**Recombinant vs purified mammal gonadotropins as maturation hormonal treatments of European eel males**

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**Abstract**

In the past three decades the European eel *Anguilla anguilla* experienced up to 99% decline in recruitment in some parts of its distribution range, thus breeding in captivity is nowadays considered key in order to save this species. With this in mind, obtaining high quality gametes is fundamental, as is the ongoing study of new hormonal treatments in order to improve current methods. Therefore, the aim of this research study was *i*) to assess the effect of two hormonal treatments (OVI, a recombinant α-choriogonadotropin; and VET, a human chorionic gonadotropin purified from female urine) on the reproductive performance of European eel males, and, after choosing the best hormone, *ii*) to compare the effects of three doses in order to cut the costs of artificial maturation.

Our results indicated that the type of hormone used (recombinant vs purified gonadotropins) significantly affected the progression of spermiation in European eel males, and that the recombinant hormone (OVI) produced better results in terms of sperm quantity and quality in most of the weeks of the treatment, remaining thus an effective treatment to induce spermiation in this species. On the other hand, in terms of the doses experiment, our results showed that from the lowest to the highest dose (0.25 to 1.5 IU/g fish) all the treatments were able to induce the whole spermiation process. However, a weekly dose of 1.5 IU/g fish of recombinant hormone (OVI) was necessary in order to provide a notable amount (volume and density) of high quality (motility and velocity) samples throughout the treatment.

Finally, the economic analysis demonstrated that the recombinant hormone (OVI, 1.5 IU/g fish) had a greater profitability than the other treatments, making it possible to obtain high-quality sperm for a lower price. In this context, and considering the fact that in the first few weeks of any hormonal treatment there is no high-quality sperm production, long-term hormonal therapies are necessary in order to lessen the cost of high-quality European eel sperm.

**Keywords**

Sperm, CASA, Motility, Hormones, Anguilla

## 1. Introduction

The European eel (*Anguilla anguilla*) is an important species for European aquaculture (5000 tonnes per year, FAO 2014), being highly appreciated both in the European and Asian markets. However, its current production still consists in the fattening of eels captured in the natural environment, due to the fact that it is not yet possible to reproduce eels in captivity. In addition, a drastic decrease has been observed in the number of wild European eels migrating from Europe and North Africa to the spawning sites in the Atlantic Ocean, leading to the species being included in the IUCN red list as critically endangered. Therefore, breeding in captivity is postulated as a key alternative in order to save this species, which will help to reduce the pressure on natural populations, it will facilitate the supply to the eel farms, and it will allow repopulation in areas where those that historically were located the eel.

Although in some fish species reproduction in captivity can be controlled exclusively by environmental factors (Rocha et al., 2008), sometimes it is impractical or even impossible to simulate the environmental conditions in which sexual maturation happens (i.e. depth, pressure, spawning migration, etc.), so the use of exogenous hormones is the only effective way of inducing reproduction (reviewed by Mylonas *et al.*, 2010, 2017). This is the case of the eel species (*Anguilla* spp.), as they do not mature spontaneously in captivity, and the maturation of both males and females must be induced with long-term hormonal treatments (Asturiano et al., 2005; Lokman et al., 2016; Ohta et al., 1997; Sorensen and Winn, 1984).

In the case of European eel males, human chorionic gonadotropin (hCG) has been the most widely used hormone for achieving spermiation, but it has been administered to the animals in several different formats (Gallego et al., 2012). The first studies date back to the middle of the 20th century, where gonadal maturation in eel males was induced by intraperitoneal injections of urine from pregnant women (Fontaine, 1936). At the end of the century, several companies were able to isolate hCG from female urine, so the induction of spermiation of this species became a much more simple and standardized process (Dollerup and Graver, 1985; Khan et al., 1987; Pérez et al., 2000). Studies from the beginning of the 21st century served to develop and optimize hormonal treatments based on purified hCG, optimazing the sperm production and sperm quality through weekly intraperitoneal injections of 1.5 IU/g fish (Asturiano et al., 2006). However, both the duration of the spermiation period (limited in time) and the interruption of the availability of hCG (in its purified form) in the market meant that new studies addressing the use of alternative hormones became necessary. In this context, the arrival of human recombinant gonadotropins ($hCG_{rec}$, produced by recombinant DNA technology) became an effective alternative due to the similar structure of the native human hormone, and throughout the last few years they have yielded good results (Gallego et al., 2012). Nevertheless, the effectiveness of treatments based on $hCG_{rec}$ apparently depends on the batch of hormones used, and sometimes it is possible to find groups of animals where although gonadal maturation occurs, the sperm quality parameters (such as motilities and velocities) are not good enough for scientific or aquaculture purposes. Recently, new studies using specific (native) European eel recombinant gonadotropins were also able to induce spermiation in eel males, but the sperm volume and motility results were low for carrying out fertilization trials (Peñaranda et al., 2018). In addition, the production of these native hormones is a tedious and sophisticated process that can only be carried out by companies, thus the end cost of the hormones is prohibitive for many research groups.

Therefore, studies into alternative hormonal treatments must be ongoing in order to improve current methods to date. With this in mind the aim of this work was *i*) to assess the effect of two hormonal treatments (recombinant vs purified mammal gonadotropins) on the reproductive performance of European eel males and, after choosing the best treatment, *ii*) to compare three different hormone doses in order to cut the costs of artificial maturation in this species both for fish farms and laboratories.

## 2. Materials and methods

### *2.1. Fish maintenance*

Eel males from the fish farm *Valenciana de Acuicultura, S.A*. (Puzol, Valencia; on the east coast of Spain) were moved to our facilities, in the Aquaculture Laboratory at the Universitat Politècnica de València, Spain. The fish were distributed into aquaria equipped with separate recirculation systems, thermostats/coolers, and covered to reduce the light intensity and fish stress. The eels were gradually acclimatized to sea water (salinity 37 ± 0.3 g/l) over the course of one week, and later once a week they were anaesthetized with benzocaine (60 ppm) and weighed to calculate the individual doses of the hormone, which were then administered by intraperitoneal injection.

The fish were not fed throughout the experiment and were handled in accordance with European Union regulations (see Ethics statement section).

*2.2. Experimental design*

*Experiment 1. Hormonal treatments: recombinant vs purified gonadotropins*

Twenty adult eel males (mean body weight 107.9±1.6 g) were equally and randomly distributed into two 150-L aquaria (10 males per treatment) where they underwent two hormonal treatments: OVI (a recombinant α-choriogonadotropin produced in Chinese hamster ovary cells by recombinant DNA technology and marketed as Ovitrelle; Merck S.L., Madrid) and VET (purified human chorionic gonadotropin marketed as Veterin Corion; Divasa-Farmavic S.A., Barcelona). The VET hormone was dissolved in a saline serum (NaCl 0.9%) to obtain a concentration of 1 IU/μL serum. The OVI hormone was diluted to obtain a similar concentration. The hormones were injected weekly at a dose of 1.5 IU/g fish and were administered for 25 weeks.

