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MILT PRODUCTION OF SEA BASS *LATES CALCARIFER* BLOCH ADMINISTERED AN ANALOGUE OF LUTEINIZING HORMONE-RELEASING HORMONE AND 17α-METHYLTESTOSTERONE

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Abstract

The milt production responses of sexually mature sea bass *Lates calcarifer* to (D-Ala^6, Pro^9-N-ethylamide) luteinizing hormone-releasing hormone (LHRHa) and 17α-methyltestosterone injections were examined.

At 24 h after injection of a low dose of LHRHa (20 µg/kg BW), the sperm count decreased significantly compared to saline-treated fish, but it returned to pre-treatment levels 48 h after injection, suggesting a possible hydration of the milt. Other milt parameters (milt volume, spermatocrit, sperm production) in LHRHa-treated fish did not vary from their controls at 24 or 48 h after injection but the overall pattern suggested a reduction in milt viscosity. Total expressible milt and spermatozoa collected over the 48-h experiment was approximately three-fold higher in LHRHa-injected fish than in saline-injected fish, indicating a stimulation of spermatozoa production, not merely milt dilution due to hydration.

In a second experiment, sperm count and spermatocrit were significantly lower than those of saline-injected fish at 17 and 48 h after a single injection of a high dose of LHRHa (80 µg/kg BW). A methyltestosterone injection combined with the LHRHa injection also resulted in a significantly lower sperm count, but the spermatocrit remained comparable to the control group, suggesting a suppression of the LHRHa-induced milt hydration response.

Results demonstrate that LHRHa stimulates milt hydration and spermatozoa production in milting sea bass and that a simultaneous methyltestosterone injection partially suppresses this response.

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Introduction

Spontaneous and hormone-induced ovarian development and spawning of sea bass *Lates calcarifer* Bloch (Centropomidae, "barramundi") have made fry available throughout the annual breeding season of June to October in the Philippines (Kungvankij *et al.*, 1986; Almendras *et al.*, 1988; Garcia, 1989a,b, 1990a,b, 1992; Toledo *et al.*, 1991; Guiguen *et al.*, 1993, 1994). Induced maturation and spawning of sea bass a month in advance of the natural breeding season has been achieved by chronic hormone therapy (Garcia, 1990a). However, sea bass spawning operations have been intermittently plagued by low fertilization rates that impact the production of hatchery fry.

Apart from sperm immotility affecting fertilization rates (Levanduski and Cloud, 1988; Dreanno *et al.*, 1999), high milt viscosity may limit milt dispersal in water in breeding operations of many warm and temperate fishes such as the mullet *Mugil cephalus* (Lee and Weber, 1986), milkfish *Chanos chanos* (Juario *et al.*, 1980), rabbitfish *Siganus guttatus* (Garcia, 1991, 1993), eel *Anguilla japonica* (Ohta *et al.*, 1996), and flounder *Pleuronectes ferrugineus* (Clearwater and Crim, 1998). To circumvent this difficulty, these workers hormonally manipulated the brain-pituitary-gonadal axis (BPG) using fish pituitary extracts, human chorionic gonadotropin (hCG), exogenous gonadotropin-releasing hormone analogues (GnRHa), or androgens to advance testicular maturation or facilitate spermiation and milt hydration.

Garcia (1990a) reported on the advancement of testicular maturation in sea bass by chronic administration of pelleted 17α-methyltestosterone (MT) and a mammalian luteinizing hormone-releasing hormone analogue (D-Ala^6, Pro^9-N-ethylamide; LHRHa). Fertilization and hatching rates were comparable with spontaneously matured control males (Garcia, 1990a), but the effects of hormone treatment on milt production parameters (i.e., sperm count, spermatocrit, and expressible milt volume) were not quantified. In the present study, therefore, we quantify the milt hydration response of mature male sea bass to injections of LHRHa and MT by measuring these parameters.

