Aquaculture
Vol. 255, Issues 1-4, 31 May 2006, Pages 522-531
http://dx.doi.org/10.1016/j.aquaculture.2006.01.009

Human chorionic gonadotropin induces spermatogenesis and spermiation in 1-year-old European sea bass (Dicentrarchus labrax): Assessment of sperm quality

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Abstract: The aims of the present study were (a) to compare sperm quality (percentage of motile spermatozoa, motility duration, density and fertility after cryopreservation) between precocious and normally maturing male European sea bass *Dicentrarchus labrax*, (b) to examine the potential of human chorionic gonadotropin (hCG) to increase spermiation in precocious males and (c) to examine the potential of hCG to induce spermatogenesis and spermiation in non-precocious 1-year-old males. One hundred precocious and 100 non-precocious fish were each randomly divided in two groups each: control (precocious saline-treated and non precocious saline-treated) and treated (precocious hCG-treated and non precocious hCG-treated). Treated groups were administered weekly with 1000 IU hCG kg^{-1} body weight while control groups were injected with physiological solution. Milt volume produced, sperm concentration, motility duration and fertilising ability were assessed every week in each group. The effect of the hormonal treatment on gonadal development was examined based on the gonadosomatic index and testicular histology. The results demonstrate that sperm produced by precocious fish has characteristics (mean value of motility class, mean maximum motility duration, concentration and fertility after cryopreservation) similar ($P > 0.05$) to those produced by 2-year-old fish. Human chorionic gonadotropin treatment in precocious fish resulted in a significant increase ($P < 0.05$) of milt volume, without affecting sperm quality. In non-precocious fish, hCG treatment resulted in greater percentage of spermiation ($P < 0.05$) compared to non-precocious saline-treated group. At the end of the trial (three weeks), 29 out of 50 non-precocious hCG-treated fish were spermiating and, within these 23 produced > 200 µl per fish of milt. No differences were observed in terms of sperm concentration, motility class, motility duration and fertilizing capacity due to hCG treatment in either precocious, or non-precocious fish. In addition, analysis of the testicular histology of fish that did not spermiate after hCG treatment, shows a significant ($P < 0.05$) enhancement of testicular development stages. The present study demonstrated that (a) precocious European sea bass males produce milt of comparable sperm characteristics to adult individuals, (b) treatment of non-precocious males with hCG induced spermatogenesis and spermiation and (c) treatment of precocious males with hCG enhanced milt volume without affecting other sperm characteristics, including fertilizing ability.

Keywords: Spermatogenesis; Spermiation; European sea bass; hCG; Dicentrarchus labrax

1. Introduction

The European sea bass Dicentrarchus labrax (L.) is an important commercial species and has been the subject of both basic and applied research. Studies have been carried out on its biology, control of reproduction, gamete quality, broodstock management and offspring quality (Carrillo et al., 1993; Pickett and Pawson, 1994; Carrillo et al., 1995). Under optimal conditions, European sea bass reproduce spontaneously in captivity. Females attain sexual maturity at 3 years of age and males at 2 years of age, but under culture conditions a large number of precocious males are observed (Carrillo et al., 1995; Asturiano et al., 2000).

The age at which fish reach sexual maturity is important for aquaculture, since in some species maturing individuals exhibit reduced somatic growth, as they divert energy from muscle growth into gonadal development (Bye and Lincoln, 1986). In these cases, sexual maturation is generally undesirable in fish production. On the other hand, fish breeders may benefit from using early maturing broodstock, to reduce generation interval, allowing faster selection of genetic characters of interest. From this point of view, the development of methods to stimulate spermatogenesis and to enhance milt production, maintaining a good sperm quality, would be beneficial to aquaculture.

It is now well established that stimulation of milt production in adult teleosts can be achieved by treatment with either gonadotropins (GtH) or gonadotropin releasing hormones and their synthetic agonists (GnRHa) (Zohar and Mylonas, 2001). Among the mammalian gonadotropins, human chorionic gonadotropin (hCG) is effective in inducing spermatogenesis and spermiation in fish (Stacey and Peter, 1979; Donaldson and Hunter, 1983). Human chorionic gonadotropin has been used successfully in goldfish Carassius auratus, rainbow trout

Oncorhynchus mykiss, gilthead bream Sparus aurata, European eel Anguilla anguilla, and Japanese eel Anguilla japonica (Donaldson and Hunter, 1983; Ohta et al., 1997), New Zealand snapper Pagrus auratus (Pankhurst, 1994) and catfish, Pangasius bocourti (Cacot et al., 2003). No information is available, so far, on the use of hCG to stimulate spermatogenesis and spermiation in European sea bass.

Zanuy et al. (1999) showed that sustained administration of testosterone (T) stimulates spermatogenesis in prepubertal European sea bass, and suggested that T could be involved in the onset of puberty, probably acting via positive feedback on the GnRH system. In adult European sea bass, GnRH treatment of mature males during the reproductive period enhanced sperm production (Sorbera et al., 1996). Since no information is available concerning the effect of hCG treatment on both spermatogenesis and spermiation in one-year old European sea bass, the aims of the present study were (a) to compare sperm quality (motility class and duration, density, fertilizing capacity and endurance to cryopreservation) between precocious and normally maturing males, (b) to examine the potential of hCG to increase spermiation in precocious males and (c) to examine the potential of hCG to induce spermatogenesis and spermiation in non-precocious 1-year-old males.

