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Sex Determination, Evolution of Gonadal Stage in Females, and Seasonal Evolution of Sperm Production in *Chelon labrosus* (Risso, 1827)

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Abstract: *Chelon labrosus* represents a promising species for current aquaculture to lean towards a more sustainable model. The control of reproduction in captivity is essential to develop the industrial production of new species. This work adds to the knowledge of the reproductive aspects of males and females of this species. We aim to (1) develop a methodology for sex identification of reproductive and prereproductive specimens of the studied species based on the plasma levels of steroid hormones—estradiol (E2) and 11-ketotestosterone (11-KT); (2) determine the maturity evolution of the females throughout the spawning season by gonadal biopsy; and (3) describe sperm quality throughout the breeding season (volume, motility, density). The results show that an 11-KT/E2 ratio > 0.4 is 100% effective in distinguishing males from females, provided that individuals present a size greater than that of first sexual maturity. A double trend was observed in the development of oocytes, with an increase in the initial diameter until the middle of the spawning season, followed by a divergence of the group, with some females continuing the maturation process and others going into regression. Sperm data show stable quality at the beginning and in the middle of the spawning season and a significant loss of quality at the end of the reproductive period. The present study also shows that, between the beginning and the middle of the reproductive season, males of this species can recover sperm volume removed by stripping.

Keywords: reproduction; *Chelon labrosus*; aquaculture; gonadal biopsy; sex hormones; sperm quality

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1. Introduction

A diversification of aquaculture production can improve the sustainability of this industry worldwide [1]. Diversification involves not only the farming of new species, but also new techniques or technologies applied to the farming of established species, farming in new areas, or even new presentation formats of established aquaculture products [2]. One of the factors driving the diversification of aquaculture is climate change [3]. In the current global warming scenario, fish farming needs to focus either on species able to adapt and grow successfully under a wide range of changing environmental conditions or on the relocation of farming areas to more environmentally friendly locations. Candidate fish species for diversification of aquaculture production at the EU level include several species belonging to the *Mugilidae* family. This family is widespread throughout the world and tolerates a wide range of temperatures and salinities in addition to omnivorous/herbivorous feeding habits, contributing to the sustainability of the current European aquaculture model by rendering it less dependent on animal ingredients. *Chelon labrosus* (*C. labrosus*; Risso,

1827) presents a remarkable osmoregulatory capacity [4]; an ability to thrive in freshwater, brackish, and marine environments; and trophic plasticity [5]. Furthermore, this species has been used as a bioindicator in ecotoxicological studies [6,7] and cultured individuals could be used as control individuals to determine changes in the natural environment through the study of biomarkers in its tissues. Its euryhaline capacity enables the monitoring of rivers and estuaries in addition to coastal areas.

C. labrosus is extensively farmed in Middle Eastern countries—Egypt, Libya, Tunisia, Iran, and Israel—where it represents a product of commercial interest, and so do preparations such as bottarga (salted and dried female gonads), which can reach high prices. The species cultivation is mostly based on wild-caught juveniles, often mixed with other mugilid species [8]. This model of aquaculture puts considerable pressure on natural populations and, depending on annual recruitment, over-capture of juveniles can lead to a collapse in natural population dynamics [9]. Domestication of the species is necessary for specimen availability for industrial production; to this end, the control of reproduction in captivity is essential. Mugilids—*C. labrosus* in particular—experience difficulties in breeding in captivity: females cannot always complete their ovarian development, and oocytes fail to undergo vitellogenesis, resulting in atresia [8]. However, partially optimized hormone induction protocols exist for different species of this family (*C. labrosus* [10], *Mugil cephalus* [11], and *C.* (*Liza*) *ramada* [12]).

In broodstock management programs, sex and maturity stage determination of specimens is essential. The best way to determine sex in fish is by gonadal biopsy. This method can be ineffective outside the breeding season because the gonads have not reached minimum maturity to obtain an identifiable sample. Other minimally invasive methods of sex identification are available, such as plasma hormone analysis [13], but the validation of its effectiveness needs to be studied at species level. During the breeding season—from February to May for *C. labrosus* [14]—maturity stage determination is important whether the aim is to obtain natural spawns or hormonally induced ones; so is the determination of the critical time for female induction in the latter case. The study of sperm quality of a species of aquaculture interest also contributes to a better knowledge of the reproductive aspects of said species and enables the development of artificial reproduction techniques which can facilitate the creation of complex genetic improvement programs. In this sense, sperm quality determination over time is vital in the design of breeding strategies in captivity and their appropriate time sequencing.