Materials and Methods

Rearing and handling of fish. Mature sea bass were selected from six to eight-year-old broodstock reared in 4 x 4 x 3 m floating net cages at SEAFDEC Aquaculture Department's Igang Marine Substation, Guimaras Island, central Philippines. Fish (1.3-3.6 kg) with expressible milt after gentle massage of the abdomen were selected. During hormonal treatment or sampling for milt, fish were anesthetized by immersion in 100-250 ppm of 2-phenoxyethanol sea water. At the start of each experiment, fish were weighed and marked with a modified Floy tag (Garcia and Gapasin, 1988). Two experiments were conducted around the natural breeding season of sea bass (May-October). Sea bass were fed trash fish once daily at approximately 5% of their body weight (BW). Water temperature and salinity were 27-32°C and 30-35 mg/l, respectively.

Milt parameters. Milt from anesthetized fish was obtained and processed as described by Garcia (1991). Milt samples were kept on ice and processed within 6 h of sampling. No significant evaporation of the milt samples was observed during the period prior to processing since samples were kept in sealed tubes. Sperm were counted using a standard hemacytometer method: milt was diluted 500X with a 0.9% NaCl solution and sperm counted under 400X magnification. Sperm count (or density) was expressed as the number of spermatozoa per µl of milt. Whenever milt was stripped to exhaustion, spermatozoa production was calculated as the product of the sperm count and the milt volume. A spermatocrit technique for sea bass was developed following Garcia (1991).

Experiment 1- Milt response to low-dose LHRHa. Experiment 1 was conducted early in the breeding season (May) to study the milt production response after acute administration of a relatively low dose (20 µg/kg BW) of LHRHa. Powdered (D-Ala^6, Pro^9-N-ethyl-

amide) LHRHa (molecular weight 1196.33; Lam Hua Dragon Co. Ltd., Hong Kong) was dissolved in a freshly prepared solution of 0.9% NaCl (saline) immediately prior to intramuscular injection near the base of the dorsal fin (Garcia, 1989a, 1990b). Five males were injected with LHRHa while five saline-injected fish served as controls. After injection, each male was held in a separate fine mesh (0.6-0.8 mm) net cage (2.3 x 2.3 x 3 m), paired with an LHRHa-injected (20 µg/kg BW) vitellogenic female having a mean oocyte diameter greater than 0.4 mm (Garcia, 1989a). Prior to injection and 24 and 48 h after injection, milt was stripped from each fish to exhaustion and milt characteristics were measured. Spontaneous milt release after injection was not monitored to prevent mortality from excessive handling. Means were compared using a general factorial ANOVA-Duncan's multiple range test or Student's paired *t* test (α=0.05).

Experiment 2 - Milt response to LHRHa and MT. We also tested the effects of acute administration of a high dose of LHRHa (80 µg/kg BW) and MT (200 µg/kg BW) on the thinning of the sea bass milt. Due to the limited number of available males, trials were replicated over four months (May-August). The preparation of the LHRHa solution was the same as for Experiment 1 while MT (17α-methyl-4-androsten-17β-ol-3-one; Sigma Chemical Co., USA) was dissolved in 2.5% ethanol in corn oil to a 2 mg/ml injection solution. Injection volumes were 0.2 ml/kg BW for LHRHa and 0.1 ml/kg BW for MT. Fish received one of four treatments: LHRHa, MT, LHRHa plus MT, or saline (control). Injected males were paired with vitellogenic females as described in Experiment 1. To minimize mortality due to handling and sampling, the hormone-treated fish were not exhaustively sampled for milt and were sampled only once or twice after injection. By combining all trials over the four months, the spermatocrit and sperm count were obtained at 17, 26 and 48 h after injection. Statistical analyses to compare means were the same as in Experiment 1.

Results

Milt parameters. The sea bass milt was highly viscous and required 60 min of centrifugation at 15,000 x g to pack the spermatozoa. Sperm count (x 10^6 sperm/µ l) and spermatocrit were highly correlated: sperm count = 0.38 (spermatocrit) + 0.87, r=0.86, p<0.0001, n=55 fish. The spermatocrit ranged from 41% (flowing milt) to 100% (solid mass of spermatozoa), whereas the sperm count ranged between 17.2 and 43.2 x 10^6 spermatozoa per µl milt. In all experiments, factorial ANOVA revealed that the milt production parameters did not significantly depend on body weight for the tested range. Therefore, statistical analyses did not consider fish body weight as a co-factor.