2. Materials and methods

2.1. Fish

The experiments were performed during January at the IFREMER station of Palavas Les Flots (France). One year old European sea bass (mean weight ± SD, 125± 34 gr) were taken from a mixed stock (rearing in natural photothermal regime during the preceding year), and were maintained under constant temperature (12 ± 1°C) and light (8L /16D) for the entire duration of the experiment. Fish were anesthetised in 2-phenoxyethanol (120 ppm) (Sehdev et al., 1963) and checked for the presence of running milt by application of gentle abdominal pressure. Males were classified as spermiating (precocious fish) if milt could be expressed. One hundred precocious and 100 non-precocious fish were selected. Fish were distributed in four identical fibreglass tanks (50 per tank). Each tank was lightproof, circular (2 m diameter) and was provided with well-aerated running seawater (35‰). Fish were fed daily to satiation with a commercially available dry diet (47% protein and 18% fat). Prior to the start the trial, fish were individually weighed and marked by tags (Fish eagle PIT tags, USA) placed in the dorsal musculature. Sperm stripped from reared adult European sea bass (2 years old, length 32-38 cm, weight 450-550 g, and maintained under natural temperature) was used as control for quality assessment of sperm obtained from precocious fish. The evaluation of the sperm quality was performed at the beginning of the trial after the selection of the groups.

2.2 Experimental protocol and sampling

Experiment 1: effect of hCG treatment on precocious fish.

Fish were divided into an experimental and a control group (see above) as follows: 50 spermiating fish, were injected weekly for three weeks with 1000 IU hCG Kg^{-1} BW (precocious hCG-treated). As control, 50 spermiating fish were injected weekly for three weeks with physiological saline (precocious saline-treated). Before each handling, the fish were anaesthetised with phenoxy-2 ethanol. All fish of each group were stripped weekly. Milt volume was assessed on all fish while sperm quality was assessed only on ten fish per group. The gonadosomatic index (GSI) and the testis histology were determined at the end of the trial (3 weeks) on ten spermiating fish randomly taken per group. The gonadosomatic index (GSI) was estimated as follows: $GSI = (G_W/B_W) * 100$, where G_W is the gonad weight and B_W is the body weight of each animal.

Experiment 2: effect of hCG treatment on non-precocious fish.

Fish were divided into an experimental and a control group as follows: 50 non-precocious fish were injected weekly for three weeks with 1000 IU hCG Kg^{-1} BW (non precocious hCG-treated). As control, 50 non-precocious fish were injected weekly for three weeks with physiological saline (non precocious saline-treated). Before each handling, the fish were anaesthetised with phenoxy-2 ethanol. All fish of each group were stripped weekly to evaluate the number of spermiating males. Fish that resulted non-spermiating, either before and/or after hCG- and saline treatment were classified as immature. Within the spermiating males after one week of hCG treatment, ten were stripped weekly to assess sperm quantity and quality. Sperm stripped from precocious saline-treated European sea bass was used, as control for quality assessment of spermiating non-precocious fish after hCG-treatment. In order to evaluate the stages of gonadal development,

GSI and testis histology were determined weekly by collecting gonads from fish that did not spermiate (immature) after the hCG treatment, and from fish non-spermiating (immature) belonging to saline-treated group (n=8-10). In addition, the GSI and the gonadal histology were also determined at the end of the trial in spermiating non-precocious fish after hCG-treatment, in spermiating non-precocious fish after saline-treatment and in non spermiating, non precocious saline-treated group (n=5-10).

2.3. Gonadal development and testes histology

Testes were quickly removed, weight to the nearest 0.001 g and fixed in Bouin's fixative. Serial 6 μm-thick parafin sections were stained with haematoxylin and eosin. Testicular development was classified using the maturation stages of Zanuy et al. (1999). Briefly: undifferentiated gonads (Stage I) contained only isolated germinal cells and somatic elements; early differentiated testis (Stage II) were organized in seminiferous lobules and differentiated spermatogonia; immature differentiated testes (Stage III) contained cysts of spermatogonia distributed to the periphery and a wide lobular lumen; early maturing testis (Stage IV) had an increased lobular size and cysts containing spermatocytes; maturing testis (Stage V) had cysts containing cells in all stages of development (spermatogonia, spermatocytes and spermatids) and same spermatozoa were released into the lumen; Spermiating fish (Stage VI) had lobules filled with sperm that was released into the seminiferous duct.

2.4. Gamete collection

For sperm collection, a gentle abdominal pressure was applied and a drop of milt was collected from the dry-blotted gonopore area into a 2 ml syringe. Urine and potentially urine-contaminated semen were discarded carefully. The semen was maintained at 4°C until used.

Females were induced to spawn by a single injection of 10 μg Kg^{-1} of [D-Trp^6]-gonadotropin-releasing hormone-agonist ([D-Trp^6]-GnRHa) and were allowed to ovulate in individual tanks (1 m^3) maintained at 13°C, about 72 h after injection. For each fertilization trial, eggs from one female were collected by stripping and were assessed for viability by their morphological features (perfect rotundity, development of a perivitelline space, yolk translucency) under a dissecting microscope, according to Fauvel et al., (1992).

2.5. Assessment of sperm characteristics, cryopreservation and insemination

Sperm concentration, percentage of spermatozoa showing forward motility, and fertilising ability were determined. In addition, the effect of cryopreservation on sperm quality was examined.

