Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual maturation.

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Abstract

Since 1960, the European eel (*Anguilla anguilla*) has suffered a dramatic reduction in natural stocks. Breeding in captivity is considered an alternative, but obtaining high quality sperm seems basic on this regard. The main objective of this study was to assess the effects of three thermal regimes (two of them variable: T10 and T15; and one of them constant: T20) and three hormonal treatments with different hormones (hCG, hCG_{rec} and PSMG) on the induction of maturation in European eel males.

In the case of the thermal regimes, our results demonstrated that the onset and progression of spermiation are strongly influenced, and perhaps closely regulated, by water temperature. T20 demonstrated the best results in all the sperm parameters (volume, density, motility, kinetic features, etc) throughout most weeks of treatment, becoming a reliable and productive method for inducing spermiation in this species. In the case of hormonal treatments, the onset and progression of spermiation in European eel males were influenced by the hormone used. In this respect, hCG_{rec} produced the best results in all the sperm parameters including volume, density, motility, kinetic features, etc., throughout most weeks of treatment, thus becoming an effective alternative treatment to the standard hCG treatment used to induce spermiation in eel species. Moreover, hCG_{rec} gave rise to the best economical profitability, making it possible to obtain good quality sperm samples at a lower price than by using the other two hormonal treatments.

Keywords

Temperature; Hormone; Spermiation; CASA; Motility; Sperm

1. Introduction

The European eel (*Anguilla anguilla*) is a teleost fish with a peculiar life cycle: prepubertal eels migrate across the Atlantic Ocean for supposedly 6–7 months to reach the spawning area, in the Sargasso Sea (Tesch, 1978; Van Ginneken and Maes, 2005). In the last years the European eel has suffered a dramatic reduction in its population mainly due to varying factors including overfishing, habitat reduction and contamination (Feunteun, 2002). Therefore, breeding in captivity is the only alternative to save this species, reducing the pressure on natural populations, and meeting the demands of eel farms.

A good tool for a breeding captivity program is obtaining high quality sperm during a large number of weeks with the aim of synchronizing the gametes maturation and fertilizing the ova produced throughout the breeding season (Jorstad and Navdal, 1996; Roldan and Gomendio, 2009).

In some fish species, reproduction in captivity can be controlled exclusively by environmental factors such as temperature, photoperiod or salinity. However, sometimes it is impractical or even impossible to simulate the environmental factors of the breeding process (i.e., spawning migration, depth, pressure, etc.) so the use of exogenous hormones is the only effective way of inducing maturation and spermiation (Boetius and Boetius, 1967; Ohta et al., 1996; Pérez et al., 2000; Asturiano et al., 2006 ; Kagawa et al., 2009;). Eels (*Anguilla* spp.) do not mature spontaneously in captivity, so the maturation of males must be induced with long-term hormonal treatments (Asturiano et al., 2005; Huang et al., 2009; Ohta et al., 1997; Pérez et al., 2000). However, in several studies has been described that the sexual maturation of non-treated males could be stimulated indirectly by treated males, suggesting the existence of chemical communication (pheromones) between them (Huertas et al., 2006). Despite the effectiveness of these long-term hormonal treatments, little attention has been paid to factors such as the duration of spermiation periods, which has been limited in time, or the variations in sperm quality parameters (Asturiano et al., 2005; Miranda et al., 2005; Mylonas et al., 1998).

A high number of environmental and procedural factors can affect the gonadal development and, consequently, the gamete quality (Mylonas et al., 2010). With regards to environmental factors, the water temperature plays a key role in the gonadal development in many fish species (García-López et al., 2006; Lim et al., 2003; Van Der

Kraak and Pankhurst, 1997). In the case of the European eel, the temperature of the hypothetical spawning area is around 20 ºC (Boetius and Boetius, 1967), and this is probably the reason why both males and females of this species have been matured at this constant water temperature (Asturiano et al., 2002, 2006; Pedersen, 2003; Pérez et al., 2000). However, it has been reported that eels undertake vertical movements during their migration across deep and cold waters (Aarestrup et al., 2009), so it seems probable that the gonadal development, that takes several months, occurs at low temperatures, and the spawning at warm temperatures. Recently it has been shown that in female European eel, variable thermal regimes induce hormonal profiles that resemble the natural ones more closely than those obtained under constant temperatures (Pérez et al., 2011).

Regarding procedural factors, both the type and dosage of hormone used are key factors in the artificial maturation of aquaculture species. Hormonal methods have evolved over time, from the use of pituitaries from mature fish to the use of various synthetic agonists of different hormones (Billard and Marcel, 1980; Rosenfeld et al., 2012; Yaron, 1995). Human chorionic gonadotropin (hCG) has been the hormone most widely used in the maturation and spermiation process in the European eel. However, due to recent problems in the availability of this hormone, new hormones should be tested. In this respect, recombinant hCG (hCG_{rec}), produced by recombinant DNA technology (Satish, 1989), could be a good alternative because it has a similar structure to the native hormone. On the other hand, pregnant mare's serum gonadotropin (PMSG), which is a priori a cheaper choice than hCG and hCG_{rec} hormones, has already been used in other fish species (Brzuska and Ryszka, 1990; Nagahama, 1994; Zakes and Demska-Zakes, 2009) to induce both spermiation and ovulation. Therefore, PMSG can be considered as another option for its use in reproduction studies for eel aquaculture.

Nowadays, the weekly administration of hCG under a constant temperature regime of 20 ºC (Asturiano et al., 2005; Peñaranda et al., 2010b; Pérez et al., 2000) has been the most widely used hormonal treatment in European eel males. Despite the good results obtained by this method, the number of weeks in which eels produce a high volume of good sperm is limited, and as such more evolved treatments are necessary to achieve shorter induction times, longer spermiation periods and/or higher volumes of quality sperm. Therefore, the study of alternative hormonal treatments must be an ongoing task in order to improve the current methods to date. In this respect, the aim of this trial was to assess the effect of the thermal regime (3 thermal regimes, including the standard

constant treatment of 20ºC), and the kind of hormone used (3 hormonal treatments, including the standard hCG treatment) on the reproductive performance of European eel males.

2. Materials and methods

2.1 Fish maintenance

Eel males from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were moved to our facilities, in the Aquaculture Laboratory at the Universitat Politècnica de València, Spain. The fish were distributed in aquaria equipped with separate recirculation systems, thermostats/coolers (to control the water temperature in the first experiment) and covered to maintain constant darkness. The eels were gradually acclimatized to sea water over the course of one week (salinity 37 ± 0.3 g/l), and once a week they were anaesthetized with benzocaine (60 ppm) and weighed before receiving the administration of hormones by intraperitoneal injection. 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).