*Experiment 2: Different doses of recombinant gonadotropin*

After choosing the recombinant gonadotropin (OVI) as the best hormone in terms of sperm quality and profitability, 30 adult eel males (mean body weight 102.3±3.7 g) were equally and randomly distributed into three 150-L aquaria (10 males per treatment). Each group (aquarium) received a different hormonal treatment doses (OVI1.5: 1.5 IU/g fish; OVI0.75: 0.75 IU/g fish; or OVI0.25: 0.25 IU/g fish; respectively) with the final aim of reducing production costs. The hormone was diluted 1:1 (IU/μl) in saline solution (NaCl 0.9%) and the doses were administered weekly for 12 weeks.

*2.3. Sperm collection and sampling*

Sperm samples were collected weekly by the application of abdominal pressure

24 h after the administration of the hormone (following the protocol described by Pérez *et al.*, (2000)), and taking special care to avoid contamination with faeces, urine and sea water. Samples were diluted 1:9 (sperm:extender) in P1 medium (D. S. Peñaranda et al., 2010) and kept in plastic tubes at 4 ºC until the sperm kinetic analyses, which were carried out in the 2 hours following sperm collection. Sperm volume was previously measured using graduated tubes and sperm density was determined by a CASA system (see next section).

### *2.4. Evaluation of sperm motility and kinetic parameters*

Samples were activated by mixing 0.5 µl of P1-diluted sperm with 4.5 µl of artificial sea water (Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2). All the motility analyses were performed in triplicate using the motility module of ISAS (Proiser R+D, S.L.; Paterna, Spain) as described by Gallego *et al.* (2013). The chamber used in all the experiments was a SpermTrack-10® (Proiser, Paterna, Spain) with a 10x negative contrast phase lens in a Nikon Eclipse (E-400) microscope.

The parameters considered in this study were total motility (MOT, %); progressive motility (pMOT, %), defined as the percentage of spermatozoa which swim forwards in 80% of a straight line; curvilinear velocity (VCL, in µm/s), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory; average path velocity (VAP, µm/s), defined as the time/average of sperm head along its average spatial trajectory; and straight line velocity (VSL, µm/s), defined as the time/average velocity of a sperm head along the straight line between its first detected position and its last position. Spermatozoa were considered motile if their progressive motility had a VSL over 10 µm/s.

In order to perform an in-depth analysis of the results, sperm samples were classified into three classes based on the percentage of motile spermatozoa provided by the CASA system: Class I (C-I) = 0-25% of motile cells; Class II (C-II) = 26-50% of motile cells; and Class III (C-III) = 51-100% of motile cells.

### *2.5. Economic analysis*

To analyse the economic profitability of each hormonal treatment (both in experiment 1 and 2) three factors were taken into account: *i*) the price of the hormone; *ii*) the total amount of hormone used (dose) throughout the whole treatment; and *iii*) the total volume of sperm of the highest motility class (C-III) produced by each treatment. The aim was essentially to relate the investment made with the level of good quality sperm produced by each hormonal treatment.

### *2.6. Statistical analysis*

The mean and standard error were calculated for all sperm parameters (volume, density, motility and the rest of the kinetic parameters). Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. A two-way ANOVA was used to analyze the sperm production and quality parameters. Significant differences were detected when *p-value* < 0.05. All statistical analyses were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

## 3. Results

### *3.1. Experiment 1. Hormonal treatments: recombinant vs purified gonadotropins*

The sperm production parameters are shown in Figure 1. Most of the OVI-treated fish (90%) started to produce sperm in the 6th week of treatment, while only 60% of VET-treated fish generated sperm in this week (Figure 1A). From the 12th to the 18th week, the VET treatment generated higher percentages of spermiating males (90-100%) than the OVI treatment (70-80%) and, finally, the decreasing percentages of spermiating males were similar in the last few weeks (19th to 25th) in both treatments.

Regarding volume, there was an increasing trend from the beginning to the end (Figure 1B) in both treatments. Volume values were generally higher in OVI treated males, although statistical differences were only found in weeks 8 and 9, probably due to the high dispersion of data found in the OVI-treated males during the last few weeks. Density values were slightly higher in VET treated males from weeks 11 to 21, but significant differences were only found in weeks 13 and 15 (Figure 1C).

Regarding the sperm quality parameters, OVI males showed higher motilities than VET males during the first few weeks of treatment (Figures 2A and 2B), reaching maximum values of 76 and 45% of MOT and pMOT, respectively. However, VET-treated males showed a marked rise from week 12 (with 77 and 35% of MOT and pMOT, respectively), and motility parameters were similar for both hormones until the end of the treatment, with values remaining over 50% in the 25th week. The sperm velocities (Figures 2C, 2D and 2E) showed a similar pattern to the motility traits: OVI-treated males showed higher velocities (VCL, VSL and VAP) than VET-treated males during the first few weeks of the treatment, but the kinetic values were similar in both hormone treatments from the 12th week until the end of the treatment.

Finally, when the volume and the sperm motility classes were considered simultaneously (Table 1, experiment 1), it was observed that the OVI treatment displayed better total volume results (with volume values over 500 mL of C-III sperm) than the VET treatment, which yielded total volume values of around 200 mL of C-III sperm. In addition, in terms of the production of high quality sperm week-by-week (Figure 3), the OVI treatment showed a higher number of weeks ($8^{th}$, $9^{th}$, $10^{th}$, $12^{th}$, $22^{th}$, $23^{th}$, $24^{th}$ and $25^{th}$) providing higher volumes of good quality sperm (C-III) than the VET treatment.
Regarding the economic analysis, the investment needed to obtain mature males was quite different in each hormonal treatment (Table 2; experiment 1). The VET treatment investment was smaller, at 0.69 €/week per male, nevertheless, although the OVI treatment required a higher investment per male (1.17 €/week per male), the total volume of class III sperm obtained from OVI-treated males was much higher than VET males (Table 1). Therefore, the final profitability of this hormone was higher in OVI treated males, where it was possible to obtain 1 mL of the highest quality sperm (C-III) for a lower price (0.44 €/mL). The other hormone (VET) produced worse economic results because it was necessary to invest 0.86 € to obtain 1 mL of good quality (C-III) sperm.

*3.2. Experiment 2: Different doses of recombinant gonadotropin*
In terms of the sperm production parameters (Figure 4), all the doses of recombinant gonadotropin (OVI) were able to induce high percentages of spermiating males (around 80%) during most of the weeks of treatment. However, the $OVI_{0.25}$ group produced the lowest percentages of spermiating males during the last few weeks (around 60%).