Sperm concentration was determined according to Fauvel et al. (1999) by spectrophotometry (Beckman DU600) at 260- nm, using the equation SC=(0.806 OD-0.032) 10^8, where SC and OD are sperm concentration (spermatoza mL^{-1}) and optical density, respectively. To assess sperm motility, sperm samples were diluted initially to 1:150 (v:v) in an isotonic non-activating medium (NAM) containing (in mg ml^{-1}) 3.5 NaCl; 0.11 KCl; 21.23 MgCl; 0.39 CaCl2; 1.68 NaHCO3; 0.08 Glucose; 10 BSA; pH=7.7 and the absence of motility was checked. Then, aliquots of 6 μl were immediately mixed with 60 μl seawater. The samples were observed for the first time 10 seconds after activation, under the

microscope (x40 magnification) connected to a camera and a video monitor. The percentage of motile cells was evaluated simultaneously by two observers in three replicates per sample using the mean for statistical analyses. Motility was categorized (motility class) according to Suquet et al. (1992a) as 0 for immotile sperm, 1 for 0-20% motile cells, 2 for 20-40%, 3 for 40-60%, 4 for 60-80% and 5 for 80-100%. The duration of motility was defined as the period of time between activation and cessation of any forward movement.

The cryopreservation protocol was applied to sperm collected from adult and precocious European sea bass. Immediately after collection, fresh sperm was diluted 1:3 in freezing diluent. The freezing diluent used was Mounib's medium complemented with 10% dimethyl sulfoxyde and 10 mg mL^{-1} bovine serum albumin (Dreanno et al., 1997). The dilution was not allowed to equilibrate and was immediately placed in straws (50 µl fresh sperm per straw) and then directly subjected to the freezing protocol. Straws were placed for 15 min on a tray in nitrogen vapour, 6.5 cm above liquid nitrogen surface. Straws were thawed in a waterbath at 35°C for 5 s (Fauvel et al., 1998). Fertilising ability of fresh and cryopreserved sperm was compared using the following experimental protocol. Before freezing, sperm was diluted 1:3 (v/v) in Mounib's medium and fresh sperm were prepared by direct dilution 1:3 (v/v) in NAM in order to keep similar insemination conditions. Aliquots of egg from the same batch (5 ml, containing about 5000 eggs) were placed in 10-ml beackers and inseminated with 150 µl of diluted sperm to obtain ~500 10^3 spermatozoa per egg as previously suggested (Fauvel et al., 1999). Frozen sperm samples were thawed just before insemination in order to avoid a possible decrease of fertility due to post-thaw delay. Sperm samples were mixed with the eggs using gentle agitation. Fertilisation was

triggered by adding 2.5 ml of seawater (38 psu; 13°C). Inseminated eggs were then transferred into 100-ml container with sea water. After 3 h at 13°C, the fertilisation success was assessed under a dissecting microscope, by examing 100 randomly chosen eggs. Eggs were assumed to be fertilised when they contained an embryo at the four-cell stage or greater.

2.6. Statistical analysis

The statistical analyses were performed using the SYSTAT statistical analysis package (SYSTAT, 1991). The means of continuous variables such as sperm volume and concentration, motility duration, GSI and fertilization success after angular transformation (when required) were compared by means of one-way or two-way analysis of variance (ANOVA) followed by a Tukey test of pairwise multiple comparisons. The comparison of motilities determined as discrete variables (classes) were achieved using a Friedmann repeated-measures ANOVA on ranks. Percentage of spermiation and frequency distribution of testicular classes were analyzed, after arcsin transformation, using a Chi-squared test. In all cases, differences were accepted at $P < 0.05$. Data are expressed as means ±S.E.M.

3. Results

3.1. Characterization of precocious European *sea bass sperm*

Fresh sperm produced by precocious fish had mean value of motility class, mean maximum motility duration, sperm concentration and fertilization ability similar (Two-way ANOVA; $P > 0.05$) to that produced by adult 2-year-old fish (Fig. 1). The motility duration of sperm collected both by adult and precocious fish was significant (Two-way ANOVA; $P<0.05$) reduced by cryopreservation process (Fig. 1).

3.2. Effect of hCG treatment on spermiation of precocious European *sea bass*

Treatment with hCG after one or two weekly injections induced a significant increase of milt volume (One-way ANOVA; Tukey's HSD, $P < 0.05$), with respect to the controls. No difference between hCG-treated and control (saline – treated) was observed after the third injection (Fig. 2A).

No significant differences were observed between precocious hCG-treated and precocious saline-treated in terms of sperm concentration, motility duration (One-way ANOVA; $P>0.05$) and class (Friedmann repeated-measures ANOVA on ranks; $P>0.05$) (Table 1). Sperm concentration ranged between 45 and 66 $x10^9$ spermatozoa ml^{-1} in the precocious hCG-treated group and from 45 to 60 $x10^9$ spermatozoa ml^{-1} in the precocious saline-treated group. The motility class ranged between 4 and 5, and the motility duration was greater than 50 sec.