2.2 Thermal treatments

A total of 317 adult male eels (mean body weight 100±2 g) were equally and randomly distributed in six 200-L aquaria (approximately 100 males per treatment) and subjected to three thermal regimes: T10, 10 ºC (first 6 weeks), 15 ºC (next 3 weeks) and 20 ºC (last 6 weeks); T15, 15 ºC (first 6 weeks) and 20 ºC (last 9 weeks); and T20, 20 ºC during the whole experimental period (Figure 1).

All the males were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of human chorionic gonadotropin (hCG; 1.5 IU g^{-1} fish; *Argent Chemical Laboratories*. USA) during 13 weeks.

2.3 Hormonal treatments

In a second experiment, and after choosing the best thermal regime (T20), a total of 54 adult male eels (mean body weight 81±7 g) were equally and randomly distributed in three 200-L aquaria (18 males per treatment) and submitted to three hormonal treatments: hCG, hCG_{rec} (recombinant hCG; *Ovitrelle.* Madrid) and PSMG (pregnant

mare's serum gonadotropin; *Sincropart*, Lab CEVA. Barcelona). All hormones were diluted 1:1 (UI/µl) in saline solution (NaCl 0.9 %) and a weekly dose of 1.5 IU g^{-1} fish was administered during 20 weeks.

2.4 Sperm collection and sampling

Sperm samples were collected 24 h after the administration of the hormone because previous studies (Pérez et al., 2000) have demonstrated that this is when the highest sperm quality is found. For the sperm collection the fish were anesthetized and after cleaning the genital area with freshwater and thoroughly drying to avoid the contamination of the samples with faeces, urine and sea water, the sperm were collected by abdominal pressure. A small aquarium air pump was modified to obtain a vacuum breathing force and to collect the sperm in a tube. A new tube was used for every male and distilled water was used to clean the collecting pipette between the different males.

To measure sperm density, samples were diluted 1:1000 or 1:10000 in P1 medium (in mM: NaCl 125, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, pH 8.5; Asturiano et al., 2004a). Ten microlitres of the dilution were taken for counting in a Thoma haemocytometer and expressed as spermatozoa $x10^9$ ml^{-1}. Sperm volume was measured using graduated tubes and samples were maintained at 4 ºC until analysis and were evaluated in the first hour after extraction.

2.5 Evaluation of motility and kinetic sperm parameters

Sperm was activated by mixing 2 µl of sperm with 200 µl of artificial sea water (Aqua Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH adjusted to 8.2; Peñaranda et al., 2010c). All the motility analyses were performed by triplicate at 30s post-activation by the motility module of ISAS (Proiser R+D, S.L.; Paterna, España) using an ISAS® 782M camera recorder (60 fps; Hz). The chamber used in all experiments was a SpermTrack-10® (Proiser, Paterna, Spain) with 10x negative contrast phase lens in a Nikon Eclipse (E-400) microscope.

The parameters considered in this study were total motility (MOT, %); progressive motility (P-MOT, %), 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 spatial average 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 straight line velocity of over 10 μm/s).

In addition, in order to perform an in-depth analysis of the evolution of sperm quality throughout the weeks of both thermal and hormonal treatments, sperm samples were classified into four classes based on the percentage of motile cells: Class I = No motile cells, II ≤ 25%, III = 25-50% and IV > 50% of motile cells.

2.6 Economical analysis

To analyze the economical profitability of each hormonal treatment (hCG, hCG_{rec} and PMSG) three factors were taken into account: sperm volume, motility class and amount of hormone used. The essential aim was to relate the level of investment with the amount of good quality sperm produced using each hormonal treatment.

2.7 Statistical analysis

The mean and standard error were calculated for all sperm parameters (volume, density, motility and rest of kinetic parameters). Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) and Student's *t*-test were used to analyze data with normal distribution. Significant differences between treatments were detected using the Tukey multiple range test ($P<0.05$). For non-normally distributed populations, Kruskal-Wallis one-way ANOVA on ranks and Mann-Whitney *U*-test were used. 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 Thermal treatments

With regards to the percentage of spermiating males (Figure 2A) the T10 treatment generated lower percentages (with maximum values around 70%) than the T15 and T20 treatments, which reached 100% spermiating males in several weeks. In addition, T10 males did not begin to produce sperm until the 10^{th} week of treatment, whereas T20 and T15 males began spermiating earlier, in the 5^{th} and 6^{th} weeks, respectively.

In all the thermal treatments, there was an increasing trend in sperm volume in the first weeks of spermiation (Figure 2B). T20 males showed higher volumes than T15 and T10 males in the majority of the weeks, with significant differences in the 8^{th} and 11^{th} week. The highest density values were observed in T20 males, with significant differences in most of the weeks (Figure 2C). Similar density patterns were observed in T20 and T15 males, while T10 males showed an increase in their first three weeks of spermiation (10^{th} to 12^{th} week) followed by a marked decrease in the last week of the experiment (13^{th} week).

Regarding total and progressive motility (Figures 2D and 2E), T20 treatment demonstrated the highest values, reaching maximum values of 75 and 35%, respectively at week 11. From this week to the end of treatment, T20 males displayed a marked decrease in total and progressive motility. Males subjected to the T10 thermal regime showed a significant but delayed increase in motility parameters from its first spermiation week (week 10) to the end of the experiment, and ended up exceeding the values obtained with the T15 and T20 treatments due to the fact this coincided with the final reduction of motility in these treatments in the last week of treatment.

The kinetic parameters (Table 1) of the sperm cells showed a similar evolution in all thermal treatments, increasing as the weeks advanced. T20 was the treatment that demonstrated the highest values in the three velocity parameters (VCL, VSL and VAP), with significant differences in several weeks.

Finally, with regards to the sperm motility classes, it was observed that the T10 and T20 treatments displayed better volume profiles (with relative volumes of maximum quality sperm (class IV) of 60% and 70%, respectively) than the T15 treatment, which showed values of around 50% in this same motility class (Figure 3). In addition, the T20 treatment resulted in a higher number of weeks (7) with good quality samples, compared to either the T10 or T15 treatments, which only induced these good sperm samples for 3 and 5 weeks, respectively (Figure 4).

3.2 Hormonal treatments

PMSG treatment induced lower percentages of spermiating males (with maximum values around 50-60%) than hCG and hCG_{rec} treatments (Figure 5A), both of which reached 100% in several weeks. In addition, PMSG males did not begin to spermiate until the 8^{th} week of treatment, while hCG and hCG_{rec} males began spermiating earlier, in the 5^{th} week.