Regarding volume, an increasing trend from the beginning to the end was seen in all three treatments (Figure 4B). The volume values were generally higher in the OVI$_{0.25}$ group, but statistical differences were only found in week 8 and 10. On the contrary, density patterns were slightly higher in males treated in the OVI$_{1.5}$ group, and significant differences were found from the $9^{th}$ and $12^{th}$ week (Figure 4C).

Concerning sperm quality parameters, OVI$_{.1.5}$-treated males provided samples with statistical higher values of MOT and pMOT throughout almost all the treatment (Figures 5A and 5B), reaching maximum values of 72 and 46%, respectively. Conversely, medium and low doses (OVI$_{0.75}$ and OVI$_{0.25}$) provided samples which only showed maximums of 30 and 20% of MOT, respectively. The spermatozoa velocities (Figures 5C, 5D and 5E) showed similar patterns to those of motility, and OVI$_{1.5}$-treated males showed higher velocities with significant differences (VCL, VSL and VAP) throughout most of the treatment.

Finally, when the volume and the sperm motility classes were evaluated simultaneously (Table 1, experiment 2), it was observed that the highest dose (OVI$_{1.5}$) was the only treatment able to produce acceptable volumes (near 100 mL) of good quality (C-III) sperm. On the contrary, medium and low doses (OVI$_{0.75}$ and OVI$_{0.25}$) provided large volumes of bad quality sperm (C-II especially and C-I), which represented more than 95% of total volume production for each treatment. In addition, when looking at the production of high quality sperm week-by-week, the OVI$_{1.5}$ treatment showed a higher number of weeks ($8^{th}$, $9^{th}$, $10^{th}$, $11^{th}$ and $12^{th}$) providing higher volumes of C-III sperm than the other treatments (Figure 6).

Regarding the economic analysis, the investment needed to obtain mature males was quite different in each hormonal treatment (Table 2, experiment 2). The $OVI_{1.5}$ treatment required the highest investment per male per week (1.17 €), while an investment of 0.58 and 0.19 € were necessary in order to mature fish with $OVI_{0.75}$ and $OVI_{0.25}$, respectively. However, the total volume of class III sperm obtained from $OVI_{1.5}$-treated males was much higher than that produced by $OVI_{0.75}$ and $OVI_{0.25}$ males (Table 1), so the final profitability of the standard dose ($OVI_{1.5}$) was the highest, with it being possible to obtain 1 mL of the highest quality sperm (C-III) for the lowest price (1.78 €/mL).

## 4. Discussion

### *4.1. Hormonal treatments: recombinant vs purified gonadotropins*

The study of alternative hormonal treatments to improve both sperm production and quality parameters must be ongoing in order to enhance gonadal maturation in fish (Mylonas et al., 2017), specifically in species with serious reproductive problems, such as the European eel (Gallego et al., 2012; Peñaranda et al., 2018). In the present study, our results indicated that the type of hormone used (recombinant vs purified gonadotropins) significantly affects the progression of spermiation in European eel males, with the recombinant hormone (OVI) producing better results in most of the weeks.

First of all, it is important to note that sperm quantity and quality have become a key factor in controlled reproduction both for aquaculture and scientific purposes, thus reasonable volumes of high quality samples are necessary in order to fertilize the maximum number of eggs (Migaud et al., 2013; Tvedt et al., 2001). In this context, although both hormones (OVI and VET) were able to induce a high percentage of spermiating males (>70%), there was a notable difference in sperm volume and density patterns between the treatments. In terms of volume, OVI-treated males produced approximately twice (even triple) the volume than VET-treated males in all the weeks, thus the final amount of sperm resulting from the OVI hormone was much higher than that produced by VET-treated males. In this context, Gallego *et al*. (2012) reported similar results in this species when using the recombinant hormone (OVI), where a purified hormone (from a different brand) used in that previous study yielded remarkable results (up to 8 mL). In addition to the volume, the density values provided by VET treated males in the present study were not high enough to compensate for the lower volumes produced by this hormone in most of the weeks, thus the recombinant hormone (OVI) was the best treatment according to both the sperm production parameters. Moreover, in addition to sperm quantity, sperm quality is a crucial factor in fertilization trials, and several kinetic parameters (characterizing sperm motility and velocity) are nowadays considered to be the best fish sperm quality biomarkers (Gallego and Asturiano, 2018). In experiment 1, both hormones yielded remarkable motility and velocity values during most of the treatment, although the recombinant hormone (OVI) was able to provide high quality samples during a greater number of weeks (18/20) than the purified hormone (12/20). In this context, it is noteworthy that an essential factor in European eel breeding captivity programs is the ability to obtain high quality sperm for a large number of weeks in order to synchronize egg production by the females (Butts et al., 2014; Tomkiewicz et al., 2013), thus the recombinant hormone (OVI) was identified as the best treatment according to the sperm quality indicators.

From a physiological point of view, the different responses found in eel males regarding the different hormonal treatments could be explained by the biological activity of each hormone: while the VET hormone was a native hCG hormone, purified and isolated from human urine (Birken et al., 1996), the OVI hormone was a recombinant version of endogenous hCG produced by recombinant DNA technology (Choi and Smitz, 2015). Even though both hormones (OVI and VET) act as analogues of the luteinizing hormone (LH), Basselt et al. (2005) reported that purified-hCG preparations contained a high number of urine derived protein contaminants as well as hCG related metabolites, whereas recombinant hCG was confirmed to be essentially intact hCG (free from contaminant proteins and with very low levels of oxidised hCG). Therefore, the different nature and origins of these hormones (with different degrees and types of glycosylation) could induce gonadal maturation in different ways, generating different patterns in sperm volume or density as seen in previous studies reported by Gallego *et al.* (2012). In addition, recents reports showed that new recombinant hCGs are available in the market (Pregnyl, Ovidrel, etc.), and they could be probably useful for gonadal maturation in fish due to the high degree of structural and functional similarity with the reference format Ovitrelle (Leao and Esteves, 2015; Thennati et al., 2018).

On the other hand, new hormonal therapies using specific recombinant gonadotropins are being developed to induce spermiation in eel species. Although results in European eel has not been good enough for applying in aquaculture purposes (Peñaranda et al., 2018), recombinant Japanese eel LH induced a much higher amount of high-quality sperm when compared to hCG injections in this species (Ohta et al., 2017). In this context, studies into alternative hormonal treatments must be ongoing in order to improve current methods for inducing the successful artificial maturation of endangered species, such as the European eel.

To sum up, our results demonstrated that the progression of spermiation in European eel males was notably influenced by the hormone used. Recombinant gonadotropin (OVI) showed the best results in terms of both sperm production and quality parameters, becoming an effective treatment to induce spermiation in the European eel.

*4.2. Different doses of recombinant gonadotropin (OVI)*

In addition to the task of pursuing new hormones in order to extend the spermiation period and enhance sperm quality, attempts to optimize hormonal therapies are also a key premise to be applied in both scientific and aquaculture sectors. In this context, and once the most efficient hormonal treatment from experiment 1 had been chosen, the effects on the induction of spermiation of several doses of the recombinant hormone (OVI) were evaluated.