No significant differences (Two-way ANOVA; $P > 0.05$) were observed in terms of GSI between precocious hCG-treated and precocious saline-treated groups (Fig. 3). The GSI values of both precocious hCG-treated and precocious saline-

269 treated are significant (Two-way ANOVA; P<0.05) different from those of
270 immature fish.
271
272 *3.3. Effect of hCG treatment on non-precocious* European *sea bass*
273 A higher percentage (Chi-squared test; P<0.05) of spermiating fish was
274 observed in the non-precocious hCG treated group compared to non-precocious
275 saline group, starting from the first week of hormone application (Table 1). At the
276 end of the trial, 29 out of 50 of the hCG-treated fish were spermiating and among
277 these 23 produced a large amount (>200 µl per fish) of milt sufficient to perform
278 the sperm quality analyses. On the contrary, at the end of the trial 5 out of 50 fish
279 of the non precocious saline-treated group were spermiating and only one of these
280 produced a large volume of milt.
281 The spermiating fish of non precocious hCG -treated group produced milt
282 continuously up to the 3rd week of the experiment although the volume declined
283 gradually (Fig. 2B). No differences were observed in terms of sperm
284 concentration, motility class, motility duration and fertilizing ability due to the
285 hormonal treatment (Table 1). Sperm concentration ranged between 46 and 60
286 x10^{9} spermatozoa ml^{-1} in both groups. The motility class ranged between 4 and 5,
287 and the motility duration was longer than 50 sec.
288 No significant differences (Two-way ANOVA; P > 0.05) were observed in
289 terms of GSI between spermiating, non-precocious hCG-treated and
290 spermiating, non precocious saline-treated fish (Fig. 3). These GSI values
291 resulted significant (Two-way ANOVA; P<0.05) different from that determined
292 in immature fish (not spermiating, non precocious saline-treated fish). In
293 addition, the GSI determined weekly in fish that were immature (belonging to

non precocious hCG-treated group) was similar (Two-way ANOVA; $P>0.05$) to the GSI of immature animals of control group (non precocious saline-treated group).

All non-spermiating fish had fully differentiated testes in which the spermatocysts contained germ cells at various stages of development (stage II, III, IV and V). The histological examination (data not shown) indicated that, the testes of immature fish, belonging to non precocious saline-treated group had a higher frequency of stages II (30%) and III (35%) with a small percentage of stage IV (18%) and stage V (20%). On the contrary, hCG treatment induced a significant (Chi-squared test; $P<0.05$) increase of testicular development (46% stage III, 42% stage IV and 40% stage V) (Fig.4A). In addition, the gonads of spermiating, non-precocious fish after hCG treatment (Fig. 4B) were in similar stage of development compared to the gonad of precocious hCG-treated fish (Fig . 4C).

4. Discussion

The present study demonstrates for the first time the ability of hCG to increase spermiation in precocious European sea bass and enhance spermatogenesis and spermiation in non-precocious one-year-old fish.

Fish breeders may benefit from using early maturing broodstock since the generation interval would be reduced, allowing more frequent selection. In order to be used for reproduction, the quality of the sperm produced by precocious animals must be similar to that of adults and its quantity must be adequate. Our data demonstrate that precocious European sea bass are reproductively functional, since the quality of their sperm is similar to that of adults in terms of concentration, motility class, motility duration and fertilizing capacity. In addition, the sperm's ability to endure cryopreservation was also similar in precocious and adult fish. Futhermore, milt volume produced by precocious European sea bass (about 1,6 ml/Kg body weight) is similar to that produced by adult fish (Sorbera et al., 1996). Previous studies carried out on stripped bass (Morone saxatilis) and rainbow trout have demonstrated that sperm stripped from precocious and adult fish posses similar motility and relative volume of milt produced but different concentration, though no information is available on their fertilizing ability (Holland et al., 1996; Liley et al., 2002).

In the present paper it was also demonstrated that the volume of milt produced by precocious animals can be increased by hCG treatment, without affecting gamete quality. In a variety of adult freshwater and seawater teleosts, it has been demonstrated that hCG both stimulates advancement of spermiation (Crim et al., 1983; Sorenson and Pankhurts, 1988) and promotes an increase in milt volume

(Takashima et al., 1984; Kreiberg et al., 1987; Yeuh et al., 1990). Our results suggest that hCG has similar effects in precocious European sea bass. The conclusion is also supported by the observation that a single injection of hCG in precocious European sea bass produced a significant increase of milt volume, similar to the situation in adult catfish (Cacot et al., 2003) and adult Rhynchocypris oxicephalus (Park et al., 2002). In many teleost fishes, plasma luteinizing hormone (LH) levels increase just prior to production of the expressible milt (Swanson, 1991) and remain stable during the spermiation period (Breton et al., 1988). Human chorionic gonadotropin is an LH-like hormone that mimics LH and, due to its relatively long retention time in circulation (Ohta and Tanaka, 1997), it stimulates the spermiation process by acting on gonadal maturation (Miura et al., 1991a). The increase of expressible milt observed after the hCG treatment in the present study is in agreement with the established role of LH in regulating the process of spermiation (Swanson, 1991; Nagahama, 1994). In adult European sea bass, the relationship between continuous stimulation of LH release and the corresponding enhancement of milt production has been demonstrated (Mañanòs et al., 2002).

Hormonal treatment induced high percentage of spermiating fish with a high milt volume produced. The sperm stripped from these fish showed similar qualitative characteristics (concentration, motility percentage and duration, and fertilisation capacity) to that of both precocious and adult fish. The increase of spermiation percentage due to the first injection of hCG, could be explained by the capacity of hCG (LH-like hormone) to induce the emptying of spermatocysts that are in an advanced stage of spermatogenesis. LH stimulates sperm hydration, migration and release (Schulz and Miura, 2002).