The present study aims to gain a better zootechnical understanding of the reproduction of *C. labrosus*, focusing on achieving breeding control in captivity conditions, and to provide useful information for the development of protocols for reproductive control of this species. First, we compare direct (gonadal biopsy) versus analytical (hormone analysis) methods for sex determination in *C. labrosus* to establish a valid technique outside the reproductive period or with prereproductive specimens. Second, we monitor the evolution of maturity development in females, performing gonadal biopsies throughout the entire breeding season. Last, we describe the evolution of sperm quality parameters during the spawning season and provide practical information for sperm management in this species.

2. Materials and Methods

2.1. Capture and Conditioning of Wild Specimens

The fish were collected from the wild in Cantabria (N. Spain) by angling during the non-reproductive seasons (June to October) in 2021 and 2022 and transported to the El Bocal Marine Aquaculture Plant facilities (Cantabria, N. Spain) in a 400 L tank with supplementary aeration and oxygenation, ensuring that oxygen saturation did not drop below 70%. Upon arrival at the facility, the fish were held in flow-through 1200 L tanks and treated with 80 ppm hydrogen peroxide at 50% baths for 3 days after arrival to minimize the risk of external infection from capture and handling; specimens were then quarantined for at least 1 month. Once the fish were fully adapted to captivity, biometric sampling (live weight) and individual identification (PIT-tag) were performed. The fish were then held

in 8 m^3 flow-through cylindrical tanks with a water renewal rate fixed at 100% per hour, under natural photoperiod and temperature at our latitude (43°29′15.6″ N, 3°49′37.1″ W). Between January 2021 and November 2022, the fish were fed ad libitum with commercial sea bream 4 mm pellets [11]. Between November 2022 and May 2023 (prebreeding and breeding season), fish were fed also ad libitum but, in this case, with crushed mussels 2 days per week and with marine polychaetes (*Hediste diversicolor* reared at our facilities) 1 day per week, alternating with sea bream pellets every day.

2.2. Sex Identification

To determine the sex of the fish outside the breeding season, we conducted a plasma steroid study and subsequently validated the use of biopsies of reproductive tissues for the same individuals during the 2023 breeding season. Blood samples of 500–1000 µL per fish (n = 25) were drawn from the caudal vein using heparinized syringes, transferred to Eppendorf vials with 10 µL heparin, and stored on ice. After 20 min centrifugation at 4 °C and 3200× g, the plasma was extracted and stored in Eppendorf vials at −80 °C until further analysis. We adapted the ELISA technique from Rodriguez et al. [15] for testosterone to analyze plasma levels of 11-ketotestosterone (11-KT; CAY-582751.96) and estradiol (E2; CAY-501890.96) in the steroid analysis following the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI, USA): 5 µL of plasma diluted in 100 µL of RB buffer. The RB buffer consisted of 100 mL of 1 M potassium phosphate buffer, 0.1 g NaN_3, 0.37 g EDTA, and 1 g bovine serum albumin; then the volume was increased to 1 L with distilled water. We extracted all steroids in three steps by adding 600, 300, and 300 µL of methanol. Following de las Heras et al. [16], we initially classified fish as male if their 11-KT levels were higher than their E2 levels and female if their E2 levels were higher than their 11-KT levels and presented an 11-KT/E2 ratio < 1.

