel (*Anguilla anguilla*) brain regions and significantly decreased the expression of *lhβ* and *fshβ*, as well as 11KT plasma levels, negatively affecting the reproductive performance of this species. In our study, circulating melatonin levels correlated with T concentration in seminal plasma for gilthead seabream, supporting the hypothesis pointing to a role of melatonin produced by the pineal organ in the regulation of the reproductive function in this species.

Among all the species analyzed, Senegalese sole is the one that still presents some reproductive bottlenecks in captivity, that need to be overcome by the aquaculture industry. In this study, interesting results were obtained when comparing wild and F1 males from this species. In blood, both groups presented higher concentrations of melatonin at MD (night) than at ML (day), although F1 males showed 2 to 2.5 times more blood melatonin than wild males, at both times of the day. However, the opposite trend was found in seminal plasma samples, in which melatonin concentration was 5 and 2 times higher in wild males at ML and MD, respectively, than in F1 males. Recently, similar results were obtained for basal cortisol levels in both groups of Senegalese sole breeders, being the wild males the ones displaying higher levels during the day, whereas F1 males did not present daily changes in either cortisol concentration or locomotor activity, when kept in aquaculture conditions (Figueiredo et al., 2020). In the present study, significant differences were found in the androgen levels of seminal plasma from wild and F1 fish, although these differences were hormone-dependent: wild individuals presented higher 11KT levels, while F1s displayed higher T levels. In addition, extremely high 11KT levels were found in F1 seminal plasma, which seems to be in accordance with the high values normally found in the bloodstream (Cabrita et al., 2011b; Chauvigne et al., 2016). However, this disparity may be compromising some reproductive traits in F1 males, since it was possible to observe a very clear unbalance between F1 and wild males regarding sex steroids. While in wild males T and 11KT levels highly correlated in seminal plasma at both times of the day, no correlation was found between those androgens in F1 males. The differences observed between sole groups, in terms of seminal plasma melatonin and sex steroid hormones, together with the higher variability found, may be an output of the imbalance in the hypothalamus-pituitary-gonadal axis already described for this species (Garcia-Lopez et al., 2006; Morais et al., 2016). Also, the circadian axis has been previously suggested to be impaired in F1 individuals of this species (Figueiredo et al., 2020).

TAS analysis in seminal plasma of Senegalese sole showed similar levels in both wild and F1 males, being statistically higher during the night for both groups. This result suggests that the antioxidant capacity of seminal plasma of F1 males is not compromised and does not seem to be the cause for the low sperm quality of these individuals, as previously hypothesized. In fact, Valcarce and Robles (2016) suggested that the spermatozoa susceptibility to oxidative stress in this species was high regardless its broodstock origin (wild or F1). Curiously, in both broodstocks we observed a relationship between seminal plasma testosterone and antioxidant status during the night, similar to what was also observed in gilthead seabream (but with different moments of the day). In a study with ram, Casao et al. (2010) reported that melatonin and T in seminal plasma were correlated and impacted sperm quality and antioxidant enzyme activity, which ultimately, affected the reproductive performance of rams. Unfortunately, in our study, for the determination

of melatonin in seminal plasma we had to work with pooled samples, due to the small volume of sperm obtained from each Senegalese sole male, which made impossible to perform correlations between levels of melatonin, T, and antioxidant status in this species. Nonetheless, the fact that we found exactly the opposite tendency between F1 and wild fish in terms of seminal plasma melatonin and sex steroids concentration, and that F1 males were missing the important interaction between T and 11KT at both times of the day, led us to believe that the reproductive dysfunction of Senegalese sole F1 males (low sperm volume, lower motility and less concentration) (Cabrita et al., 2006) may be related to an impairment in this brain-pituitary-gonad axis, wherein melatonin plays a pivotal role (Falcón et al., 2007; Migaud et al., 2010; Takahashi and Ogiwara, 2021), rather than being due to low sperm antioxidant capacity of these individuals.

A holistic interpretation of the present data also allowed the identification of physiological differences between species. Regarding seminal plasma melatonin, its presence or absence seems to be related with different reproductive strategies adopted by the species, as mentioned before for European seabass, which did not present seminal plasma melatonin. Moreover, European seabass was the species showing lower TAS levels in seminal plasma samples, which may explain the high susceptibility of this species sperm to oxidative stress (Martinez-Paramo et al., 2012). Differently, in gilthead seabream and Senegalese sole, species in which melatonin was detected in seminal plasma, they presented higher TAS and it would be of interest to investigate the possibility of melatonin production by the gonads itself, or the existence of a balance between blood and gonadal melatonin levels. Furthermore, the determination of T and 11KT in seminal samples showed different day/night patterns in the three species, which might point to differences in the circadian regulation of reproduction. Thus, the dual species European seabass and the diurnal gilthead seabream presented higher levels of androgens during the day, when T and 11KT concentrations correlated. However, in Senegalese sole, a nocturnal species, both sex steroids were higher during the night, with levels correlating only in wild males.

5. Conclusions

In face of the results from this study, it is possible to infer that melatonin levels in fish seminal plasma present day/night oscillations, at least in those species in which melatonin was successfully detected (gilthead seabream and Senegalese sole). Furthermore, we observed that melatonin levels in seminal plasma were species-specific. However, the origin of the seminal plasma melatonin remains to be determined, since no correlation was found with the circulating blood levels in gilthead seabream. The presence of melatonin in seminal plasma showed a positive influence on the antioxidant status, which suggests a melatonin role in fish reproductive antioxidant system. A great step forward was taken to understand Senegalese sole reproductive dysfunction by determining similar TAS levels in both F1 and wild males. However, in F1 males a lack of correlation between T and 11KT androgens was found during the day and at night, which may be crucial for the proper reproductive performance of the species. To conclude, this study contributed to the fundamental knowledge on melatonin distribution in fish reproductive system, and its relationship with sex steroids levels and antioxidant capacity of seminal plasma, encouraging further studies in this area. More investigation is needed to determine whether melatonin is produced by fish gonads, if this organ has melatonin receptors, as well as the seasonal variations of this hormone in seminal plasma.

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CRediT authorship contribution statement

Francisca Félix: Methodology, Investigation, Validation, Data curation, Formal analysis, Writing – original draft, Visualization. **Victor Gallego:** Investigation, Writing – review & editing. **Ana Mendes:** Investigation, Resources. **Florbela Soares:** Investigation, Resources. **Luisa M. Vera:** Methodology, Validation, Formal analysis, Writing – review & editing. **Elsa Cabrita:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Project administration, Funding acquisition. **Catarina C.V. Oliveira:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

Author's declaration indicates no conflict of interest for this manuscript.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739578.

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