Our results showed that from the lowest to the highest dose of the recombinant hormone (0.25 to 1.5 IU/g fish), all the treatments were able to induce the whole spermiation process. Previous studies reported that a single injection of hCG was enough to induce spermatogenesis and spermiation both in European and Japanese eel species (Khan et al., 1987; Miura et al., 1991), but a continuous supply of hormone was necessary to maintain both the sperm production and the sperm quality throughout the weeks (Asturiano et al., 2005). In this context, our results agree with these previous studies, and a periodic supply of hCG (even using the lowest doses) was able to maintain the spermiation process over the weeks.

Concerning the sperm production rates (volume and density), the $OVI_{0.25}$ group surprisingly yielded the highest sperm volumes throughout the treatment, with values reaching close to 8 mL in the 10$^{th}$ week. However, sperm density was lower in this group ($OVI_{0.25}$) compared to the other two groups where higher recombinant hormone doses were injected ($OVI_{0.75}$ and $OVI_{1.5}$), thus the total amount of spermatozoa (volume x density) produced weekly was similar for all treatments. This density-volume pattern has already been described in other species, and this effect seems to be controlled by the maturation inducing steroids (MIS) which regulate the final stages of sperm maturation (Asturiano et al., 2002; Schulz et al., 2010). In this context, high densities would usually be linked to small volumes and conversely, low densities would need to be compensated by high sperm volumes. In addition, the density data yielded in this study using the standard doses of recombinant hCG (1.5 IU/g fish) agree with previous values obtained by administering the recombinant hormone in this species (Gallego et al., 2012), but density data were significantly higher than those obtained using purified hormone a decade ago (Asturiano et al., 2006; Pérez et al., 2000).

On the other hand, and concerning the sperm quality parameters, notable differences were found between the treatments. In this context, only the group with the highest dose ($OVI_{1.5}$) was able to generate samples with acceptable motility values from the 8$^{th}$-9$^{th}$ weeks until the end of the treatment, while $OVI_{0.25}$- and $OVI_{0.75}$-treated males produced bad quality sperm (<35% of motility) in all the weeks. This low response in terms of motility in the groups receiving the lowest doses could be due to a hormonal failure in the maturation process. In this sense, an insufficient weekly dose of gonadotropin could generate a deficient production of steroidogenic enzymes, which in turn would produce a low production of the steroids involved in gonadal maturation, therefore causing a poor production of good quality sperm (Jamalzadeh et al., 2014; Peñaranda et al., 2010; Schulz and Miura, 2002). Throughout the bibliography, the most common dose applied in fish has been 1 IU/g fish, but doses are usually species-dependent, varying from 0.15 IU/g fish in pikeperch (*Sander lucioperca*) (Falahatkar and Poursaeid, 2014) to 50 IU/g fish in silver perch (*Leiopotherapon plumbeus*) (Denusta et al., 2014). Considering European eel references, previous experiments carried out a decade ago also showed that doses of 0.75 IU/g fish were unable to provide high quality samples throughout the treatment, as per this study. However, a dose of 1.5 IU/g fish of purified hCG administered every 2 weeks provided a greater number of samples but of a similar quality (Asturiano et al., 2005), given more chances for carrying out hatchery operations related to fertilization trials.

To sum up, our results have demonstrated that in order to achieve a successful maturation process in the European eel, a minimum dose of 1.5 IU/g fish of recombinant hCG administered weekly is necessary, inducing the production of reasonable volumes of high quality sperm samples.

### *4.3. Economic analysis for the different hormones and doses*

From a biological point of view, the best hormonal treatment should provide a large amount (volume and density) of high quality (motility and velocity) samples for as many weeks as possible. However, from an economic point of view, a reduction in the costs of hormonal therapies is essential in order to obtain affordable and more effective treatments (Mylonas et al., 2017).

In the present study, the economic performance of the treatments was assessed by taking into account both the economic investment made (type, price and dose of hormone) and the total volume of high-quality sperm generated by each treatment. In experiment 1 and concerning high-quality sperm price, the recombinant hormone (OVI) generated the best results throughout the experiment, improving the performance yielded by the purified hormone (VET). In this context, and despite the fact that the investment required for maturating males using the recombinant hormone was almost double that of the purified gonadotropin (1.17 vs 0.69 €/male per week, respectively), the large amount of high-quality sperm produced by OVI-treated males (triple that of VET-treated males) meant a greater profitability, making it possible to obtain high-quality sperm for half the price (0.44 €/mL) of the purified hormone (0.86 €/mL). These results agree with previous studies carried out by Gallego *et al*. (2012), where recombinant hCG also generated better economic performances (0.5 €/mL) than gonadotropins purified from pregnant women and mares (0.72 and 1.8 €/mL, respectively).

Moreover, the analysis of the economic return of the different doses used in experiment 2 yielded interesting results. Although the highest ($OVI_{1.5}$) and the lowest dose ($OVI_{0.25}$) of recombinant hormone generated similar economic performance in terms of high-quality sperm price (1.78 and 1.85 €/mL, respectively), the total C-III volume generated by $OVI_{0.25}$ was too low (2.5 mL/week) for a sustainable application in eel aquaculture, including large-scale fertilizations. In addition, when comparing the results of the economic profitability of the same hormone (OVI) and dose (1.5 IU/g fish) from experiments 1 and 2, the results were notably different: the recombinant hormone showed a much better economic performance in experiment 1 (0.44 €/mL) than in experiment 2 (1.78 €/mL). This difference can be explained by the large-scale production concept, where the cost advantages obtained by applying a different scale of operation (in this case 25 vs 12 weeks for experiment 1 and 2, respectively) decrease the cost per unit of output (high-quality sperm). In fact, when the economic profitability of experiment 1 was calculated just for the first 12 weeks, the economic performance of OVI was lower (1.06 €/mL) than for values obtained in the same experiment for 25 weeks (0.44 €/mL). Thus, because during the first few weeks of any hormonal treatment there is no high-quality sperm production, long-term hormonal therapies are necessary in order to lessen the cost of production of high-quality European eel sperm.

Finally, and linking the production of large amount (volume and density) of high-quality (motility and velocity) sperm samples to the essential hatchery tasks such as *in vitro* fertilization trials (IVF), Butts *et al*. (2014) showed that the sperm to egg ratio became a critical step towards establishing successful *in vitro* fertilization protocols. In this context, and taking into account the optimum value of sperm:egg ratio reported in this species (1:25,000), a large amount of eggs (approx. 10 million per week) could be fertilized using a batch of 10 males induced with recombinant hCG (1.5 IU/g fish).