255 An increasing trend in sperm volume (Figure 5B) was displayed in all the hormonal
256 treatments and hCG- and hCG_{rec}-treated males showed higher values than PSMG-
257 treated males, with significant differences in the 10^{th}, 11^{th}, 14^{th} and 18^{th} weeks.
258 Maximum values for PMSG, hCG and hCG_{rec} treatments were obtained in the 15^{th}, 19^{th}
259 and 20^{th} week, respectively.
260 Sperm density showed high variability under all the hormonal treatments (Figure 5C).
261 Samples from males treated with hCG_{rec} demonstrated an increase in the first 5 weeks
262 of treatment reaching maximum values of 18 x 10^9, followed by a decrease from the
263 10^{th} week until the end of treatment. However, in the five first weeks of spermiation
264 hCG-treated males displayed a progressive decrease and a gradual decline until the end
265 of the treatment, reaching minimum values in the 19^{th} week. PMSG-treated males
266 generated the highest values at the end of the treatment, with significant differences in
267 the 15^{th}, 17^{th}, 18^{th} and 19^{th} weeks.
268 With regards to motile and progressive motile cells (Figuress 5D and 5E), hCG_{rec}
269 treatment generated the highest values, reaching maximums of 70 and 35% (motile and
270 progressive motile cells, respectively) in the 9^{th} week. Males treated with hCG showed a
271 similar motility pattern to hCG_{rec} males in the first weeks of treatment, but displayed a
272 decrease in motile and progressive motile cells from the 11^{th} week to the end of
273 treatment. PMSG-treated males showed an upward trend from the 11^{th} week, reaching
274 motility and progressive motility values similar to hCG_{rec}-treated males in the last 7
275 weeks of treatment.
276 The hCG_{rec} and hCG treatments produced the highest values (with significant
277 differences) in VCL, VSL and VAP throughout the first weeks of spermiation (Table 2).
278 However, from the 14^{th} week of treatment hCG-treated males displayed a sharp decline
279 in these parameters, whereas values of hCG_{rec}-treated males remained constant until the
280 end of treatment. PMSG males showed an increasing trend over the weeks reaching
281 values similar to those of hCG_{rec} males in the last weeks of treatment.
282 Finally, with regards to the sperm motility classes, hCG_{rec} and PMSG treatments
283 produced better volume profiles (with class IV sperm volumes around 70% and 60%,
284 respectively) than hCG treatment, which resulted in values of less than 30% for the
285 same motility class (Figure 6). hCG_{rec} treatment induced good quality samples (class
286 IV) in every week of treatment (except in the 5^{th} week), while hCG and PMSG
287 treatments demonstrated some weeks without good sperm samples (week 6 and 3,
288 respectively) throughout the treatment (Figure 7).

3.3 Economical analysis

The investment needed to obtain mature males was quite different in each hormonal treatment (Table 3). hCG_{rec} treatment signified the highest investment per male and an amount of 19.5 € was necessary in order to mature each animal throughout the 21 weeks of treatment. hCG and PMSG treatments represented a smaller investment per male (7.5 and 10.6 €, respectively). However, the total volume of class IV sperm obtained from hCG_{rec}-treated males was much higher, therefore the final profitability of this hormone was the best, as it was possible to obtain one millilitre of the highest quality sperm for the lowest price (0.5 €/mL). The other hormones produced worse economic results, and PMSG was found to be the most expensive treatment (1.8 €/mL).

4. Discussion

4.1 Thermal treatments

Temperature is one of the most important environmental factors affecting aquatic wildlife organisms, where seasonal changes in this parameter, interacting with the photoperiod signal, can regulate the sexual maturation process (Dorts et al., 2011; Van Der Kraak and Pankhurst, 1997).

In terms of aquaculture production, obtaining spermiating males weeks in advance means minimising costs and risks in fish handling. In this respect, T20 males began spermiating earlier and also demonstrated higher percentages of spermiating males in all weeks, compared to the alternative thermal treatments (T15 and T10). In addition, it was observed that warm temperatures were strictly necessary in inducing sperm production in European eel males: fish that underwent both T10 and T15 thermal regimes did not begin to produce sperm until they had spent 1-2 weeks at 20 ºC. Therefore, it seems that the lower temperatures used during the first weeks of T10 and T15 treatments were capable of preventing the spermiation process and thus, the production of sperm. In a previous study about the temperature effect in the sexual maturation on the European eel, Boetius and Boetius (1967) could obtain males in an earlier stage of maturity, in which the lumen of their spermatic tubules is being filled of spermatozoa, from a wide temperature range of 13 to 25.5 ºC after 10 weeks of treatment. However, the sperm

volume obtained in this trial was not good, and a mathematical analysis of the temperature/maturation period data revealed an optimum temperature of about 20°C .

In this respect, it is well known that water temperature can modulate the enzymatic activity necessary for the synthesis of steroids and its receptors and so, the different thermal treatments applied in this study could be regulating all stages of spermatogenesis and spermiogenesis throughout the gonadal development (Billard et al., 1982; Schulz and Miura, 2002; Peñaranda et al., 2011). Previous studies have demonstrated the effects of temperature on the spermatogenesis of fish: in rainbow trout (*Salmo gardnierii*) low temperatures stimulate the first stages of this process, while warm temperatures stimulate the latter stages (Breton and Billard, 1977); in Nile tilapia (*Oreochromis niloticus*) higher temperatures accelerate spermatogenesis, whereas at lower temperatures it takes longer (Vilela et al., 2003).

On the other hand, parameters such as volume and density are usually analysed in sperm studies in order to report information on the amount of sperm available for use in reproductive events like artificial gamete fertilizations, etc. In the present study, the volume data agrees with values obtained by other authors of European eel studies (1-4 mL $100g^{-1}$ fish; Asturiano et al., 2005, Pérez et al., 2000), and the sperm density values obtained were significantly higher than those obtained in these same experiments (values around 1-2 $x10^{9}$ ml^{-1}). In this respect, it would be important to find out the minimum sperm-to-egg ratio needed for successful fertilization, but this parameter is only known for a limited number of species and few studies have been developed with regards to the European eel (Sorensen et al., unpublished results).

On the other hand, total motility and progressive motility are recognized as important sperm traits for male fertility and sperm competition, because significant correlations were found between the number of motile spermatozoa and fertilization rates in some fish species (Liu et al., 2007; Ottesen et al., 2009; Rurangwa et al., 2004). In our study, T20 was the treatment that demonstrated the best values throughout most of the weeks, whereas alternative thermal regimes (T10 and T15) did not reach such high values, except for T10 males that did show values of around 70% of motile cells in the last week of treatment. Therefore, T20 was the most effective treatment with regards to these parameters (total and progressive motility), resulting in good quality samples (class IV) in almost every week of treatment, from the 5^{th} week onwards.