We performed gonadal biopsies by cannulating the females with feeding cannulas; Klinik Health L/RX probe CH-05 50 cm (Well Lead Medical Co., Ltd., Guangzhou, China). For males, we applied gentle abdominal pressure to obtain sperm samples. For all sampling procedures—blood sampling, female cannulation, and sperm collection—specimens were previously sedated with clove oil (eugenol) dissolved in 96% ethanol (20 ppm) in the holding tanks and transferred to a 400 L auxiliary tank; then, they were anaesthetized one by one with the same anaesthetic (40 ppm) in a 100 L tank. At the time of sexing by plasma hormone analysis (2021/2022 non-breeding season), the fish weight range was between 0.27 and 2.02 kg. When these same individuals were sexed by gonadal biopsy (2023 breeding season), the weight range was between 0.54 and 2.19 kg. The success in sexing with the analytical method is expressed as a percentage of the sexing obtained by biopsy.

2.3. Gonadal Development Monitoring and Sperm Analysis

Throughout the 2023 breeding season (January to May), we studied relevant reproductive variables on males and females held in tanks as described above. We used 14 females (0.9–2.1 kg) and 6 fluent males (0.6–1.7 kg) sexed as such in the first sampling.

Female oocyte development was assessed on a biweekly basis by gonadal biopsies, as described above. The oocytes obtained were analyzed immediately after collection for diameter measurement under a microscope Nikkon Eclipse Ts2R (Nikkon, Tokyo, Japan), using 20 eggs per female. We measured and processed the images with NIS-Elements Imaging Software v 4.60.00, (Nikkon, Tokyo, Japan). To avoid any alterations in egg diameter due to overhydration, the eggs were resuspended in seawater at the beginning of the microscopic measurements. We discarded nonspherical eggs from the measurements. The development of egg diameter over the breeding season was plotted against time.

We used 4 mL syringes to collect sperm through the genital pore of anaesthetized fish, aiming to collect all available volume from each individual. Sperm in the sampling syringes was then kept on a bed of ice until analysis. Each sperm sample was evaluated for total sperm volume, density, and motility (as a percentage of motile cells) after activation with seawater (35‰): sperm volume was titrated; 10 µL were preserved in 40 µL of

Ringer's 200 [17] and further 10 µL were preserved in 190 µL of buffered 4% formalin for subsequent motility and density analyses, respectively. Activation for motility assessment was performed by placing 0.5 µL of sperm-Ringer 200 in 19 µL of seawater at 4 °C in a Makler® chamber (Selfi-Medical Instruments, Haifa, Israel). We used the computer-assisted CASA system (Proiser SL, Valencia, Spain) for sperm analyses. The loss of motility after activation was subsequently assessed by measuring the percentage of motile cells at 15 s intervals, taking motility at 15 s as the initial motility. We also performed activation tests at different salinities, testing sterile seawater (autoclaved) mixed in different proportions with ultrapure laboratory water (filtered 0.4 µm and UV irradiated) until the following salinities were obtained: 35, 26, 18, and 9‰. We evaluated the percentage of motile cells and the following kinetic parameters: the percentage of progressive cells; the percentage of fast, medium, and slow cells; and the mean velocity. Table 1 presents the salinity, pH, and osmolarity of the resulting activation media.

Table 1. Chemical characteristics of the activation media used in the activation tests at different salinities.

Salinity (‰)	pH	Osmolarity (mOsm)
35	8.0	1054
26	7.9	804
18	7.7	547
9	7.5	263

2.4. Statistical Analyses

We used the chi-square test to compare sex identification by plasma hormone analysis with sex identification by biopsy. Total sperm volume, density, and motility (as a percentage of motile cells) data were compared between sampling events (beginning, middle, and end of the breeding season) by paired *t*-tests for related samples. Simple regression analysis was used to evaluate the effect of time after activation on the loss of sperm motility. The influence of salinity on sperm activation was tested using a general linear model with time (beginning, middle, and end of the breeding season) as a repeated measure factor, followed by a Tukey HSD post hoc test. Normality and variance homogeneity had been previously assessed with the Kolmogorov–Smirnov and Levene tests, respectively.