## 5. Conclusions

In conclusion, this study shows that the type of hormone used significantly affected the progression of spermiation in European eel males, and the recombinant hormone (Ovitrelle at 1.5 IU/g fish) produced the best results in terms of sperm quantity (volume and density) and quality (motility and velocity). In addition, the economic analysis demonstrated that the recombinant hormone had a greater profitability than the other treatments, hence becoming an effective method to induce the spermiation process in this species with the aim to provide high quality samples during a great number of weeks

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**Declaration of interest**

The authors declare no conflict of interests

**Ethics statement**

This study was carried out in strict accordance with the recommendations given in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 regarding the protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV) and final permission was given by the local government (Generalitat Valenciana, Permit Number: 2014/VSC/PEA/00147). The fish were sacrificed using anesthesia and all efforts were made to minimize suffering. The fish were not fed throughout the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

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**Table legends**

**Table 1.** Total sperm volumes (mL) recovered from the different hormonal treatments of experiment 1 (OVI and VET; 1.5 IU/g fish) and experiment 2 ($OVI_{1.5}$: 1.5 IU/g fish; $OVI_{0.75}$: 0.75 IU/g fish; and $OVI_{0.25}$: 0.25 IU/g fish) for each sperm motility class (CI-CIII).

**Table 2.** Profitability of hormonal treatments of experiment 1 (OVI and VET; 1.5 IU/g fish) and experiment 2 ($OVI_{1.5}$: 1.5 IU/g fish; $OVI_{0.75}$: 0.75 IU/g fish; and $OVI_{0.25}$: 0.25 IU/g fish) in relation to economic investment and production of high-quality (Class III) sperm.

**Table 1.**

| Sperm Class | *Experiment 1 (25 weeks)* | | *Experiment 2 (12 weeks)* | | |
|---|---|---|---|---|---|
| | OVI | VET | $OVI_{1.5}$ | $OVI_{0.75}$ | $OVI_{0.25}$ |
| C-I | 29.7 | 14.7 | 29.9 | 199.6 | 206.5 |
| C-II | 45.1 | 85.0 | 26.5 | 15.8 | 94.4 |
| C-III | 544.9 | 201.4 | 99.0 | 13.7 | 15.9 |

**Table 2.**

| | | *Experiment 1 (25 weeks)* | | *Experiment 2 (12 weeks)* | | |
|---|---|---|---|---|---|---|
| | | OVI | VET | $OVI_{1.5}$ | $OVI_{0.75}$ | $OVI_{0.25}$ |
| Dose | IU/g fish | 1.5 | 1.5 | 1.5 | 0.75 | 0.25 |
| Hormone price | €/IU | 0.008 | 0.005 | 0.008 | 0.008 | 0.008 |
| [a]Dose price | €/g fish | 0.012 | 0.007 | 0.012 | 0.006 | 0.002 |
| [b]Investment/male | €/male | 1.17 | 0.69 | 1.17 | 0.58 | 0.19 |
| [c]C-III sperm price | €/mL | 0.44 | 0.86 | 1.78 | 6.44 | 1.85 |

[a] Dose x Hormone Price

[b] Investment to maturate one male (100 g approx.) per week.

[c] Total investment (€) / Total C-III sperm volume (mL).

## Figure captions

**Figure 1.** Evolution of sperm production parameters throughout the hormonal treatments (OVI and VET; 1.5 IU/g fish): A) Percentage of spermiating males; B) Sperm volume; and C) Sperm density. Data are expressed as mean ± SEM and asterisks indicate significant differences between treatments at each week of treatment.

**Figure 2.** Evolution of sperm quality parameters throughout the hormonal treatments (OVI and VET; 1.5 IU/g fish): A) Percentage of motile cells; B) Percentage of progressive motile cells; C) Curvilinear velocity; D) Rectilinear velocity; and E) Average path velocity. Data are expressed as mean ± SEM and different letters indicate significant differences between treatments at each week of treatment.

**Figure 3.** Percentage of sperm volume from each motility class (I-III) in each week throughout the different hormonal treatments: A) OVI and B) VET.
Motility classes: Class I = 0- 25%; Class II = 26-50%; and Class III >50% of motile cells.

**Figure 4.** Evolution of sperm production parameters throughout the different hormonal doses of OVI treatment ($OVI_{1.5}$: 1.5 IU/g fish; $OVI_{0.75}$: 0.75 IU/g fish; and $OVI_{0.25}$: 0.25 IU/g fish): A) Percentage of spermiating males; B) Sperm volume; and C) Sperm density. Data are expressed as mean ± SEM and different letters indicate significant differences between doses at each week of treatment.

**Figure 5.** Evolution of sperm production parameters throughout the different hormonal doses of OVI treatment ($OVI_{1.5}$: 1.5 IU/g fish; $OVI_{0.75}$: 0.75 IU/g fish; and $OVI_{0.25}$: 0.25 IU/g fish): A) Percentage of motile cells; B) Percentage of progressive motile cells; C) Curvilinear velocity; D) Rectilinear velocity; and E) Average path velocity. Data are expressed as mean ± SEM and different letters indicate significant differences between doses at each week of treatment.

**Figure 6.** Percentage of sperm volume from each motility class (I-III) in each week throughout the different hormonal doses OVI treatment: A) $OVI_{1.5}$: 1.5 IU/g fish; B) $OVI_{0.75}$: 0.75 IU/g fish; and C) $OVI_{0.25}$: 0.25 IU/g fish.

Motility classes: Class I = 0- 25%; Class II = 26-50%; and Class III >50% of motile cells.