In addition to percentage of motile spermatozoa as a good tool to predict fertilization ability, kinetic sperm parameters (VCL, VSL or VAP) provided by CASA software

may also serve as prognostic indicators of the fertilization potential of sperm (Donnelly et al., 1998; Gage et al., 2004; Rudolfsen et al., 2008). In our study, T20 males showed higher velocity values than T15 and T10 males in most of the weeks of treatment, and VCL values were comparable with data previously reported in European eel: 134 μm/s (Gibbons et al., 1985), 160 μm/s (Woolley, 1998) or 125 μm/s (Asturiano et al., 2005). However, VSL values obtained under these thermal treatments were significantly higher than those reported in the cited literature. With VSL being one of the most important kinetic parameters (probably because spermatozoa with faster straight line speeds have more chance of contacting an oocyte in the natural environment), sperm samples induced in the present study showed better quality spermatozoa than those demonstrated in previous reproduction studies of this same species (Gibbons et al., 1985; Woolley, 1998).

In summary, our results demonstrate that the onset and progression of spermiation in European eel males are strongly influenced, and perhaps closely regulated, by changing water temperature. The T20 regime showed the best results in all of the sperm parameters (volume, density, motility, kinetic features, etc) throughout most weeks of treatment, becoming a reliable and productive method for inducing spermiation in this species.

4.2 Hormonal treatments

Our results indicate that the type of hormone used significantly affected the onset and progression of spermiation in European eel males. Recombinant hCG (hCG_{rec}) produced the best results in almost all the sperm parameters throughout the weeks of treatment, becoming an effective and alternative treatment to that of the standard hCG used to induce the spermiation in European eel at 20 ºC.

With regards to to the onset of spermiation, both hCG- and hCG_{rec}-treated males began to produce sperm in 5^{th} week of treatment, and both treatments induced a high percentage of spermiating males in the following weeks. Previous studies have reported similar results with this hormonal treatment both in European eel, where eel males usually begin producing sperm in the 4^{th}-5^{th} week of treatment (Asturiano et al., 2006; Pérez et al., 2008, Peñaranda et al., 2010a,b), and in Japanese eel, where males usually begin producing sperm in the 5^{th}-6^{th} week of treatment (Ohta et al., 1996, 1997). However, males induced by PMSG began to spermiate later, in the 8^{th} week, showing a

lower percentage of spermiating males throughout all the treatment. It appears that this hormone caused a delay in the gonadal development and thus, late spermiation.

These different responses found in eel males regarding the different hormonal treatments could be explained by the biological activity of each hormone. This bioactivity depends on dimerization and glycosylation, which are processes occurring in the rough endoplasmic reticulum and Golgi apparatus (Ulloa-Aguirre et al., 2001). There are different degrees and types of glycosylation, and depending on these types, gonadotropins will show more or less bioactivity (Hearn and Gomme, 2000; Ulloa-Aguirre et al., 1999). In the present study, the hormones used to induce the maturation in the European eel had different characteristics and origins: hCG is a hormone produced during human pregnancy and purified from urine, while hCG_{rec} is a recombinant version of endogenous hCG produced in Chinese hamster ovary (CHO) cultured cells by recombinant DNA technology. Both hormones (hCG and hCG_{rec}) are analogues of the luteinizing hormone (LH). On the other hand, PMSG is a complex glycoprotein obtained from the serum of pregnant mares and acts like a follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Therefore, considering the different nature and origins of these hormones, it is possible that each hormone has different degrees and types of glycosylation and thus performs the stages of maturation in a different way.

On the other hand, sperm volume and density are important parameters that have traditionally been used for the assessment of semen quality. Customarily, sperm volume obtained from the artificially maturated eel males has been unsuitable compared with the volume of eggs obtained from eel females (Ohta and Unuma, 2003). In this trial, milt volume increased gradually under all the treatments as the number of injections increased, probably due to the cumulative effect of hormones in the first weeks (Asturiano et al., 2005; Ohta et al., 1996; Pérez et al., 2000) and finally, due to the hydration controlled by the maturation-inducing steroids (MIS) in the last weeks (Asturiano et al., 2004b). hCG_{rec}- and hCG-treated males showed the highest volumes throughout the treatment (>5 mL 100 g^{-1} fish from the 15^{th} week onwards), exceeding previous values obtained both in European (Pérez et al., 2000) and Japanese (Ohta and Unuma, 2003) eel males. A reverse trend was evidenced in density and volume from the 10^{th} week of treatment: as the sperm volume increased, the density values decreased. This opposite pattern may be explained by the fact that high densities are probably necessary in order to compensate for the small volumes and, in the

opposite case, low densities need to be compensated by high volumes of sperm production.

On the other hand, with regards to motility and progressive motility, hCG_{rec} was the treatment that produced the best values (both high and stable) in every week. In eel species, the potential of sperm motility is usually acquired during the period between the 7^{th} and 9^{th} injection (Pérez et al., 2000; Ohta and Unuma, 2003). In this trial hCG_{rec}- and hCG-treated males showed a similar trend, while PSMG-treated males did not showed high percentages of motile cells until the 15^{th} week. hCG_{rec} treatmentgenerated good quality samples for the largest number of weeks, displaying samples of this kind (class IV) in almost every week of treatment. The kinetic parameters showed a similar trend to that of the motility data and hCG_{rec} was the treatment that induced higher velocities throughout all the sampling weeks. hCG- and PMSG-treated males also showed good values in these parameters, but only at the beginning and end of treatment, respectively.

Our results demonstrate that the onset and progression of spermiation in European eel males are influenced by the hormone used. In this respect, hCG_{rec} showed the best results in all the sperm parameters (volume, density, motility, kinetic features, etc…) throughout most weeks of treatment, becoming an effective and alternative treatment to the standard hCG treatment used to induce spermiation in eel species.

4.3 Economical analysis

From a practical point of view, the best hormonal treatment is one which is able to provide samples with high values of volume, density, motility and kinetic parameters for as many weeks as possible. However, due to the current economic crisis, the aquaculture sector is going through a delicate situation and cheaper and more effective treatments are becoming more and more necessary. In the present study, hCG_{rec} treatment generated the best results, improving on the results obtained by hCG hormone, which nowadays is the most widely used method of inducing male maturation in eel species. In addition, despite the greater investment required for hCG_{rec}-treated males, the final profitability of this hormone was demonstrated to be the best, making it possible to obtain one millilitre of good quality sperm for a lower price than possible using either of the other two hormonal treatments (hCG and PSMG).

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Table legends

Table 1. Kinetic parameters (VCL, VSL and VAP) of sperm cells throughout the thermal treatments (T10, T15 and T20). Data are expressed as mean (SEM) and different letters indicate significant differences between treatments.

Table 2. Kinetic parameters (VCL, VSL and VAP) of sperm cells throughout the hormonal treatments (hCG, hCG_{rec} and PMSG). Data are expressed as mean (SEM) and different letters indicate significant differences between treatments at one sampling time.

Table 3. Profitability of hormonal treatments (hCG, hCG_{rec} and PMSG) in relation to economic investment and production of high-quality (class IV) sperm.