3. Results

3.1. Sex Identification

After steroid hormone analysis, 12 fish were considered female according to the de las Heras [16] method; most of them showed 11-KT values close to zero (Figure 1). The gonadal biopsy confirmed that these were all female (12/12: 100% success). All females used in the present work presented a size greater than that at first sexual maturity (35 cm length; >0.7 kg weight), according to Ben Tuvia [18]. After hormone analysis, 13 individuals were classified as male according to de las Heras [16]; 7 of those showed low values of both hormones, though higher for testosterone (Figure 1). Of the 13 males, 8 were confirmed as such by gonadal biopsy (8/13: 61.5% success). The chi-square test revealed that the differences between the two methods were not significant ($\chi^2 = 0.64$; $p = 0.4227$). Using a ratio of 11-KT/E2 < 0.4 instead of 11-KT/E2 < 1, the success in identifying males increased to 76.9% (10/13) without affecting the identification of females. In this case, the chi-square test also shows no significant differences between the two methods, but with a higher probability of success when modifying the value of the 11-KT/E2 ratio ($\chi^2 = 0.20$; $p = 0.6542$). The erroneous determinations occurred in those male individuals (38.5%) which had not yet reached the size of first sexual maturity (35 cm length, <0.7 kg weight; [18]). By excluding these specimens from the comparison, the success in sex determination by the analytical method also reaches 100% for males.

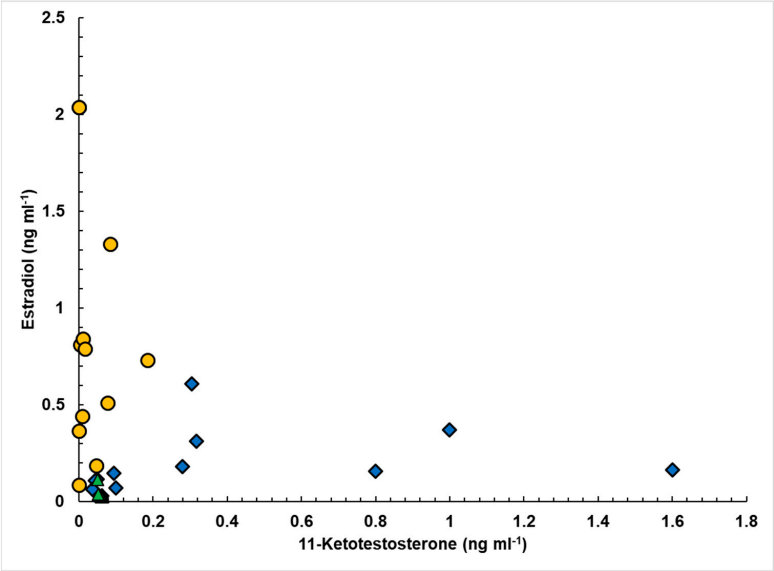

Figure 1. Biplot showing the levels of estradiol and 11-ketotestosterone measured in each specimen. Orange circles indicate those identified as females with the proposed ratio 11-KT/E2 < 0.4. Blue diamonds indicate those identified as males. Green triangles correspond to the specimens misidentified as males with the ratio 11-KT/E2 < 0.4.

3.2. Maturity Evolution Monitoring

We identified a clear trend towards increasing oocyte diameter as the reproductive season progressed (Figure 2). Around midseason, 66% of the females produced oocytes with a diameter between 500 and 600 µm. From this time until the end of the season, gonadal maturation was observed to continue in part of the females, for which the diameter of the oocytes continued to increase until reaching a maximum of around 800 µm at the end of the season (May 2023). For other female specimens, oocytes decreased in diameter and either became atresic or failed to provide a suitable sample.