Experiment 1 - Milt response to low-dose LHRHa. The expressible milt volume varied from less than 0.1 ml (considered 0 during statistical analysis) to 2.6 ml per fish. When the pre-treatment volumes were minimal (0.2-0.4 ml/fish), more milt was collected at 24 h than at the pre-treatment sampling among the LHRHa-treated fish (LHRHa20). However, when the pre-treatment volumes were high (1.2-1.6 ml/fish), the expressible milt volumes at 24 h were lower than at the pre-treatment sampling (data not shown).

A general decline in expressible milt volume was noticed in all fish during the 48 hours of the trial but it was significant only among saline-injected fish starting at 24 h (Fig. 1a). The expressible milt volume of the LHRHa20-injected fish tended to be higher than that of the saline-injected fish 24 h after treatment (p=0.08) but it declined to a comparable volume at 48 h. Nevertheless, the mean (±SEM) total expressible milt volume of the LHRHa20-injected fish (1.2±0.3 ml/fish) over the 48 h period was significantly higher than that of the saline-injected fish (0.3±0.1 ml/fish; Fig. 2a). A similar pattern was observed in the number of spermatozoa which, starting at 24 h, declined significantly only in saline-injected fish (Fig. 1d). Although the mean number of spermatozoa collected from the LHRHa20-treated group was higher than in the saline-treated group at 24 and 48 h, the two groups did not significantly differ from each other.