Figure legends

Figure 1. Thermal regimes applied for each treatment (T10, T15 and T20). Arrows indicate the first injection with human chorionic gonadotropin (hCG).

Figure 2. Evolution of sperm quality parameters throughout the temperature treatments (T10, T15 and T20): A) Percentage of spermiating males; B) Sperm volume; C) Sperm density; D) Percentage of motile cells and E) Percentage of progressive motile cells. Data are expressed as mean ± SEM and different letters indicate significant differences between treatments at each week of treatment.

Figure 3. Percentage of total volume for each motility class (I-IV) in thermal treatments. Motility classes: Class I = No motile cells; II ≤ 25%; III = 25-50% and IV > 50% of motile cells.

Figure 4. Percentage of spermiating males from each motility class (I-IV) in each week throughout the thermal treatments: A) T10; B) T15 and C) T20. Motility classes: Class I = No motile cells; II ≤ 25%; III = 25-50% and IV > 50% of motile cells.

Figure 5. Evolution of sperm quality parameters throughout the hormonal treatments (hCG, hCG_{rec} and PMSG): a) Percentage of spermiating males; b) Sperm volume; C) Sperm density; D) Percentage of motile cells and E) Percentage of progressive motile cells. Data are expressed as mean ± SEM and different letters indicate significant differences between treatments at each week of treatment.

Figure 6. Percentage of total volume of each motility class in hormonal treatments. Motility classes: Class I = No motile cells; II ≤ 25%; III = 25-50% and IV > 50% of motile cells.

Figure 7. Percentage of spermiating males from each motility class (I-IV) in each week throughout the hormonal treatments: A) hCG; B) hCG_{rec} and C) PMSG. Motility classes: Class I = No motile cells; II ≤ 25%; III = 25-50% and IV > 50% of motile cells.

690 **Table 1**

	VCL						VSL						VAP					
Week	**T10**		**T15**		**T20**		**T10**		**T15**		**T20**		**T10**		**T15**		**T20**	
5					65.5	(12.0)					25.5	(6.2)					39.6	(7.3)
6			127.8	(43.6)	95.9	(16.2)			61.0	(31.7)	39.0	(9.2)			83.0	(37.8)	56.1	(10.4)
7			24.1	(24.1)	77.2	(16.8)			5.4	(5.4)	27.6	(6.0)			12.1	(12.1)	43.8	(9.5)
8			82.2	(23.5)	140.0	(9.0)			36.3	(12.3) b	63.3	(6.2) a			48.6	(14.8) b	85.7	(5.9) a
9			85.6	(22.1) b	142.6	(9.0) a			39.9	(10.3) b	65.7	(5.3) a			53.7	(13.4) b	88.1	(5.9) a
10	33.5	(33.5)	117.7	(20.9)	126.0	(10.8)	6.7	(6.7) b	51.2	(14.4) ab	63.9	(6.0) a	15.7	(15.7) b	69.9	(15.8) ab	83.0	(7.1) a
11	122.1	(13.6)	122.7	(17.5)	149.9	(6.4)	49.1	(7.1)	57.5	(11.2)	72.2	(4.8)	69.8	(8.3)	76.0	(13.0)	94.3	(5.6)
12	119.2	(9.8)	123.1	(11.4)	121.0	(8.4)	57.8	(4.7)	52.8	(9.5)	57.0	(4.2)	77.0	(6.3)	71.5	(9.5)	73.5	(5.0)
13	133.2	(13.6) a	113.6	(12.4) ab	74.1	(5.1) b	56.4	(7.8)	49.1	(8.8)	30.2	(4.2)	79.7	(9.4) a	67.9	(9.4) ab	45.8	(3.8) b

691 **Table 2**

	VCL						VSL						VAP					
Week	**hCG**		**hCG_{rec}**		**PMSG**		**hCG**		**hCG_{rec}**		**PMSG**		**hCG**		**hCG_{rec}**		**PMSG**	
5	121.8	(0.0)	113.4	(14.1)			63.4	(0.0)	55.3	(13.7)			77.4	(0.0)	67.2	(14.1)		
6	86.5	(6.6)	108.1	(12.9)			30.4	(5.1) b	50.4	(7.1) a			49.9	(5.0)	67.5	(8.8)		
7	111.3	(14.9)	127.7	(13.2)			48.7	(7.5)	66.6	(7.1)			66.5	(9.4)	85.3	(8.6)		
8	129.7	(8.5)	141.2	(10.5)	109.1	(0.0)	60.6	(5.7)	72.5	(7.0)	50.7	(0.0)	80.9	(6.4)	91.6	(7.9)	66.5	(0.0)
9	141.3	(18.7) ab	166.6	(4.1) a	36.5	(36.5) b	63.4	(8.5) ab	86.2	(3.9) a	14.9	(14.9) b	85.3	(11.3) a	110.9	(3.6) ab	21.9	(21.9) b
10	96.5	(24.4) ab	147.5	(7.8) a	67.1	(29.6) b	46.3	(12.2) ab	72.3	(5.0) a	31.0	(14.7) b	59.7	(15.4)	94.9	(5.7) a	41.2	(18.9) b
11	119.2	(13.4) a	137.0	(7.0) a	65.9	(14.2) b	52.9	(9.2) ab	70.0	(5.4) a	31.2	(8.3) b	73.6	(10.2) a	90.6	(6.0) a	42.9	(10.4) b
12	118.1	(5.2) ab	130.5	(10.7) a	82.0	(19.5) b	59.2	(5.5)	68.9	(6.9)	40.0	(10.9)	74.8	(5.6)	86.9	(7.8)	53.1	(13.5)
13	112.3	(10.4) ab	145.6	(7.6) a	107.2	(10.9) b	43.9	(6.5) b	75.3	(5.7) a	48.3	(8.3) b	63.4	(7.1) b	94.9	(5.9) a	66.2	(8.8) b
14	129.8	(9.5)	159.5	(7.2)	132.7	(12.3)	54.6	(5.0) b	76.8	(4.9) a	56.4	(6.7) ab	75.9	(6.1) b	100.9	(5.0) a	78.9	(8.0) ab
15	71.9	(33.2) b	171.8	(7.7) a	130.0	(20.9) ab	32.2	(16.0) b	86.1	(5.8) a	57.3	(10) ab	44.2	(20.8)	110.0	(5.9) a	80.1	(13.8) ab
16	56.2	(34.9) b	148.3	(15.6) a	150.2	(11.4) a	26.9	(16.8)	73.6	(8.3)	68.6	(8.5)	35.7	(22.3)	95.6	(9.9)	90.8	(9.1)
17	127.7	(16.2)	155.4	(12.8)	153.3	(8.3)	59.5	(10.4)	71.2	(7.0)	69.9	(5.3)	78.4	(11.5)	94.8	(8.4)	95.7	(6.3)
18	108.9	(23.6)	156.4	(12.1)	133.5	(15.0)	39.6	(9.7) b	83.4	(8.9) a	59.2	(10.6) ab	60.9	(13.8)	103.4	(9.2) a	81.2	(11.7) ab
19	109.7	(54.9)	116.0	(15.7)	150.6	(7.8)	48.3	(26.6)	56.0	(8.2)	71.2	(5.5)	65.2	(33.8)	73.9	(10.3)	94.4	(6.5)
20	0.0	(0.0) b	140.7	(9.5) a	123.1	(26.2) a	0.0	(0.0) b	69.2	(7.4) a	60.0	(13.8) a	0.0	(0.0) b	90.2	(7.7) a	78.1	(17.2) a