**Figure 1.**

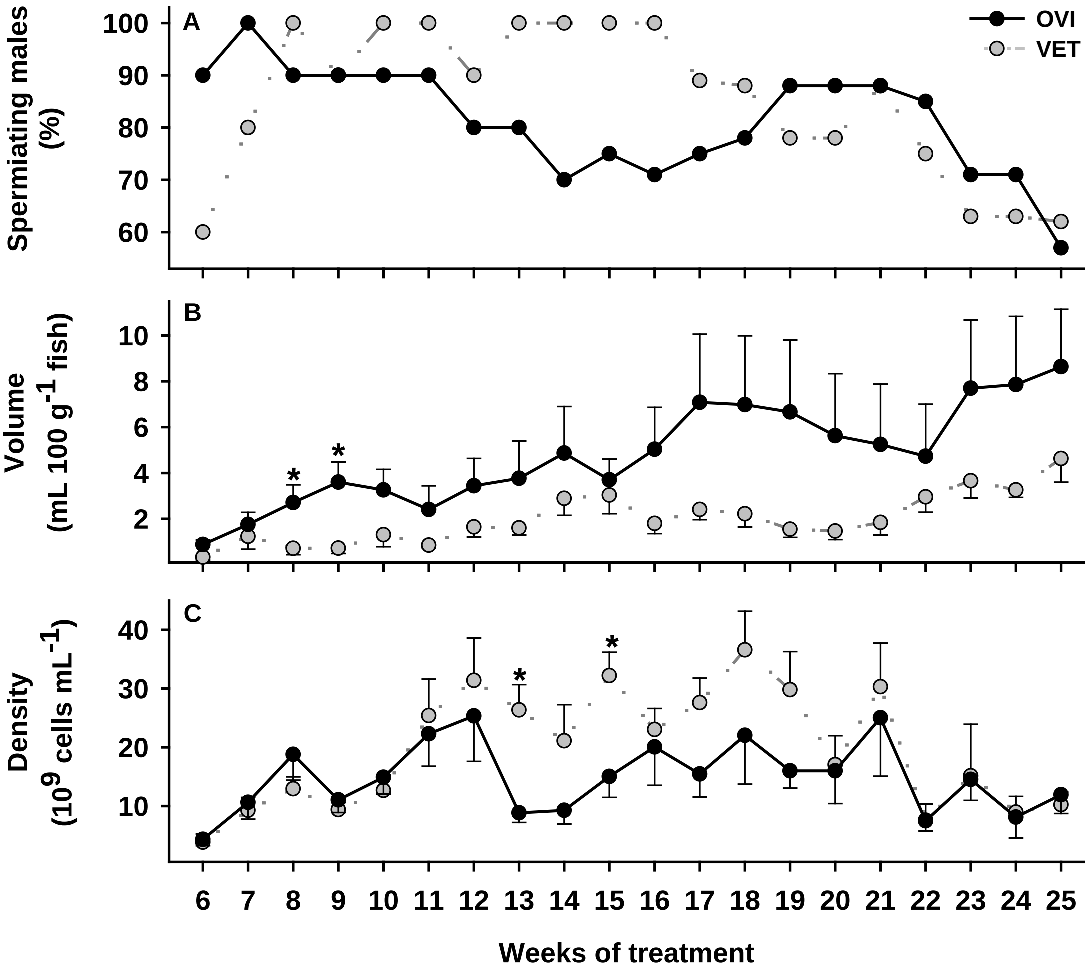

A
B
C
OVI
VET
Spermiating males (%)
Volume (mL 100 g-1 fish)
Density (109 cells mL-1)
Weeks of treatment
*