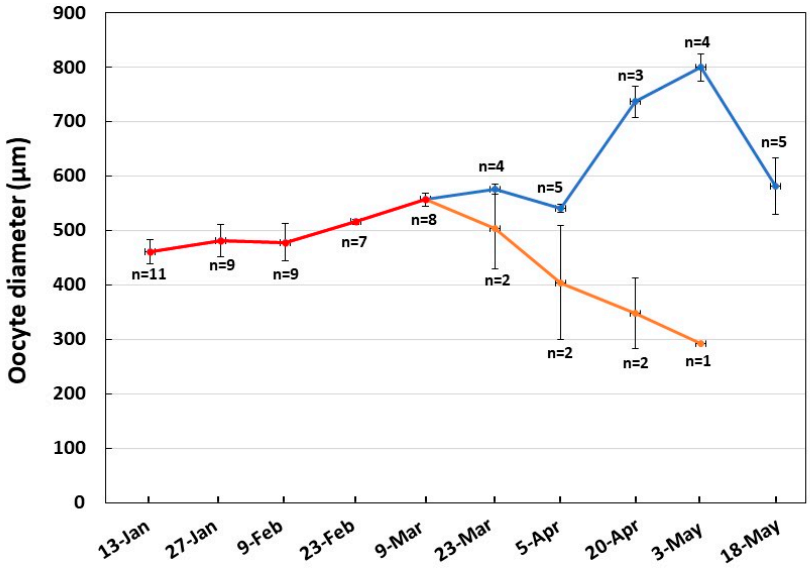

Figure 2. The development of oocyte diameter (mean ± SE) in *Chelon labrosus* females during the 2023 breeding season. n indicates the number of females from which a sample was obtained by gonadal biopsy. The red section of the line indicates the period with all samples increasing in diameter. The blue section indicates the samples that continued to increase the diameter. The yellow section indicates the samples that began to decrease in diameter.

3.3. Sperm Analysis

Our results show sperm quality varying over the course of the breeding season (Figure 3). No significant differences ($p > 0.05$) in sperm volume were detected between initial and middle sampling, despite the maximum possible volume of sperm being extracted in each sampling. Sperm volume was significantly reduced at the end of the season ($p < 0.05$), but three out of the six males were still fluent (Figure 4). Sperm motility decreased as the breeding season progressed, although differences were not significant ($p > 0.05$). Sperm density increased significantly ($p < 0.05$) from the beginning to the middle of the season and then decreased significantly ($p < 0.001$) at the end.

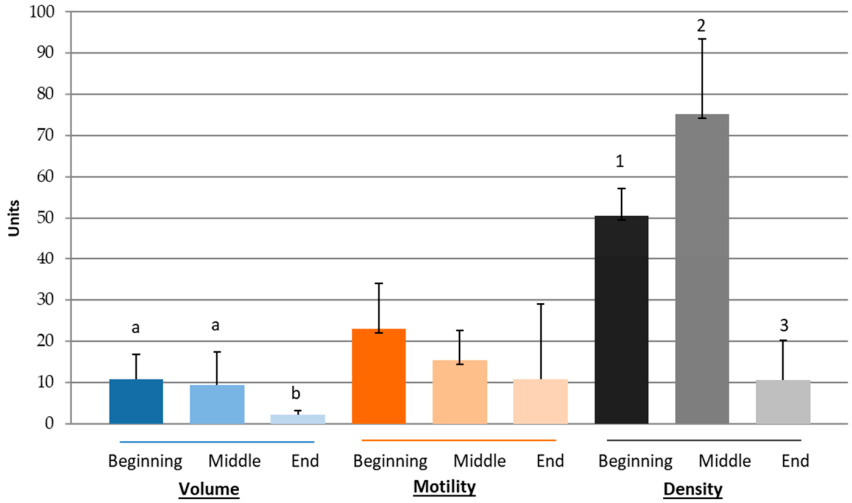

Figure 3. Mean ± SE at the beginning, middle, and end of the 2023 breeding season for volume, motility, and density of the analyzed samples. The different letters and numbers in superscript over the error bars indicate significant differences for the volume and density, respectively ($p < 0.05$; paired *t*-test for related samples). Volume is expressed in 10× mL, motility in %, and density in 10^9 cells per mL.

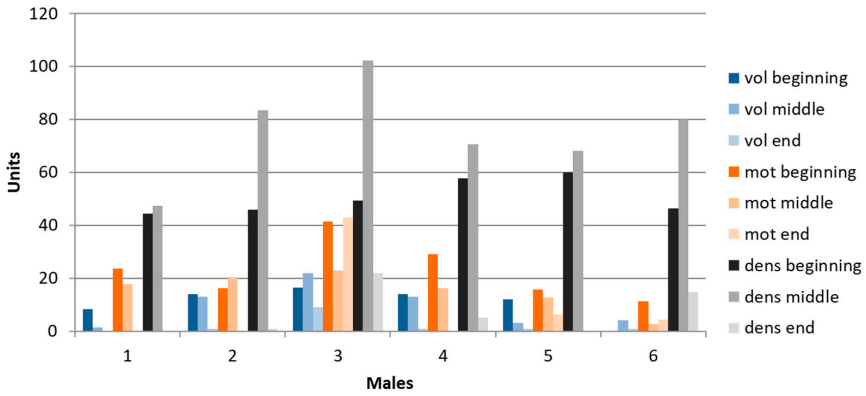

Figure 4. Volume, motility, and density values studied at the beginning, in the middle, and at the end of the spawning season for each male. Volume is expressed in 10× mL, motility in %, and density in 10^9 cells per mL.

