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Long-term feeding restriction in prepubertal male sea bass (*Dicentrarchus labrax* L.) increases the number of apoptotic cells in the testis and affects the onset of puberty and certain reproductive parameters

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ABSTRACT

In sea bass (*Dicentrarchus labrax*), the onset of puberty under intensive farming conditions is likely related to the availability of food. Therefore, a close connection between feed intake, lipid storage and onset of reproduction can be established. This study reports the effects of long-term feed restriction on the reproductive performance of male sea bass entering their first breeding season. A control group (C) was fed to apparent satiety, while experimental groups were restricted to 1/4 (1/4 C) or 1/8 (1/8 C) of the ration administered to the control group. It was determined that restricted feed regimes influenced the weight, length, growth rate and gonadosomatic index (GSI) of the fish. Specifically, the 1/8 C group showed lower GSI values than the control animals. In the experimental animals, the histological analysis revealed a moderate delay in testes development and a larger number of apoptotic bodies at onset of gametogenesis. The plasma level profiles of 11-ketotestosterone (11-KT) were comparable among the groups. Additionally, certain sperm parameters, including sperm count, motility and expressible milt volume, were evaluated throughout the reproductive season, and long-term fasting was found to affect sperm volume and counts, curvilinear velocity (VCL), average path velocity (VAP) and percentage of static and medium motility sperm of sea bass sperm. However, puberty was not suppressed; at most, gonadal development was reduced and progression of gonadal stages delayed as well as the duration of the production of sperm. Unexpectedly, some characteristics of their motility experienced a significant increase.

1. Introduction

Fish puberty is a complex process in which an individual acquires the capacity to reproduce for the first time. This life stage is characterized by the activation of the two main gonadal functions: the production of germ cells and the synthesis of reproductive hormones. This progression culminates with the first spermiation in males and the first ovulation (or first spawning) in females (Mylonas et al., 2010; Taranger et al., 2010). In the wild, as part of the life strategy, the onset of reproduction in fish is associated with favorable environmental conditions (the availability of food, absence of predators, etc.), enabling them to invest surplus energy in reproduction rather than in somatic growth (Ware, 1982). However, farmed fish reared under controlled favorable conditions accompanied by a surplus of food (overeating) show accelerated growth and maturation patterns (Taranger et al., 2010; Thorpe, 2004). Therefore, the effects of nutrition and food composition on reproductive performance in commercial fish species have been widely studied (Izquierdo et al., 2001). However, little attention has been devoted to the impact of food deprivation on immature fish entering their first sexual reproduction cycle, in an attempt to mimic the conditions to which the fish are exposed in the wild. Among the few studies on this subject are those carried out with male Atlantic salmon (*Salmo salar*) and goldfish (*Carassius auratus*), in which it was demonstrated that a reduction in feeding rate causes a disruption of gonadal maturation (Berglund, 1995; Clemens and Reeds, 1967; Luquet and Watanabe, 1986; Trombley et al., 2014). Nowadays, European sea bass is an important species for intensive marine fish farming; however, there is a significant lack of knowledge about the effect of food rations on the onset of puberty in males, subsequent testicular development and sperm quality parameters at the second year of life. Previous work done with female sea bass (Cerdá et al., 1994) indicated that long-term reduction in food rations to half of the amounts received by the control animals (1/2 C), reduced the size of the eggs and larvae and delayed the spawning time. However, fecundity, egg quality (i.e. buoyancy, hatching rates and biochemical composition) and plasma

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vitellogenin levels were not affected, except for the 17β-estradiol plasma levels, which were lower in fish under restricted food rations. Moreover, these fish exhibited a higher number of spawns per female and larger spawning span than control fish, thus suggesting a beneficial compensatory effect of feed restriction on spawning performance.

Computer-Assisted Sperm Analysis (CASA) has proven to be a useful tool in the study of fish reproduction, as it provides accurate information on sperm motility and concentration and offers an approach to predict the sperm fertilizing capacity (Cabrita et al., in press; Gallego et al., 2013; Kime et al., 2001; Rurangwa et al., 2004). In addition, it is known that the nutritional status prior to the onset of gametogenesis can be critical to build up a fat storage capable of ensuring the ongoing supply of nutrients and energy to properly maintain this process (Luquet and Watanabe, 1986; Taranger et al., 2010; Thorpe, 2004). With this in mind, we studied the effect of two restricted feed protocols consisting of either 1/4 or 1/8 of the ration received by the control animals (therefore more restrictive rations than that used by Cerdá et al. (1994) for female sea bass) in prepubertal male sea bass six months before the onset of gametogenesis. Accordingly, and for the first time, we report both the effects of a restrictive feeding regime on the onset of puberty and reproductive performance of male sea bass during the first reproductive season, as well as on certain sperm parameters.

2. Material and methods

2.1. Fish and rearing conditions

Three hundred one-year-old prepubertal European male sea bass (127.6 ± 0.5 g and 21.74 ± 0.03 cm) purchased from Aquanord (Gravelines, France) were maintained under natural photoperiod and seawater temperature (19 ± 6 °C) conditions at the facilities of the Instituto de Acuicultura de Torre de la Sal (Castellón, Spain, 40°N 0°E). The experiment lasted for a total of nine months, starting in July, six months before full reproduction, and ending in late March, coinciding with the end of the reproductive period in this species.