The histology of the testis of non-precocious fish spermiating after hCG treatment showed similar characteristics to those of precocious treated fish. Furthermore, in fish which did not become spermiating after treatment, we observed a high degree of incidence of stages IV and V of testicular development. These results suggest an involvement of the hCG (LH-like) hormone in the induction of spermatogenesis and are in agreement with the results reported for adult European sea bass (Rodrìguez et al., 2000; Mañanos et al., 2002) and other species (Weil and Crim, 1983; Pankhurst et al., 1986; Pankhurst, 1994).

Treatment of non-precocious European sea bass with hCG produced both completion of spermatogenesis and initiation of spermiation. Our results are consistent with those reported for Japanese and European eel, in which both a single administration or weekly injections of hCG induced spermatogenesis **(**Khan et al., 1987; Miura et al., 1991a; Ohta et al., 1996a; Ohta and Tanaka, 1997; Perez et al., 2000). The failure of the hCG treatment to produce spermiation in some non-precocious European sea bass could be explained at least partially, by the individual variability of gonadal development observed at the time of the first injection. Human chorionic gonadotropin administration to fish at advanced stages of development could induce spermiation, while when administered to fish at early developmental stages, could only stimulate advancement in gonadal maturity but not spermiation. Other possible reasons could be differences in plasma hCG concentration (after the injection) or differences in the androgen production in response to the hormonal stimulation, as also reported for Japanese eel (Ohta and Tanaka, 1997). The observation that the GSI was similar in animals which were spermiating naturally and in hCG-treated animals suggests that hCG stimulate spermatogenesis and spermiation in a physiological manner.

Artificial induction of testicular maturation by hormonal treatment could affect the quantity and the quality of milt produced (Zohar and Mylonas, 2001). For example in Japanese eel hCG treatment led to a production of a small sperm volume (Ohta et al., 1997), while injections of pituitary extracts in adult carp Cyprinus carpio and rainbow trout decreased sperm concentration, by increasing seminal fluid production but not spermatozoa production (Clemens and Grant, 1965). However, more recently, it has been demonstrated that a decrease of sperm concentration does not occur in European sea bass and white bass Morone chrysops (Sorbera et al., 1996; Mylonas et al., 1997). In precocious and non-precocious European sea bass, our results also demonstrated that hormonal treatment with hCG produced an increase of milt volume production without affecting concentration or quality.

Milt production in some fishes is affected by stripping frequency. For example, in turbot Scophthalmus maximus and European sea bass during the first reproductive season, an increase in stripping decreased the period of spermiation (Suquet et al., 1992b; Fauvel et al., 1999). Similar to these findings, we observed that in precocious and spermiating non-precocious hCG-treated European sea bass, the milt volume stripped decreased during the trial. In adult European sea bass at the end of the reproductive period, GnRHa implants also failed to prevent decrease due to successive stripping (Rainis et al., 2003), while both in GnRH-treated and control fish, the frequency of stripping had no deleterious effect on length of the spermation period (Sorbera et al., 1996). Therefore, our results suggest that the effect of stripping on sperm production in precocious European sea bass was similar to that of fish that are in the first reproductive cycle.

In conclusion, it was demonstrated that precocious European sea bass are reproductively functional, since the quality of their sperm is similar to that of adults in terms of concentration, motility class, motility duration and fertilizing capacity. Our results demonstrate that hCG treatment could be used to increase the sperm produced by precocious 1-year-old European sea bass males without altering sperm quality, and that hCG treatment also enhanced spermatogenesis and spermiation in non-precocious fish.

Acknowledgements

The authors wish to thank Mrs. Nicolette S. James for her language assistance.

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Table 1

The effect of weekly treatment of non-precocious and precocious European sea bass with hCG on percentage of spermiation, sperm motility, density and fertilizing ability in an in vitro insemination trial.

Weeks	I	II	III	I	II	III
	Precocious saline-treated n=10			Precocious hCG-treated n=10		
Fertilizing rate (%)	65 ± 5	68 ± 10	60 ± 18	66 ± 2	65 ± 5	68 ± 5
Spermatozoa (x10^9 ml)	55 ± 5	53 ± 4	50 ± 5	58 ± 8	57 ± 6	53 ± 8
Motility class	4.5	4.3	4.2	4.6	4.6	4.2
Motility duration (sec)	80 ± 18	70 ± 15	68 ± 18	75 ± 5	83 ± 5	67 ± 10
	Non precocious saline-treated			Non precocious hCG-treated		
Spermiation (%)	[a] 6 % (3/50)	[a] 2.7 % (1/37)	[a] 3.8 % (1/26)	[b] 44 % (22/50)	[a] 5 % (1/20)	[b]60 % (6/10)
	Precocious saline-treated n=10			Non precocious hCG-treated n=10		
Fertilizing rate (%)	62 ± 2	60 ± 20	65 ± 18	58 ± 1	60 ± 5	68 ± 5
Spermatozoa /ml x10^9	53 ± 5	50 ± 4	52 ± 7	55 ± 7	54 ± 6	58 ± 8
Motility class	4.4	4.5	4.3	4.4	4.2	4.5
Motility duration (sec)	78 ± 20	70 ± 20	75 ± 18	68 ± 5	70 ± 5	68 ± 5

Values are means ± SE. Within row, different letters indicates statistically significant differences (Chi-squared test; $P<0.05$). The ratios in brackets represent the number of spermiating fish on total. The absolute value of the denominator decrease since: 1) each week fish (8-10) that were not spermiating were used to perform histological studies and 2) it does not include fish which spermiated during the previous week.