692 **Table 3**

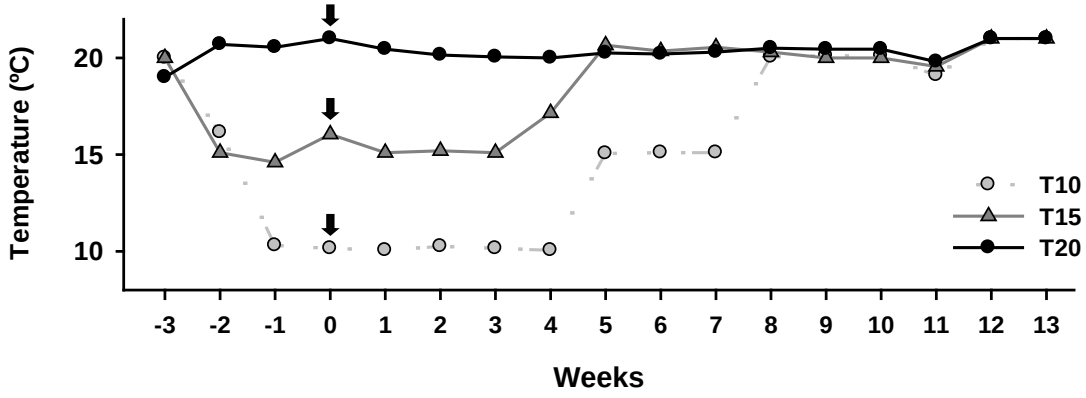
		hCG	hCG_{rec}	PMSG
Hormone price	€/IU	0.003	0.008	0.004
[a]Dose price	€/g $_{\text{treated fish}}$	0.005	0.012	0.007
[b]Investment per male	€/male	7.5	19.5	10.6
cSperm (class IV) price	€/mL	0.7	0.5	1.8

693
694 [a] Hormone Price x 1.5 IU g^{-1}

695 [b] Investment to maturate one male during 21 weeks of treatment.

696 [c] Total Investment / Total Volume of Sperm (class IV)

697 **Figure 1**



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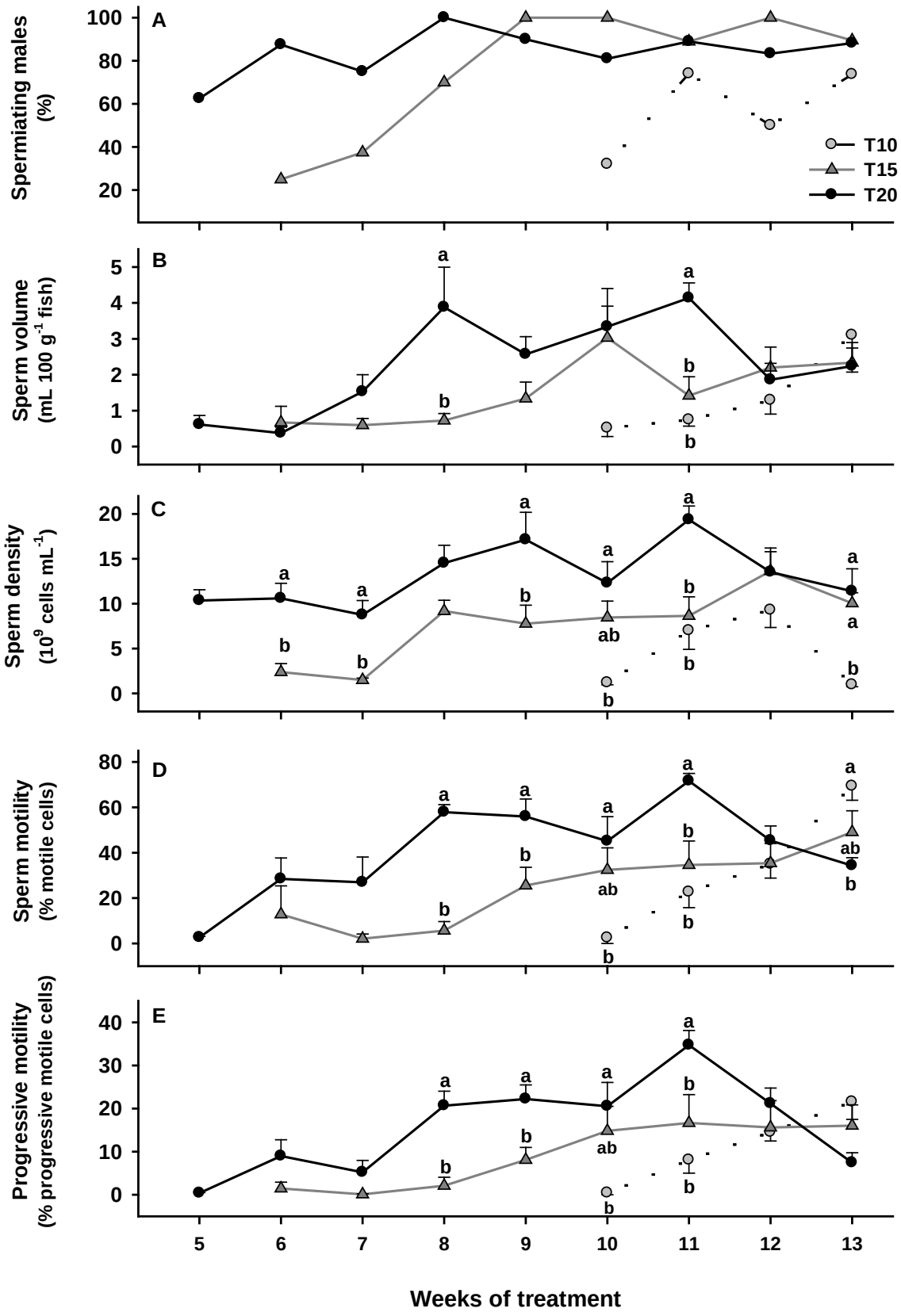
699 **Figure 2**