Fish were randomly placed in six 500 L tanks, 50 per group in duplicate, and fed a commercial dry pellet diet provided by Proaqua Nutrición, S.A. (Palencia, Spain). The composition was as follows: 44% protein, 20% lipids, 20% carbohydrates, 8% ash and 1–3% humidity. Control groups (C) were fed until apparent satiety twice during natural daylight hours, at 9:00 AM and 14:00 PM. The food provided to the experimental groups was measured in grams of dry pellets, representing a 1/4 C or 1/8 C proportion of the daily amount given to the control groups. The feeding schedule for the experimental groups was the same as for the control groups.

2.2. Fish sampling, histological and hormonal analysis

Animals were handled and sacrificed in accordance with Spanish and European legislation concerning the protection of animals used for experimentation or other scientific purposes (Royal Decree 53/2013 and 2010/63 EU, respectively). Fish were anesthetized with ethylene glycol monophenyl ether (0.5 ml × l^{-1} water), and body weight (Bw) and fork length (Bl) were recorded on a monthly basis. The condition factor (CF) was calculated as the ratio between weight and fork length (Bw/Bl^3) × 100. Additionally, specific growth rate (SGR) was calculated in terms of weight as follows: $SGRw = (\ln Wf/\ln Wo) \times \Delta t^{-1}$; in which ln = natural logarithm; Wf = final weight in grams; Wo = initial weight in grams and Δt = time period in days. In July, September, November, February and March, 9 fish per group were sacrificed to obtain blood samples and estimate several somatic indexes, including the gonadosomatic index ($GSI = \text{gonad} \times Bw^{-1}) \times 100$; mesenteric fat index ($MFI = \text{mesenteric fat} \times Bw^{-1}) \times 100$; hepatosomatic index ($HSI = \text{liver} \times Bw^{-1}) \times 100$; and carcass index ($CI = \text{carcass} \times Bw^{-1}) \times 100$. Blood samples were collected from the caudal vein using 1-ml heparinized syringes and then transferred to 0.5 ml Eppendorf tubes treated with heparin and placed on ice. Tubes were subsequently centrifuged at 3000 rpm for 30 min at 4 °C. The plasma was stored at −20 °C until analysis.

A small piece of gonadal tissue was fixed by immersion in 4% formaldehyde:1% gluteraldehyde buffered saline and embedded in 2-hydroxyethyl methacrylate polymer resin (Technovit 7100, Germany) to stage the testis. Another small piece of testis was fixed in 4% paraformaldehyde in 0.2 M phosphate buffer (pH = 7.2) and embedded in paraffin (Paraplast, Fisher Scientific™) for TUNEL performance. Gonadal tissues were sectioned 3 and 4 μm, respectively and stained according to the method described by Bennet et al. (1976). Plasma levels of 11-ketotestosterone (11-KT) were determined by a specific immunoassay (EIA) according to Rodríguez et al. (2000).

2.3. Gonadal development classification and apoptotic body count

Testicle development was staged according to the procedure described by Begtashi et al. (2004) as follows: Stage I (immature), Stage II (spermatogonial or proliferative phase), Stage III (meiotic phase), Stage IV (differentiation phase), Stage V (full spermiation phase) and Stage VI (post spermiation phase). The identification and counting of apoptotic bodies (*ab*) were performed by light microscopy (NIKON Eclipse E600) in nine males sampled in November, coinciding with the highest level of testicular mitotic activity in this species. The number of apoptotic bodies (*ab*) was evaluated at 50× magnification in twelve optical areas (2500 μm^2) per fish. Additionally, and due to the fact that the *ab* counting may provide false positives, we used the TACS® Blue Label Kit (TREVIGEN) in the testes to confirm the *ab* previously identified by light microscopy. The immunohistological identification of *ab* was performed by detection of nuclear DNA fragmentation using terminal deoxynucleotidyl transferase enzyme (TdT) TUNEL method (Gorczyca et al., 1993). This protocol is based on standard streptavidin conjugated to horseradish peroxidase (HRP), in conjunction with a biotinylated primary antibody (anti-BrdU). According to the manufacturer's instructions the sections were dewaxed in xilene at room temperature (2 × 10 min) and dehydrated through decreasing concentration of ethanol. Subsequently, slides were washed twice in 0.1 M phosphate-buffered saline (PBS) and then were incubated with 50 μl of Proteinase K for 30 min at 37 °C. Sections were washed in phosphate-buffered saline with 0.01% Tween-20 (PBST) (2 × 10 min) and immersed in 0.35% hydrogen peroxide for 10 min to quench endogenous peroxide activity and incubated for 5 min in TdT 1× solution and then immersed in 50 μl of labeling reaction mix (B-dNTPs) for 30 min in humidity chamber at 37 °C. Reaction was stopped using stop buffer for 5 min at room temperature and samples were washed twice in PBS 1×. Using the biotinylated anti-BrdU, samples were incubated for 30 min at 37 °C and revealed using 50 μl of Streptavidin-HRP solution for 10 min at room temperature. The *ab* identification was performed by immersing sections in TACS Blue label for 5 min. Finally, nuclei were counterstained with Fast Red for 30 s and sections were mounted with DPX (Panreac). Sections that were not incubated with primary antibody served as negative controls.

2.4. Sperm collection and automatic analysis

Males were identified by milt production at the beginning of the breeding season, and were subsequently tagged and tracked over the entire reproductive season (December–March). Fish were anesthetized and the genital area was then cleaned with fresh water. A gentle stripping massage was performed and total expressible milt was collected in a sterile tube for further volume registration. The percentage of running males was recorded and calculated by determining the percentage of the male population which was able to release sperm from the total number of male fish in each group. To assess certain milt parameters, sperm samples from randomly chosen fish (n = 9) were processed as previously described by Felip et al. (2006). The nine randomly chosen