Figure captions

Fig. 1. Mean (± SEM) sperm motility class and duration (upper panel), density and fertilizing ability (lower panel) of precocious and adult European sea bass (n=10). Asterisks indicate significant differences (Two-way ANOVA; $P< 0.05$) between fresh and cryopreserved sperm.

Fig. 2. Mean (± SEM) volume of expressible milt (ml/Kg $^{-1}$ body weight) produced by precocious fish (A; n=50) and non-precocious fish (B) after one, two or three hCG injections. In B; n=10 for hCG-treated fish (chosen within those become spermiating after the first treatment), and n= 3 for saline-treated (all fish become spermiating after the first week). Asterisks indicate significant differences (One-way ANOVA; Tukey's HSD, $P< 0.05$) between saline-treated and hCG-treated fish, at each sample time.

Fig. 3. Mean (± SEM) gonadosomatic index (GSI) determined after three determined after three weekly hCG injection in precocious (Saline-treated and hCG-treated) and in spermiating non-precocious fish after treatment (Saline-treated and hCG-treated) (n=5-10). The GSI was determined at the end of the trial in fish stripped every week. Asterisks indicate significant differences between immature and both precocious and non-precocious fish (Two-way ANOVA; $P< 0.05$).

Fig. 4. Micrographs of testis belonging to: (A) immature, (B) spermiating non-precocious hCG-treated fish, and (C) precocious hCG-treated fish sampled at the end of the experiment. (A) Maturing Testis. All stages of germ cell development

593 are present: Primary spermatogonia (sgA), primary spermatocytes (sc1),

594 secondary spermatocytes (sc2), spermatids (spt) and spermatozoa (sz). (B, C)

595 spermiating fish . Lumen of the lobules are filled with sperm. Scale bars= 30 µm.

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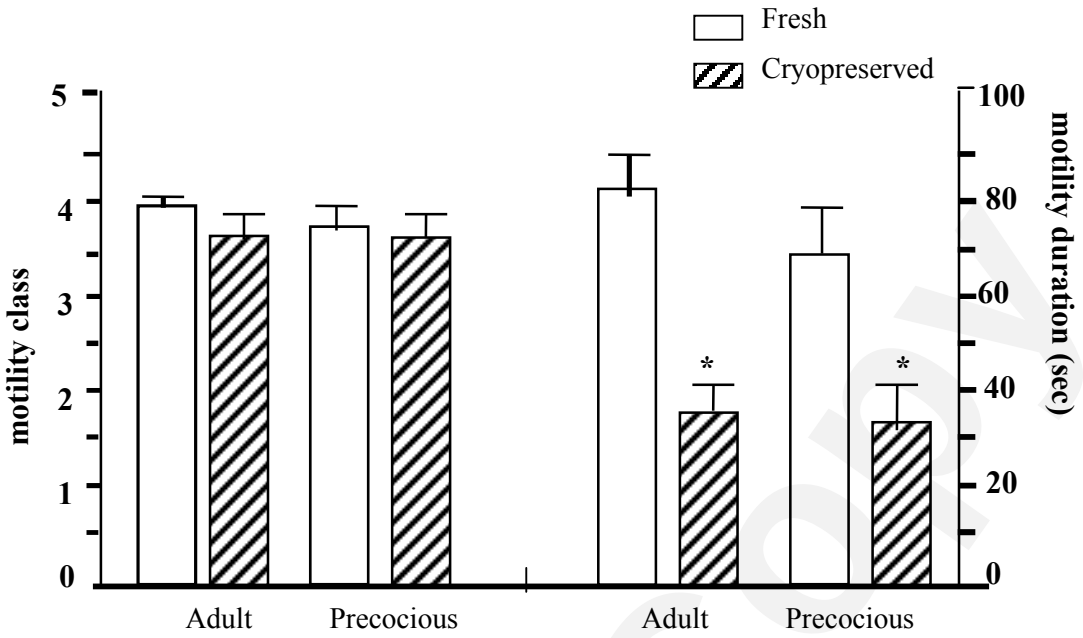