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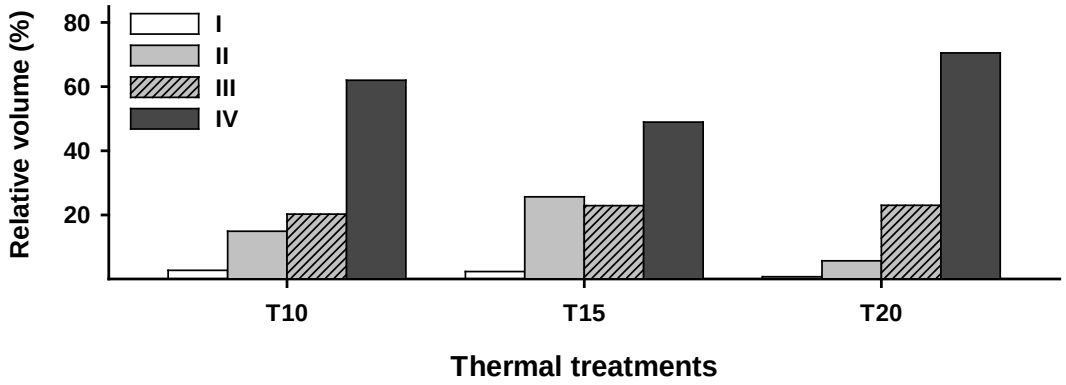
701 **Figure 3**



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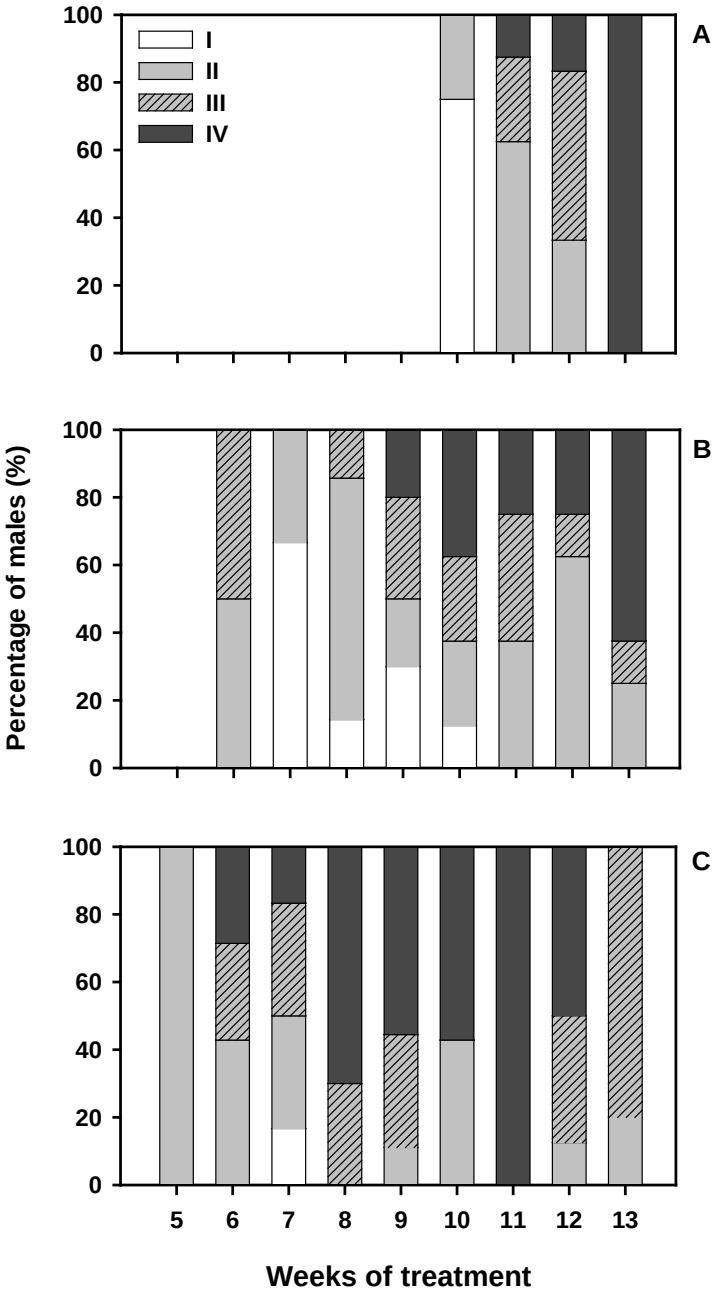
703 **Figure 4**



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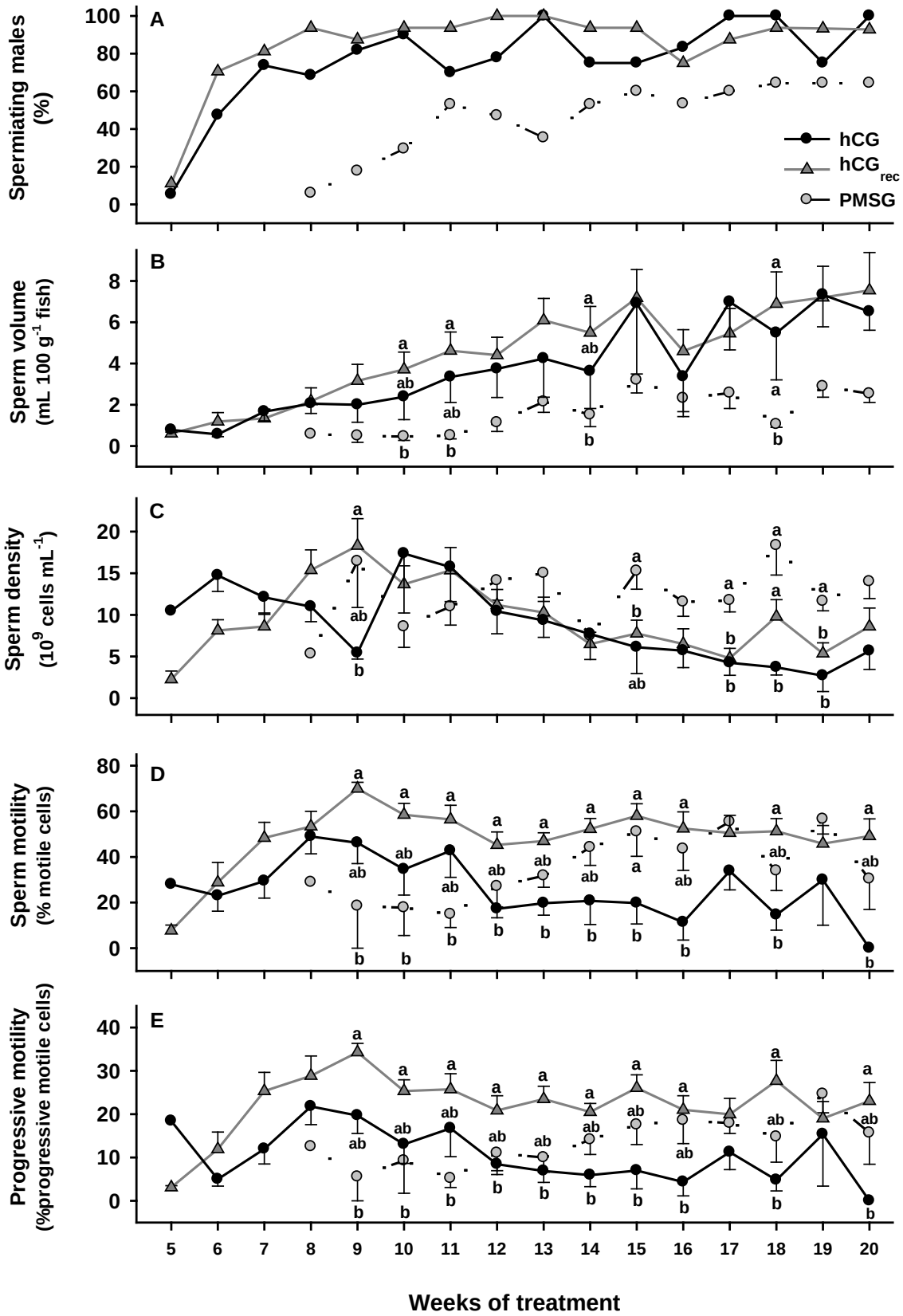