In 3 out of the 6 males (males 2, 3, and 4), individual results show maintenance of or an increase in sperm volume between the first and subsequent stripping (Figure 4). For male 3, this maintenance was accompanied by a 50% motility loss compensated by an increase in density in the same range, resulting in the same number of motile cells. This was partially also the case for male 4.

Simple regression analysis shows a significant ($R^2 = 0.9882$; $p < 0.001$) progressive loss of sperm motility after initial activation. Within 15 s of activation, about 25% of the sperm

becomes motile; after 1 min, motility is reduced by about 50%; within 2 min after activation, motility falls below 5% (Figure 5).

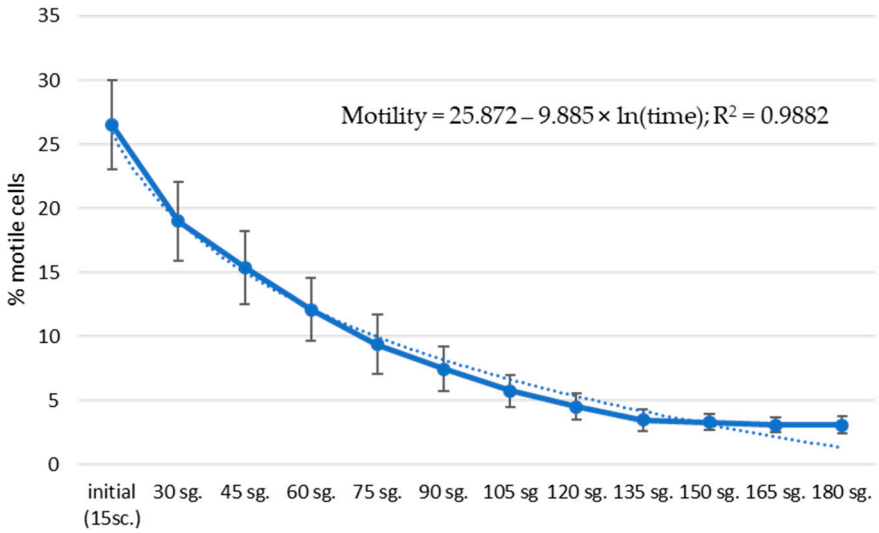

Figure 5. Mean ± SE motility loss of *C. labrosus* sperm over time after activation with seawater 35‰. The dotted line corresponds to the projection of the logarithmic function model associated with motility loss.

As per the results of sperm activation at different salinities, no motility was detected for the lower salinity tested (9‰); hence, this level was not included in the tests. The analysis of variance (ANOVA) with repeated measures revealed the percentage of medium-speed cells and mean velocity to be the only sperm variables showing significant differences ($p < 0.05$; Figure 6). Although activation at 35‰ salinity resulted in a higher percentage of cells with medium velocity, activation at 26‰ salinity showed a trend with non-significantly higher values in the motility, the percentage of progressive movement cells, and the percentage of fast cells, and a higher mean velocity than that obtained after activation at 35‰ or 18‰ salinity. Mean sperm cell velocity after activation at 18‰ salinity was significantly similar to that obtained after activation at 26‰ salinity.