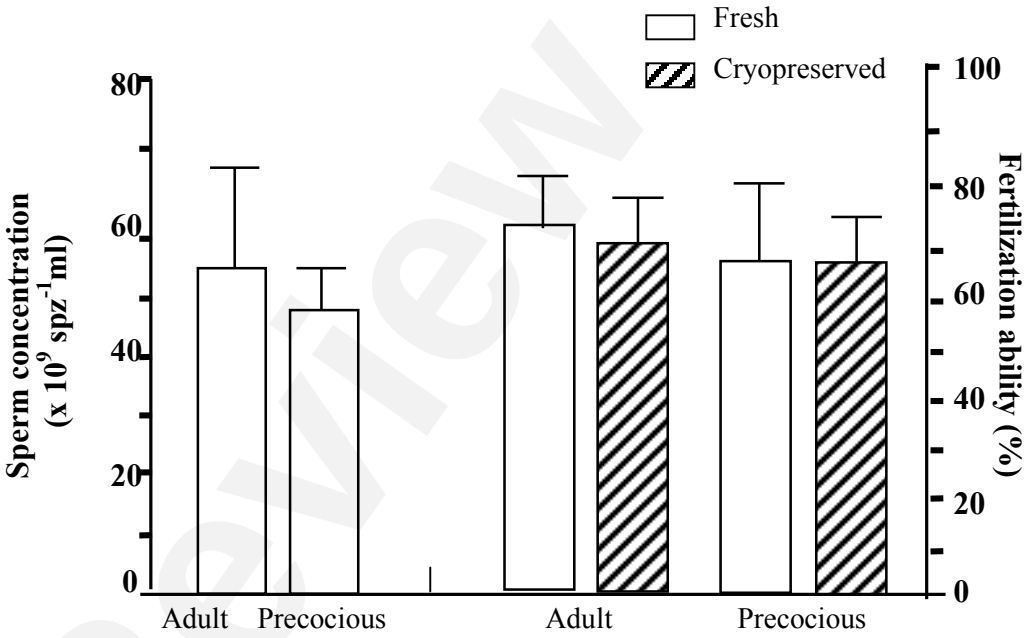
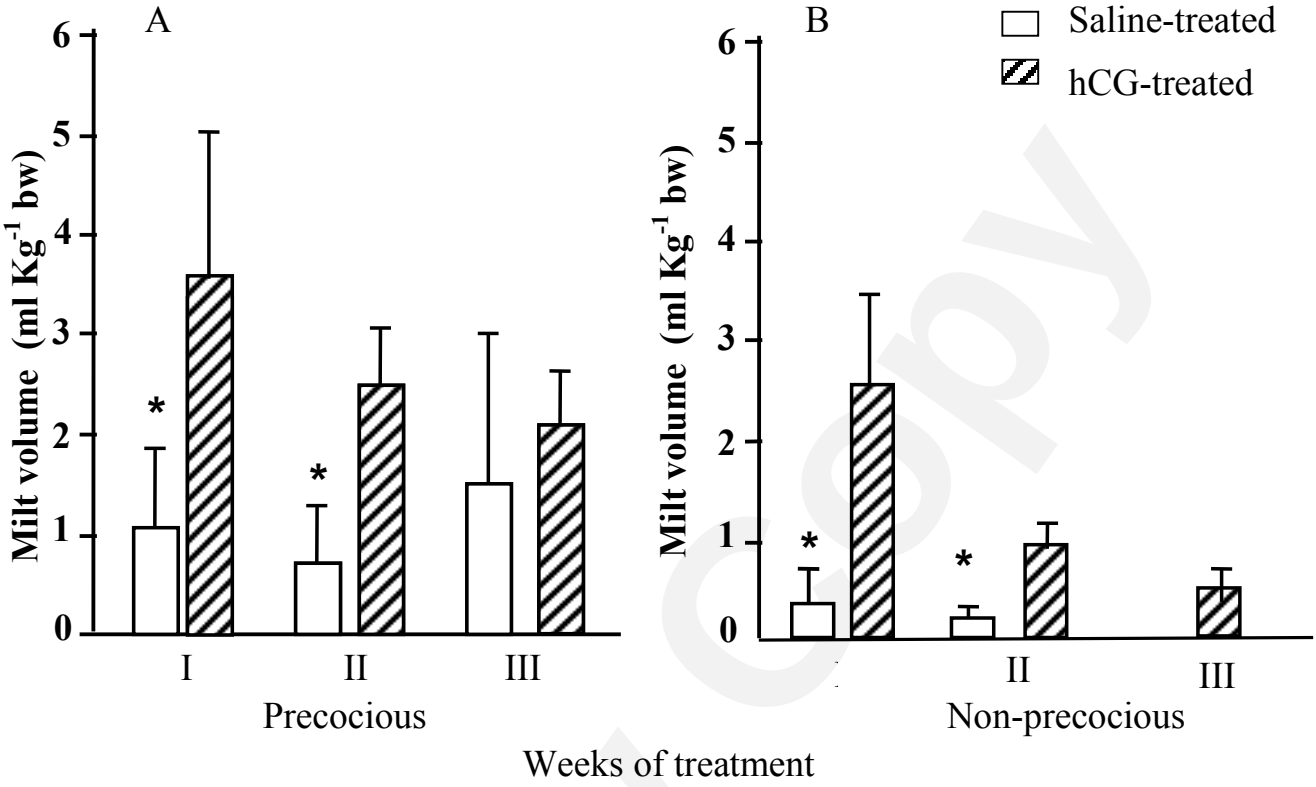


Fig. 1.