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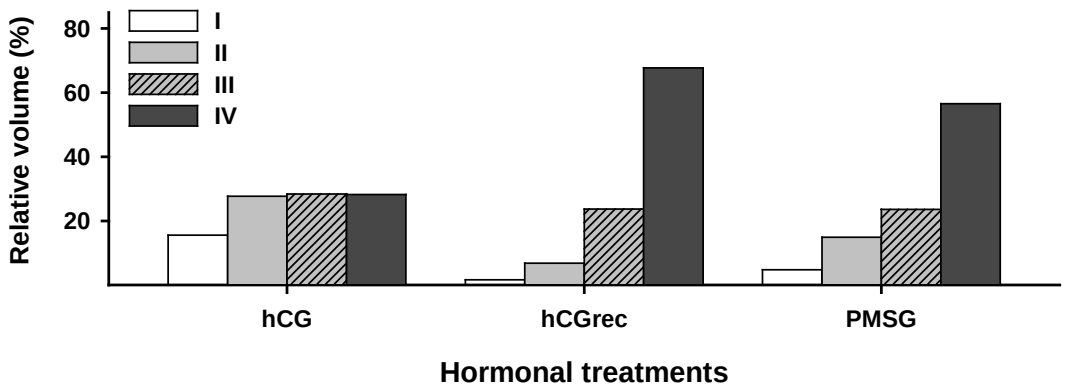
708 **Figure 6**



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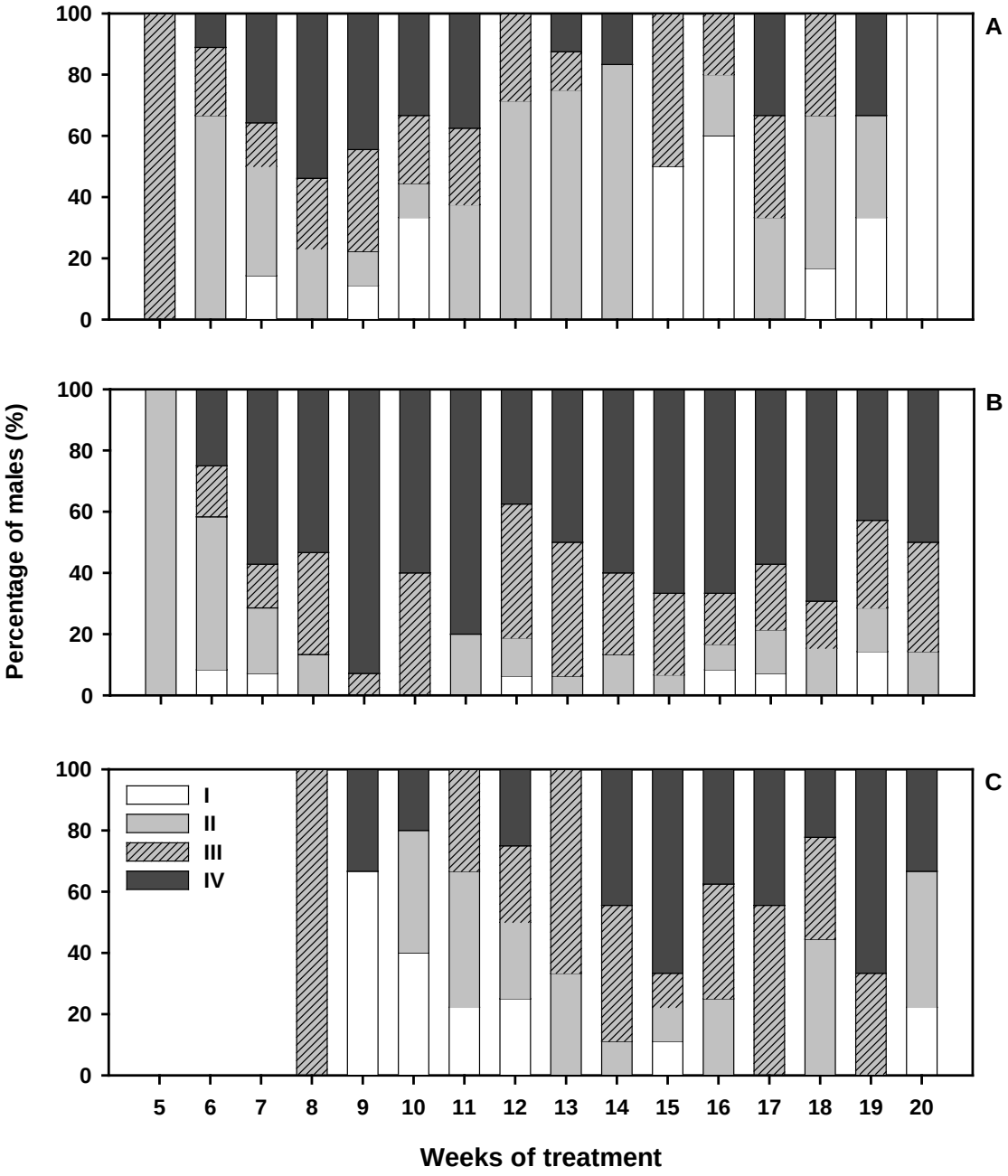
710 **Figure 7**



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