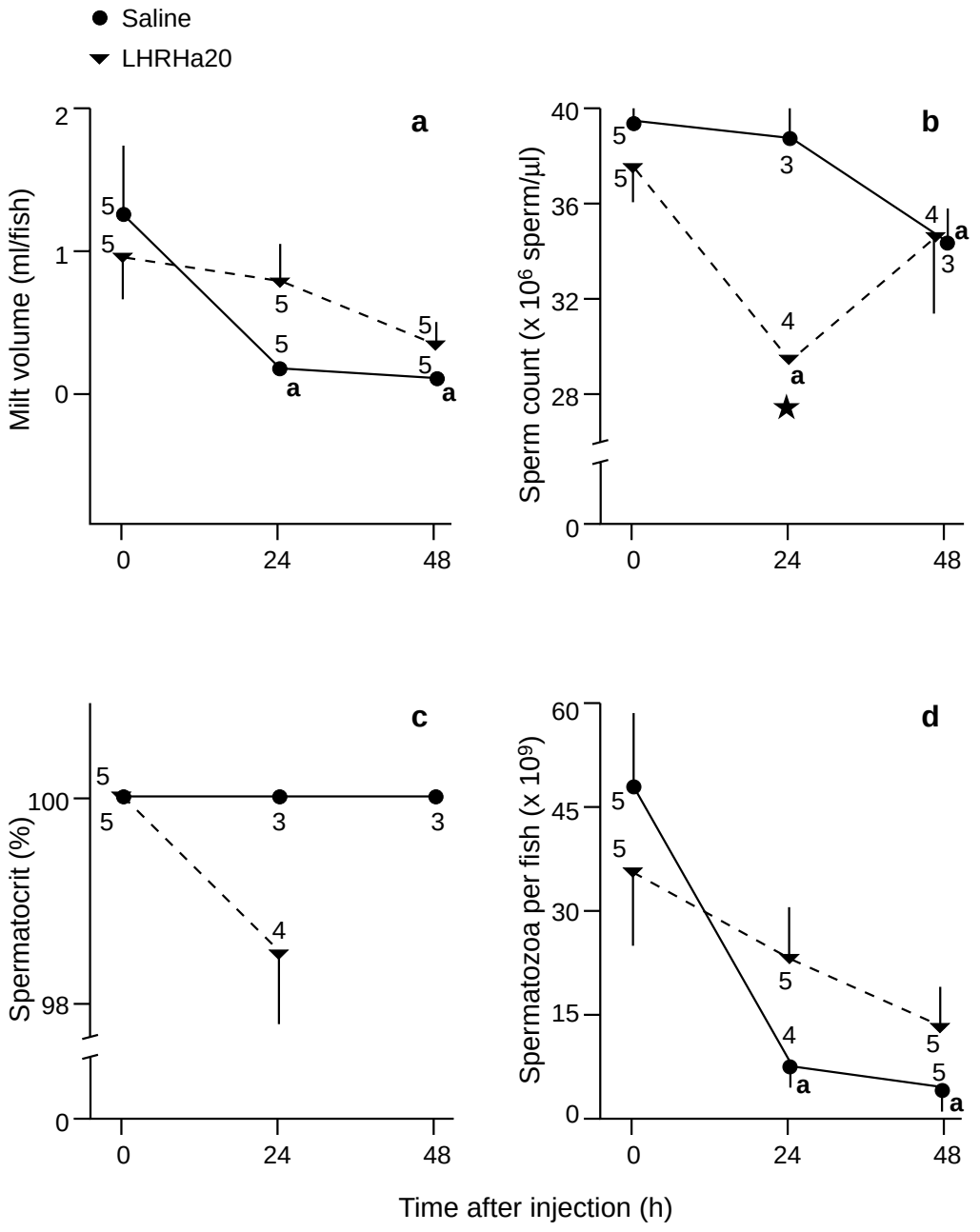

Fig. 1. Volume of expressed milt (a), sperm count (b), spermatocrit (c), and spermatozoa (d) in sea bass injected with a low-dose of LHRHa (20 µg/kg BW) or saline (Experiment 1). Time 0 refers to the pre-treatment expression of milt. The short vertical lines indicate SEM. Numbers indicate sample size. The letter "a" denotes a significant difference from the pre-treatment value ($p<0.05$). A star indicates a significant difference from the saline control ($p<0.05$).