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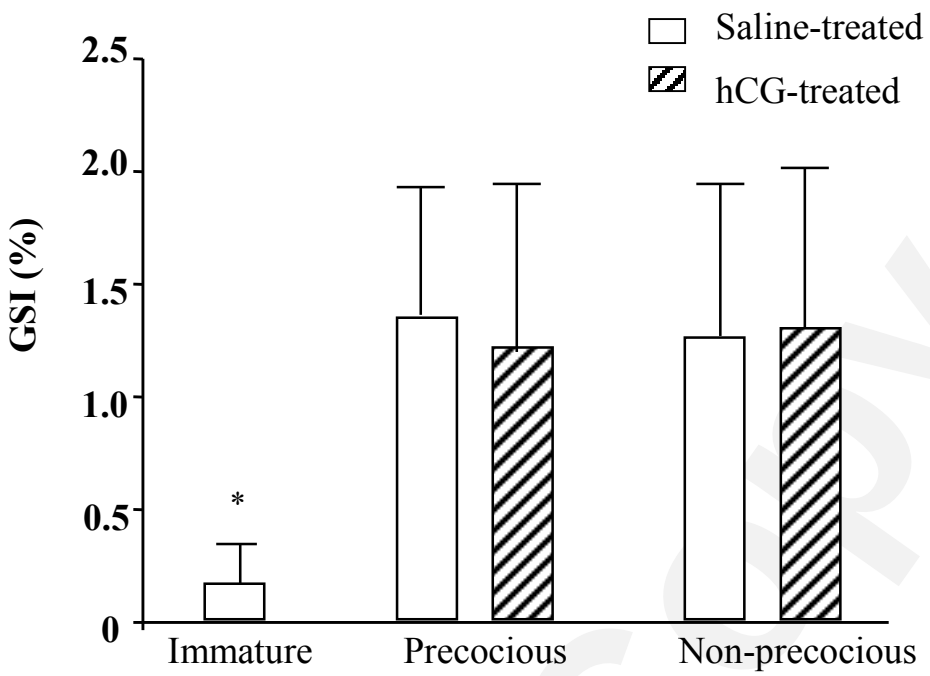

Fig. 3.

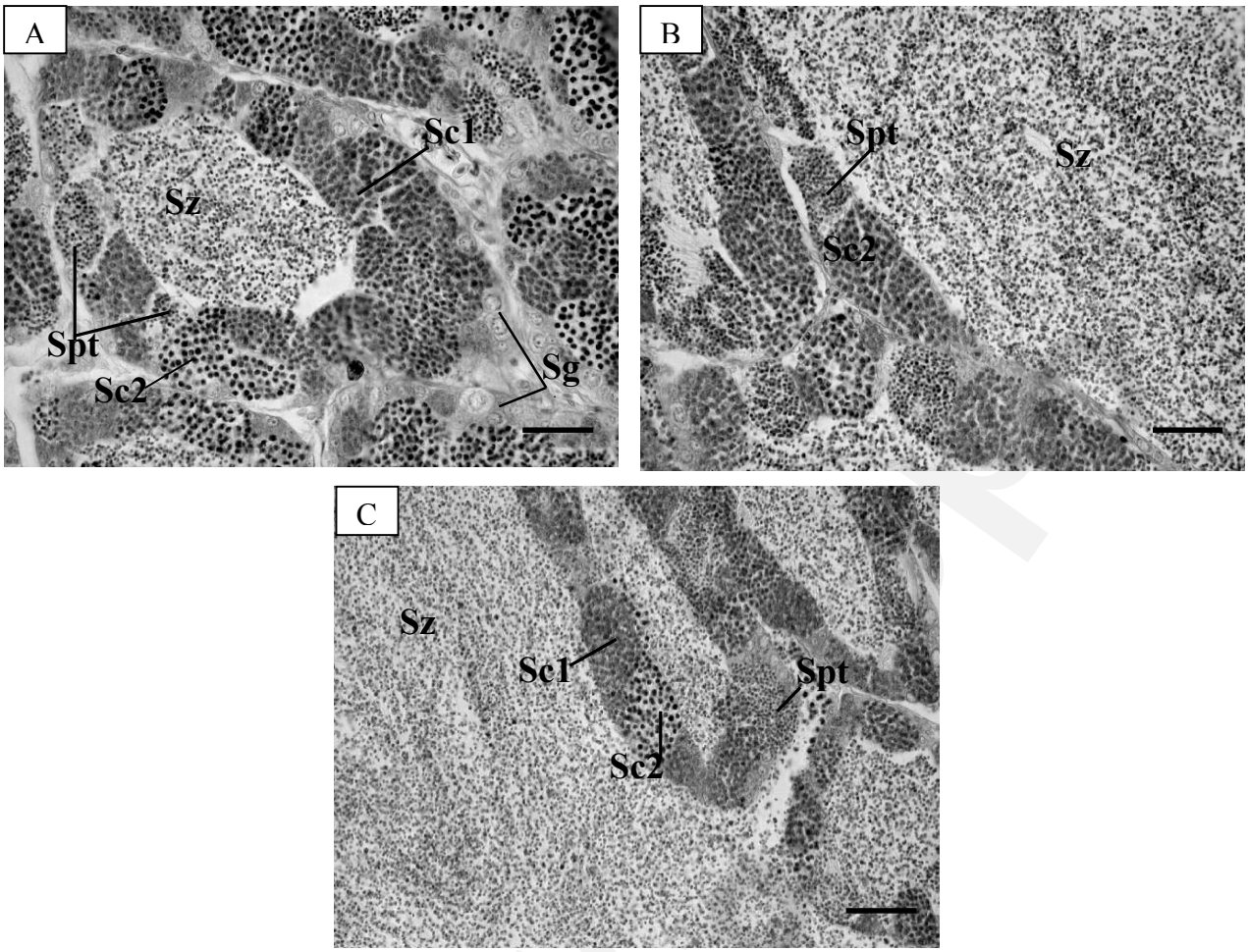

Fig. 4