**Figure 2.**

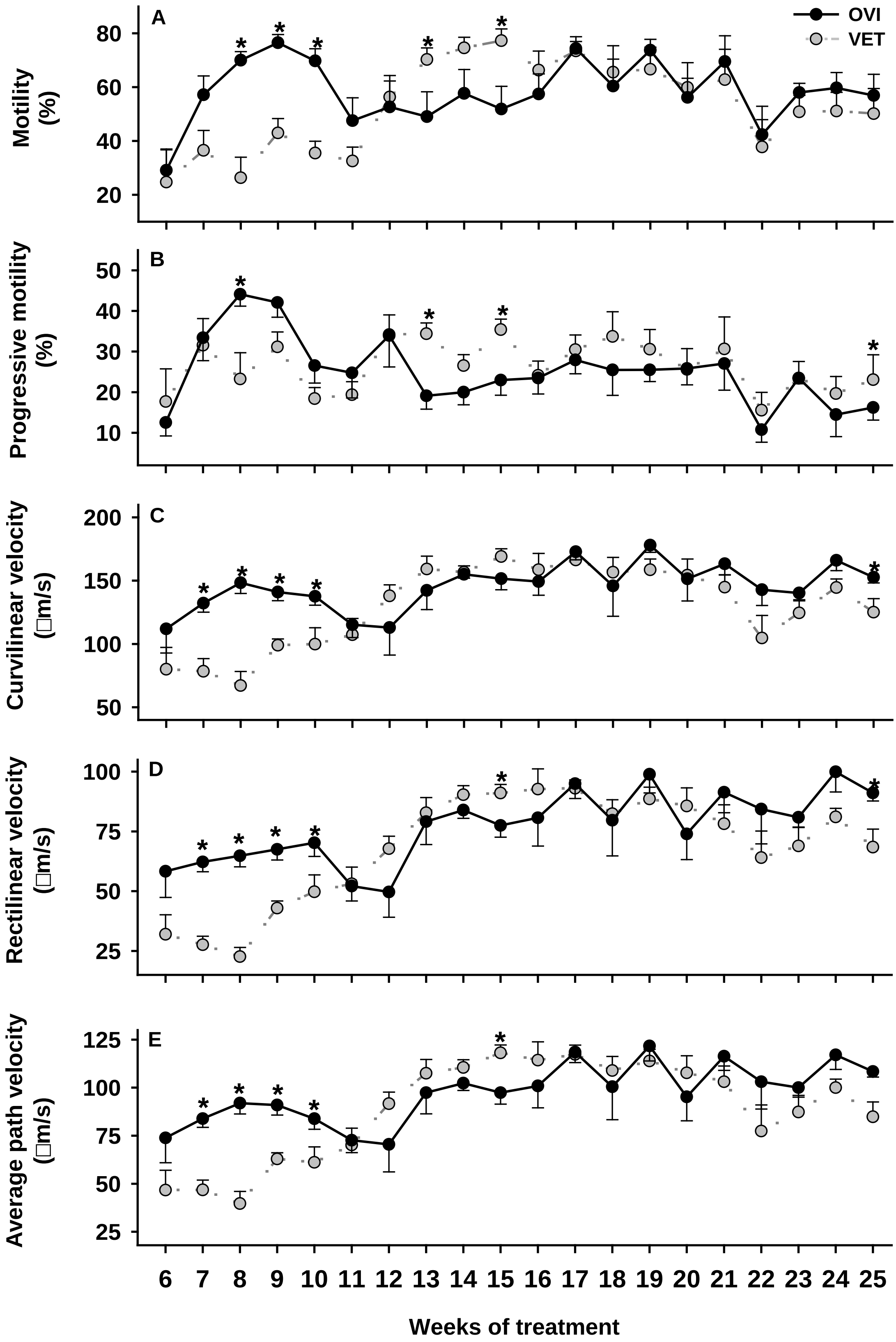
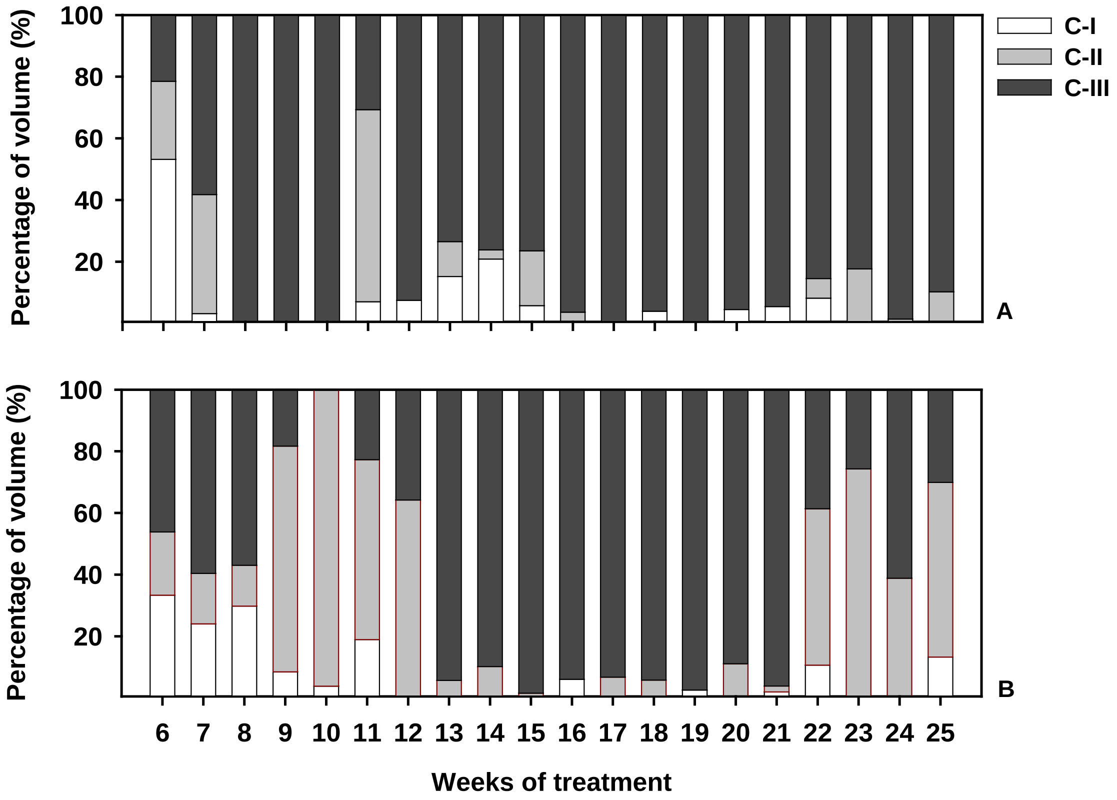
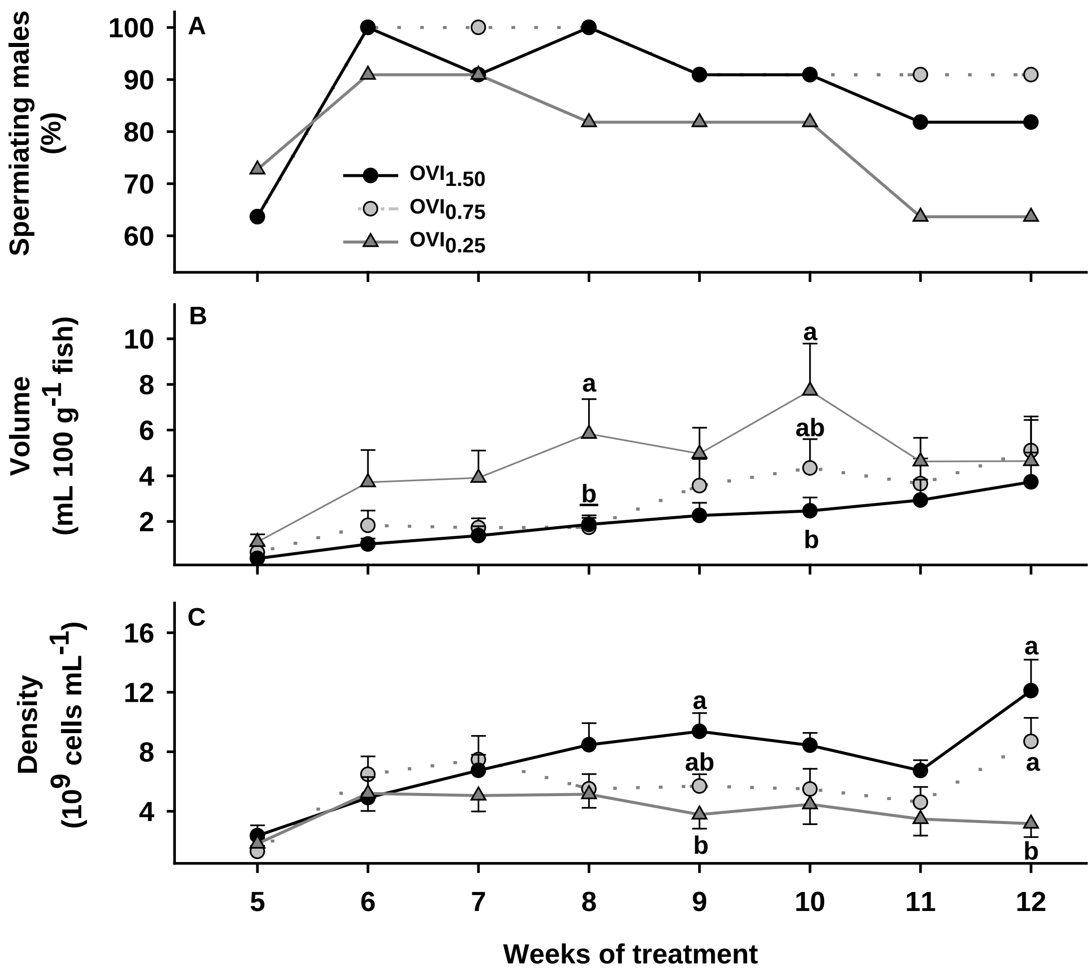

A
B
C
D
E
OVI
VET
Motility (%)
Progressive motility (%)
Curvilinear velocity (□m/s)
Rectilinear velocity (□m/s)
Average path velocity (□m/s)
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Weeks of treatment