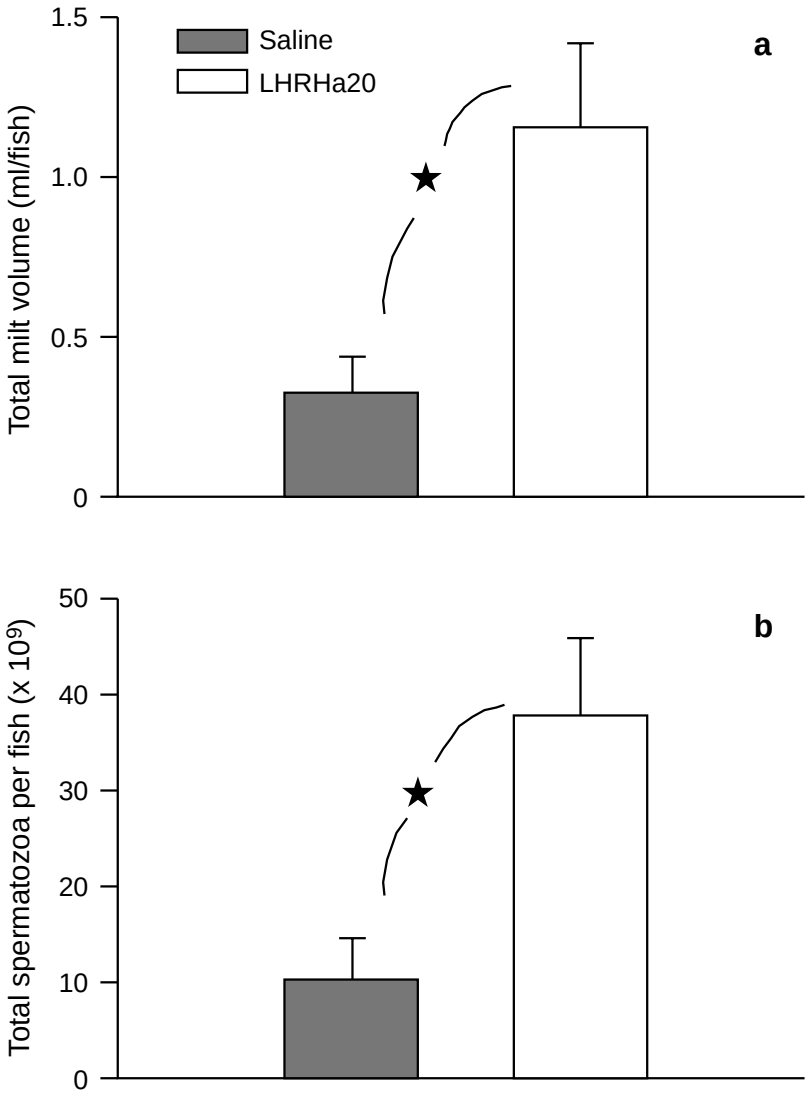

Fig. 2. Total milt volume (a) and spermatozoa (b) collected over the 48 h trial from sea bass injected with a low-dose of LHRHa (20 µg/kg BW) or saline. Values are the sums of exhaustive strippings (n=5 per group) at 24 and 48 h. The vertical lines indicate SEM. A star indicates a significant difference between the groups ($p<0.05$).

Nevertheless, the total spermatozoa collected over the 48-h trial was significantly higher in hormone-injected fish (Fig. 2b).

The sperm count and spermatocrit profiles were similar to each other. With expressible milt volumes decreasing through time, the number of replicates available for determining these two parameters likewise decreased. At 24 h after injection, the sperm count of the LHRHa20 fish was significantly lower than the initial value and that of the saline-treated cohorts (Fig. 1b). However, the sperm count

of the LHRHa20 fish regained its pre-treatment level at 48 h while the sperm count of the control fish declined further. There was no difference between the sperm counts of the two groups at 48 h.

Experiment 2 - Milt response to LHRHa and MT. The sperm count and spermatocrit responses to LHRHa and MT were determined thrice after injection (at 17, 26, and 48 h). The saline controls maintained a high sperm count and spermatocrit (means ranged 36.4-41.4 x 10^6/µl milt and 90.3-100%, respectively) throughout the experiment (Fig. 3). In contrast, a high dose of LHRHa (80 µg/kg BW; LHRHa80) resulted in a significant thinning of the milt such that the sperm count and spermatocrit were below pre-treatment levels during all three samplings. At 17 h after treatment, the combination of LHRHa80 and MT (LHRHa80+MT200) lowered the sperm count to significantly ($p<0.05$) below the level of the saline control but slightly above the LHRHa80 level. The spermatocrit was significantly maintained at a high level in the LHRHa80+MT200 group. Most of the MT-injected fish in both the MT200 and the LHRHa80+MT200 groups did not express milt at 26 h and, at 48 h, no milt could be collected from any fish injected with MT.

Discussion

Milt of untreated sea bass is highly viscous with a majority of spermatocrit values being over 88%. This is in contrast to salmonids, which express thin milt (23.4-27.1%) that requires only 15 min of centrifugation (Bouck and Jacobson, 1976; Aas *et al.*, 1991). Because of the close correlation between spermatocrit and sperm count ($r=0.86$), a technique to estimate sperm density according to spermatocrit may be a relatively rapid alternative to directly counting sperm in sea bass. However, the difficulty of drawing milt into microhematocrit tubes from minute and highly viscous samples is problematic, along with the prolonged centrifugation required. Perhaps, diluting extremely viscous samples prior to centrifugation and correcting spermatocrit values by the appropriate dilution factor will circumvent these difficulties.

Treatment with exogenous GnRH analogues stimulates secretion of pituitary gonadotropin (GtH) in sexually recrudescent and mature teleosts such as carp (Yaron, 1995), rainbow trout (Crim *et al.*, 1988), white bass *Morone chrysops* (Mylonas *et al.*, 1997), and African catfish *Clarias gariepinus* (Schulz *et al.*, 1994). In sea bass, solubilized or pelleted LHRHa has been used to induce maturation and spawning in females during the breeding season (Garcia, 1989a,b, 1990a,b, 1992). In these experiments, males were selected simply if milt could be expressed by gentle abdominal pressure. The milt production response to exogenous hormones was not described, but hatching data indicated the efficacy of hormonal therapy in inducing spermiation/milt hydration.