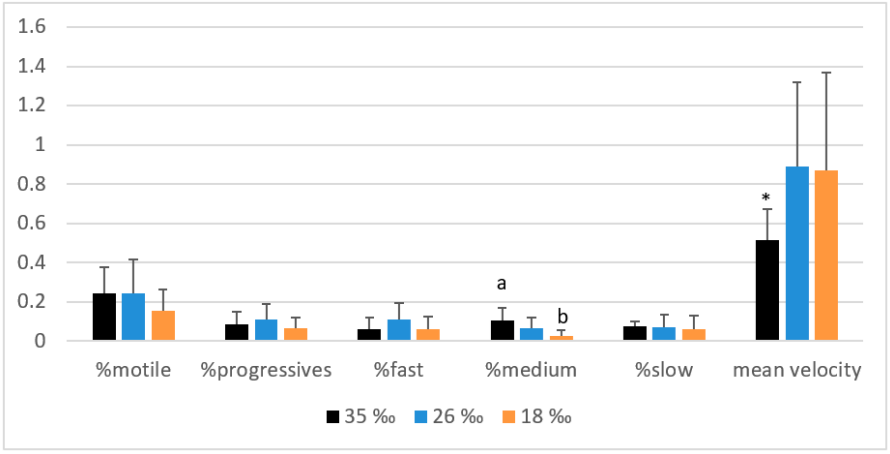

Figure 6. Sperm quality parameters after activation of samples with seawater diluted with ultrapure water in different ratios (35, 26, and 18‰). Percentages are represented in percentage per one and average velocities in µm/sg. The different letters and symbols in superscript over the error bars indicate significant differences in % medium speed cells and the mean velocity, respectively.

4. Discussion and Conclusions

The present study reveals that an accurate sex identification outside the breeding season is feasible for *C. labrosus* by determining steroid hormone levels in plasma, although males must first reach first sexual maturity. More than half of the studied females were observed to fail ovarian development completion in captivity from the middle of the breeding season onwards, at least during the first period of reproduction from their capture. The results also show the ability of males to recover sperm volume after a complete extraction of sperm available, with half of the males maintaining or even increasing sperm volume between the first and the subsequent extraction, with minimal or nonexistent loss of sperm quality, also revealing that sperm activation is enhanced at a salinity of 26‰.

Regarding sex determination, our work demonstrates that hormone analysis allows for the identification of specimens in prereproductive stages or specimens for which biopsies cannot be obtained. The technique described was 100% effective in sexing females and failed in only fewer than 25% of males. The common factor in the erroneous cases was the specimen size, which had not reached first sexual maturity, according to Ben-Tuvia [18]: two weighed less than 350 g when blood samples were taken and the other was sexed as a male after hormone analysis when it weighed less than 0.7 kg, being identified as a female by gonadal biopsy two seasons later when weighing 1.9 kg. Therefore, sex identification by steroid hormone analysis in *C. labrosus* outside the breeding season is possible and provides a 100% success rate in females and males, provided these present a greater size than that corresponding to first sexual maturity, avoiding, for instance, any handling manipulation during the breeding/spawning season that could interfere with the reproductive success. In addition, reducing the threshold of the 11-KT/E2 ratio to 0.4 instead of 1, as suggested by de las Heras [16], would improve sex determination outside the breeding season by hormone analysis.

We identified a clear tendency for oocytes to increase in diameter as the reproductive season progressed. In the middle of the season, approximately 60% of females provided oocytes with a diameter between 500 and 600 µm. This size has been indicated as adequate for hormonal inductions in this species [10]. From that moment until the end of the reproductive season, gonadal maturation continued in part of the females, while in others, the oocytes decreased in diameter, becoming atresic and eventually disappearing. Inhibition of the final oocyte maturation process is one of the most common reproductive dysfunctions in captive fish: vitellogenesis is completed correctly but postvitellogenic oocytes stop their final maturation and undergo atresia [19]. In the present study, a possible cause could be the inhibitory reproductive response associated with stress caused by the fortnightly cannulation, which has been described for other fish species [20]. This is an important handicap that limits the reproduction in captivity of *C. labrosus* and which must be overcome by artificial induction unless the management of other environmental variables involved is improved. To the best of our knowledge, only one spontaneous fertilized spawn has been achieved in captivity [21] according to the literature, and most reproductive issues of this species have been tackled by using hormonal induction methods [10]. Our results show that gonadal maturation in females does not progress equally in all of them and that females with oocytes in advanced stages of vitellogenesis can be observed from the middle of the reproductive phase (March at our latitude) until the end of the period. Therefore, planning the inductions according to the specific stage of development of each individual would be beneficial to increase the success in the completion of gonadal development, the release of mature oocytes, and their fertilization. However, further research is needed to establish other parameters besides oocyte diameter that could correlate with spawning success with or without hormonal induction, such as egg morphology and its structures and/or the relative position of cell organelles [22,23] and other external indicators that could improve reproductive strategies for this species [24].