**Figure 3.**



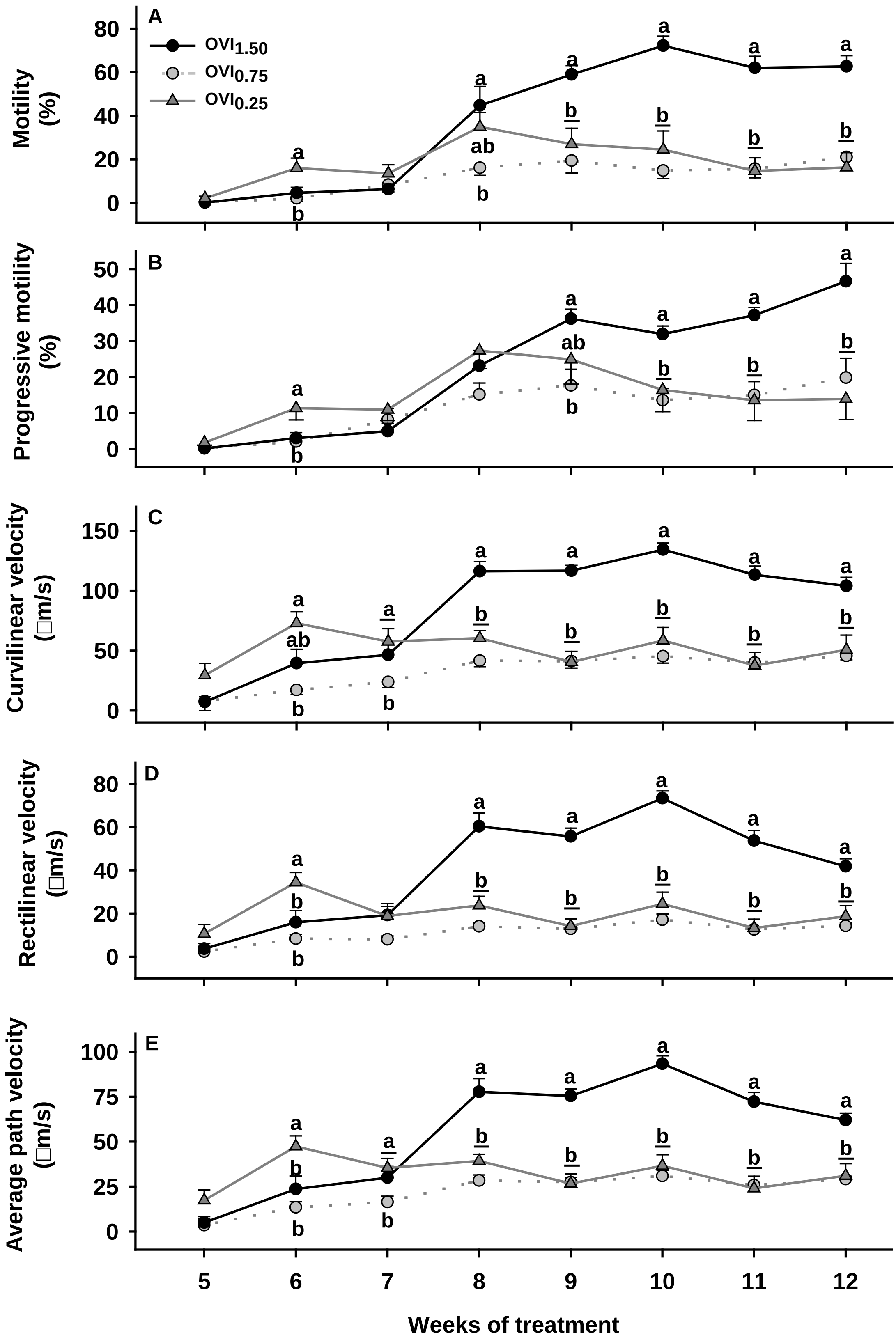
Percentage of volume (%)
100
80
60
40
20
C-I
C-II
C-III
A
B
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Weeks of treatment

**Figure 4.**

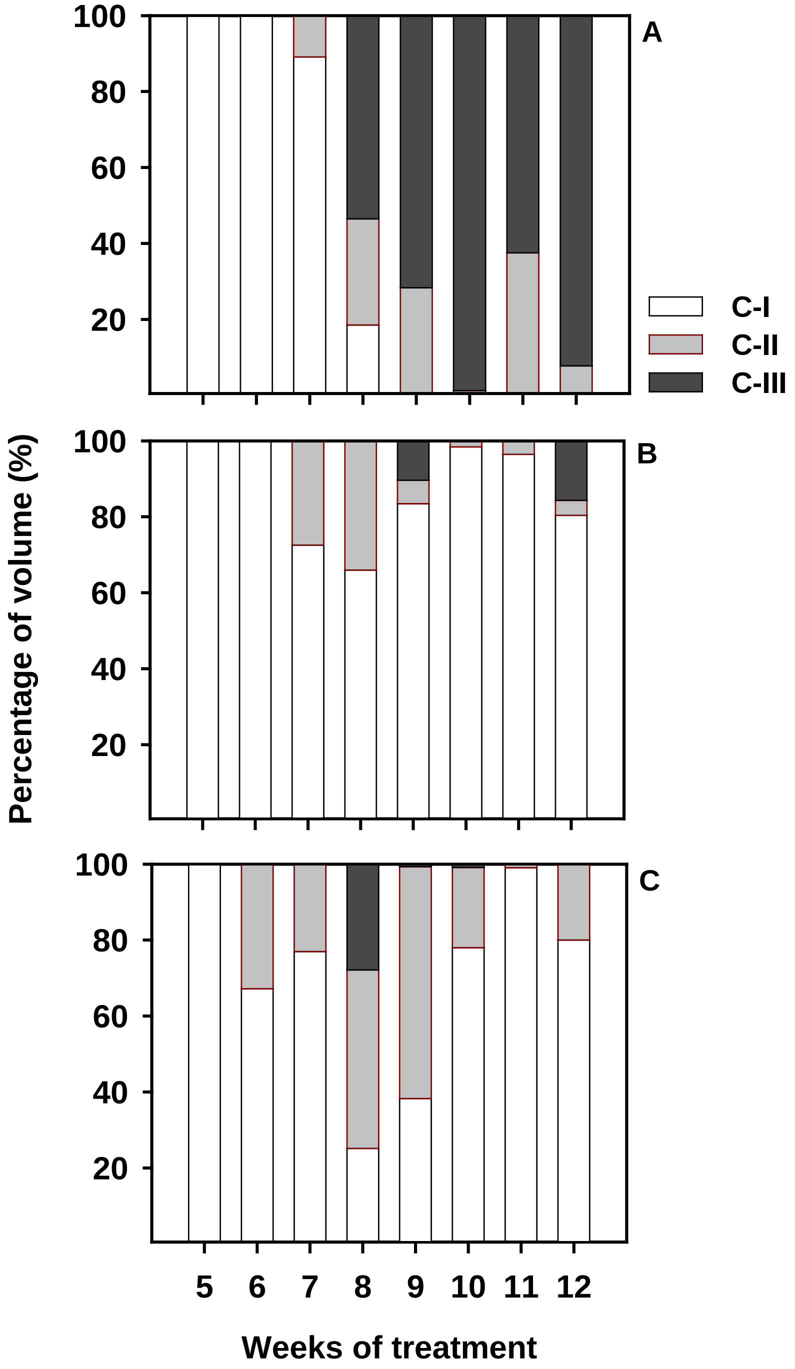


A
Spermiating males (%)
100
90
80
70
60
OVI1.50
OVI0.75
OVI0.25
B
Volume (mL 100 g-1 fish)
10
8
6
4
2
a
b
a
ab
b
C
Density (109 cells mL-1)
16
12
8
4
a
ab
b
a
a
b
5
6
7
8
9
10
11
12
Weeks of treatment

**Figure 5.**


A
B
C
D
E
OVI1.50
OVI0.75
OVI0.25
Motility (%)
Progressive motility (%)
Curvilinear velocity (□m/s)
Rectilinear velocity (□m/s)
Average path velocity (□m/s)
Weeks of treatment
5
6
7
8
9
10
11
12

699 **Figure 6.**

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