Our present results support these earlier observations. In Experiment 1, the lower sperm count, lower spermatocrit and higher expressible milt volume in LHRHa-injected fish indicate hydration of the milt. Likewise, the higher dose of injected LHRHa in Experiment 2 also lowered both the sperm count and the spermatocrit. In many teleosts, during the final stages of testicular maturation, hydration of the seminal plasma along the reproductive tract in response to endogenous hormonal cues is required to produce expressible milt (Billard *et al.*, 1982; Yamauchi and Yamamoto, 1982; Loir and Billard, 1990). Note that this 17-24 h latency in milt hydration allows the accumulation of milt prior to its expression, and is timed with the onset of LHRHa-induced spawning of females that occurs 34-40 h after hormone injection (Garcia, 1990b).

At 24 and 48 h post-injection, milt volume and spermatozoa were only slightly higher in the LHRHa-injected fish than in the control. However, total milt and spermatozoa collected over the 48 hours were significantly higher. This observation may reflect stimulation of spermatozoa production and not merely milt dilution due to hydration. Sea bass exhibit a continuous mode of gametogenesis (Guiguen *et al.*, 1994) wherein spermatozoa may be recruited readily from queued batches of maturing spermatogenic cells through GtH-

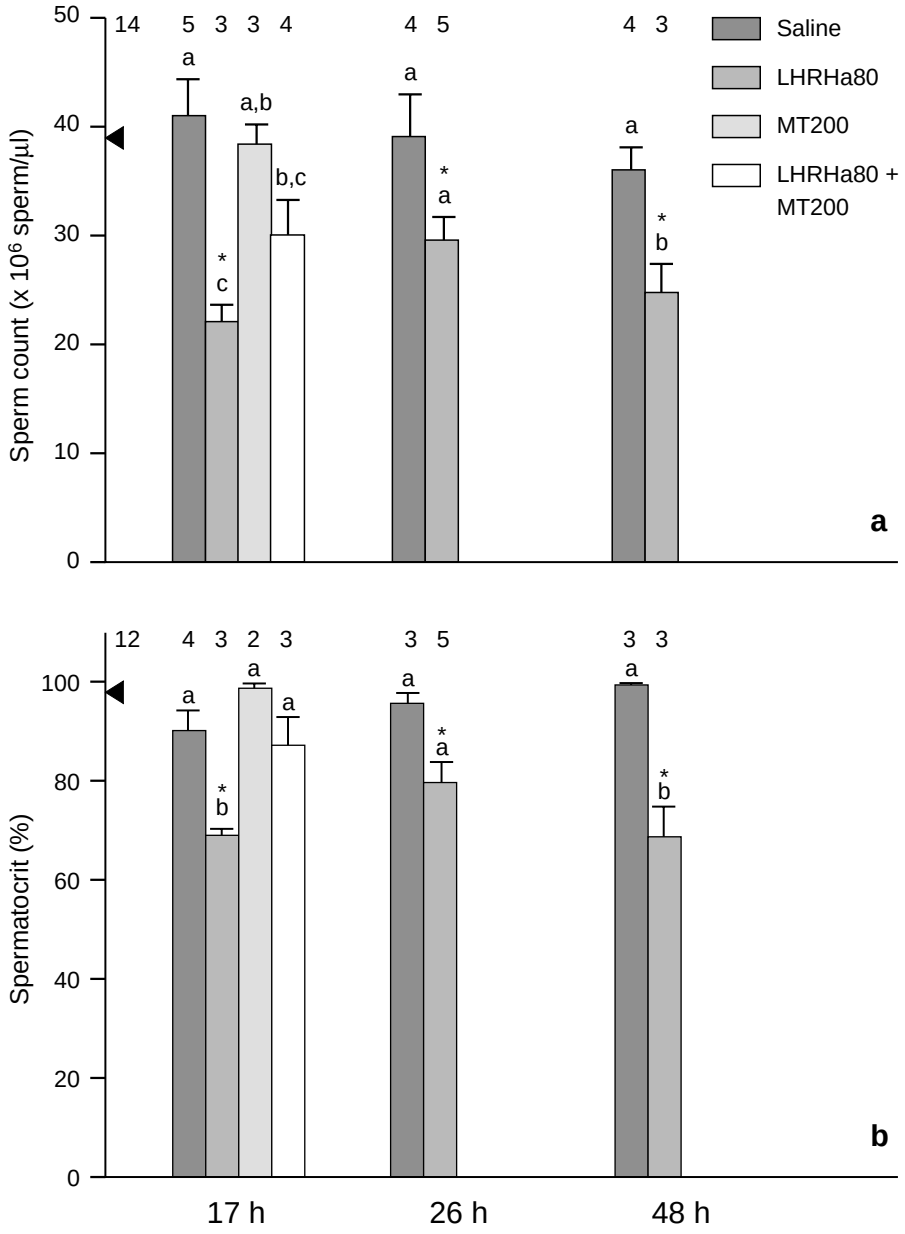

Fig. 3. Sperm count (a) and spermatocrit (b) of sea bass injected with saline, a high dose of LHRHa (80 µg/kg BW), methyltestosterone (200 µg/kg BW), or a combination of LHRHa and methyltestosterone (Experiment 2). Arrowhead on vertical axis indicates the pre-treatment value of a representative sample of the fish. Vertical lines and numbers show SEM and sample size, respectively. An asterisk indicates a significant difference from the pre-treatment value ($p<0.05$). Within a sampling event, values with different letters are significantly different ($p<0.05$). Most methyltestosterone (MT)-injected fish did not express milt at 26 h. No MT-injected fish expressed milt at 48 h.

mediated LHRHa action, resulting in the significantly higher total spermatozoa collected from the LHRHa injected fish.