Several studies have described sperm quality in *Mugil cephalus* [25,26] and other mugilid species [27], but references in relation to *C. labrosus* are limited to grey literature [28] and are absent in indexed literature. The results obtained in the present work represent

a first description of sperm quality throughout the breeding season of wild specimens adapted to captivity and provide data on volume, motility, density, and loss of motility as well as differences obtained after sperm activation with water at different salinities.

We first proved the effectiveness of the collection method by stripping in obtaining volumes between 0.1 and 2. mL and a mean value of 1.01 ± 0.16 mL during the period studied. Similar volumes have been obtained for *M. cephalus* [25]. This indicates the potential production of the males of this species. Sperm quality throughout the breeding season has been studied in other species [29–31] and is of great importance when planning breeding strategies on a commercial scale, as is in sperm cryopreservation campaigns for the implementation and development of on-farm genetic improvement programs. The results obtained herein show the adequate period to obtain maximum quality values to be between the beginning and the middle of the breeding season accepted for this species [14], not exceeding the final breeding season (May under the conditions described in the present work). Nevertheless, these values may vary depending on light regimes, temperature, and/or other factors such as salinity. Our results suggest the potential of evaluating the onset of the breeding season with sperm quality samplings several months before the breeding season studied in the present work to determine from what point in time maximum sperm quality values appear. This would also apply to the peak maturity of females, which could also be influenced by the same environmental variables as males.

Regarding individual sperm production, 50% of the specimens were able to maintain sperm volumes between the first and second sampling and even increase density. Little literature is available on the frequency of stripping in relation to the loss of sperm quality in fish [32]. Our results show the possibility of a bimonthly sampling of this species without loss of quality within the breeding period. As mentioned above, further investigation is advisable to assess whether or not this period can be extended due to the onset of spawning and whether or not a higher frequency can be achieved—as it occurs in turbot (*Scopthalmus maximus* [33]) or sole (*Solea senegalensis* [34]), for which potential frequencies of 1 month or 15 days within the spawning season have been reported, respectively.

Given the euryhaline capacity of the species [4] and its frequent presence in fresh and brackish waters [35], salinity could play a prominent role in the reproduction of *C. labrosus*. The study of this variable is important when handling the sperm of a species for the design of immobilizing solutions, short-term preservatives, or cryoprotectants [30]. Our results show a salinity limit between 9 and 18‰ that greatly inhibits sperm motility and should be considered as a threshold for the reproduction of the species, as it occurs in *Mugil cephalus* [36]. In addition, we detected a slight rebound, although not significant, in the quality variables associated with a decrease in salinity up to 26‰, except in the percentage of cells with medium velocity. The mean velocity was significantly increased by this decrease in salinity. Based on the results obtained, the study of the effect of salinity between 35 and 26‰ could be convenient to obtain a significant improvement in sperm quality. Even testing this osmotic variable makes it possible to trigger and induce natural spawning in the species together with the standard environmental variables (temperature and photoperiod). In addition, acclimatization of specimens to different salinities could modify the sperm tolerance ranges to activation salinities, as it occurs in other euryhaline species such as *Oryzias latipes* [37].

The results obtained in the present work as a whole provide relevant knowledge for improving the reproduction of *C. labrosus* in captivity and a basis for future research, in particular with regard to the sex identification of individuals, refinement of peak of maturity determination in males and females, development of specific protocols for sperm cryopreservation, and consideration of salinity as a potential variable influencing the reproduction of this species.

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Institutional Review Board Statement: The fish were always handled (routine management and experimentation) according to the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 53/2013) for the use of laboratory animals. Moreover, all the people involved in the experiments had the required FELASA accreditations for each procedure (ECC556/2015). The project was evaluated by the official ethics committee with favorable report number NTS-ES-285239.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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