In caged sea bass, plasma testosterone and 11-ketotestosterone (KT) slowly rise during testicular recrudescence and peak during spermiation (Guiguen *et al.*, 1993). Testosterone is a precursor of KT synthesis. Its action may be directed at the hypothalamus and/or pituitary which, depending on the stage of sexual maturity, is sensitive to aromatizable steroids such as MT (Khan *et al.*, 1999). In several teleosts, testosterone and other aromatizable androgens feedback positively on the BPG when testes are immature, recrudescent or regressed, and negatively when testes are ripe (Crim and Evans, 1983; Borg *et al.*, 1986; Antonopoulou *et al.*, 1999; Khan *et al.*, 1999; Schulz and Goos, 1999; Zanuy *et al.*, 1999). Thus, chronic treatment with sustained-release MT implants advances maturation of sexually quiescent sea bass males, perhaps by stimulating pituitary GtH secretion (Garcia, 1990a). However, MT implants may cause a decline in spermiation and milt production once sea bass attain full maturity. Although the present experiment involved injections of MT instead of sustained-release implants, the spermatocrit profile shows that the MT suppressed LHRHa-induced milt hydration 17 h after injection.

Milt production in sexually mature sea bass primed weekly with MT (100 mg/kg BW) during the breeding season declined three weeks after the priming injection (Hilomen-Garcia, unpublished data). However, in several fish, it increased during the sixth week following the acute administration of LHRHa80 and MT. Data on MT injections in sea bass have been inconsistent as little milt was expressed from MT-injected males 26 h after injection in the current study.

Both experiments had a similar effect on sperm count despite a four-fold difference in dose used: the sperm count declined to about 30×10^6 per µl milt at 24-26 h post-injection. On the other hand, there was a notable difference in spermatocrit 24-26 h after injection. The high dose of LHRHa (80 mg/kg BW) lowered the spermatocrit to 80% while the low dose (20 mg/kg BW) lowered it only to 98%. The experimental design precludes comparison of the two doses, because the low-dose group (Experiment 1) was exhaustively stripped of milt while milt from the high-dose group (Experiment 2) was sampled more conservatively. Nevertheless, from our limited results, we recommend LHRHa injection at a dose of 80 mg/kg BW. At this dose, the spermatocrit substantially drops while the sperm count drops no more than after the lower dose. Future experiments should study dose/response relationships and fertilization rates to determine the lowest effective LHRHa dose and reduce the cost of sea bass breeding.

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