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Hormonal sex reversal in Atlantic cod, *Gadus morhua*

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ABSTRACT

Pre-harvest sexual maturation, a major constraint to aquaculture operations, commonly occurs in both sexes of Atlantic cod (*Gadus morhua*). Production of all-female triploids would alleviate this problem because they are sterile. The objective of this project was to develop effective endocrine manipulations to produce monosex stocks of cod, as the first step towards producing all-female triploids, using dietary 17β-estradiol (E2) and 17α-methyldihydrotestosterone (MDHT) fed during the labile period of gonadal differentiation. In the first experiment, triplicate treatments of E2 at 5, 10 and 20 mg/kg feed and MDHT at 0.67, 2 and 6 mg/kg feed were fed to fish during the growth interval from 17 ± 2 to 43 ± 4 mm standard length (SL). The only treatment which affected sex ratio was the highest MDHT dose, with a significant reduction in proportion of females compared to controls. Based on these results and a thorough histological examination of gonadal development, a follow-up experiment used duplicate treatments of E2 at 20, 40, 80 and 120 mg/kg and MDHT at 3, 6, 12 and 18 mg/kg fed to fish during the growth interval from 7.8 ± 1.2 to 45 ± 2 mm SL. This resulted in a dose-dependent shift in sex ratio in both estrogen and androgen treated groups, with no females observed in the groups fed the two highest doses of MDHT and a significantly reduced male ratio in E2 groups at the two highest doses. These results indicate that successful sex reversal can be achieved through dietary hormonal manipulation when the treatment starts at around 8 mm SL.

1. Introduction

Pre-harvest sexual maturation is a major constraint to the commercialization of Atlantic cod aquaculture (Taranger et al., 2010). Among the options available to delay or eliminate sexual maturation in fishes, triploidy induction is currently the most promising solution (Piferrer et al., 2009). However, similar to other species, gonadal development is reduced to a much greater extent in triploid female cod than in triploid males (Feindel et al., 2011). Such a sexually-dimorphic effect implies a demand for the use of all-female triploid stocks in cod aquaculture. The commercial-scale production of all-female diploid populations can be accomplished by exposing fish to exogenous estrogen (direct feminization) or by crossing sex-reversed fish with regular fish (indirect feminization) (Devlin and Nagahama, 2002). Although it is possible to produce all-female triploid populations by direct feminization (e.g., Piferrer et al., 1994), this is not a suitable option when the end product is destined for human consumption. However, for species having female homogamety, as is the case for Atlantic cod (Haugen et al., 2011; Otterå et al., 2011; Whitehead et al., 2012), indirect feminization of triploids can be achieved by crossing functionally sex-reversed females ('neomales') with regular females and then inducing triploidy shortly after fertilization to create all-female triploid populations (Benfey, 2009).

The effects of sex steroids on gonadal differentiation have been shown to be species-, timing- and dose-specific (Piferrer, 2001). The timing of optimum hormonal treatment is based on the timing of gonadal differentiation into ovaries and testes. Treatments for complete sex reversal are normally applied through the labile period, when primordial gonads are most sensitive to hormones, until the end of gonadal differentiation, with histology commonly used to determine the labile period. Previous research suggested that gonadal differentiation begins in Atlantic cod at 19 mm total length (TL) (Chiasson et al., 2009) or 16 mm standard length (SL) (Haugen et al., 2011).

Sex steroids that have been used successfully to sex reverse fish include the natural estrogen 17β-estradiol (E2) and the synthetic androgen 17α-methyltestosterone (MT) for feminization and masculinization, respectively (Devlin and Nagahama, 2002). However, due to the endogenous conversion of MT to E2 via the enzyme cytochrome P450 aromatase, 'paradoxical feminization' occasionally occurs when MT is used for masculinization (Hackmann, 1971). Therefore, the non-aromatizable synthetic androgen 17α-methyldihydrotestosterone (MDHT) is frequently used as an alternative to MT.

In addition to influencing the course of gonadal differentiation, sex steroids can also have negative effects on reproduction, growth and survival (reviewed in Piferrer, 2001). In a practical sense, successful hormonal treatments should achieve the highest percentage of sex

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reversal with the least negative impact. The aim of this research was to develop effective protocols for hormonal sex reversal of Atlantic cod, as the first step towards large-scale production of all-female populations of triploids for aquaculture. Both feminization and masculinisation were attempted since the genetic mechanism of sex determination was not known for any gadoid species when this research began.

2. Methods

2.1. Fish culture

Fertilized eggs were obtained from gametes manually stripped from broodstock held at the St. Andrews Biological Station (SABS; St. Andrews, New Brunswick), using 8 females and 2 males for Experiment 1 (in 2009) and 12 females and 8 males for Experiment 2 (in 2010). Embryos were initially reared in 40 L upwelling, conical incubators supplied with oxygenated, UV-treated and filtered (20 μm) sea water at 6 °C and 33 ppt salinity. When 80% hatch was estimated, larvae and remaining eggs were pooled (within an experiment) and divided among eight 450 L larval rearing tanks at an average density of 53 larvae/L. Larval rearing tanks received similarly treated water as the incubators, but with temperature increasing from 10 °C (at transfer) to 14 °C by 192 days post-hatch (dph). Because families were pooled at the larval transfer stage it was not possible to investigate family effects on any subsequent results.

Beginning at 2 dph in both experiments, hatched larvae were fed rotifers enriched with Protein Selco Plus (INVE Aquaculture, Dendermonde, Belgium). In Experiment 1, larvae were then co-fed rotifers and enriched (Ori-Green, Skretting, Bayside, New Brunswick) *Artemia* (1.1–2.2/mL) from 46 to 51 dph, followed by co-feeding of *Artemia* and untreated 0.3 mm microdiet (Gemma Wean Diamond, Skretting) until 71 dph, and then solely the 0.3 mm microdiet until the end of hormone treatment (see Section 2.3 below). In order to start the steroid treatments earlier in Experiment 2, larvae were co-fed rotifers and hormone-treated *Artemia* replacement diet (150 Gemma Micro-Diamond, Skretting) from 26 to 31 dph, then solely the treated *Artemia* replacement diet until 50 dph, followed by co-feeding of treated *Artemia* replacement diet and treated 0.3 mm microdiet until 58 dph and finally just the microdiet until the end of hormone treatment. In both years, fish were reared in flow-through systems with an average temperature of 12.0 °C (10.0 °C at the lowest in March and 13.9 °C at the highest in September) and oxygen saturation of 103% (ranging from 86 to 120%). Larval growth was monitored by weekly measurement of SL of 10 fish per tank. Five (Experiment 1) or all 10 (Experiment 2) of these fish were preserved in 5% phosphate-buffered formalin for histology.

2.2. Histology

For larvae between 7 and 15 mm SL, the heads of fixed fish were removed prior to processing. For larger larvae, a section cut from approximately 5 mm anterior to 2 mm posterior of the urogenital papilla was used. Standard histological procedures were used to obtain 7 μm sections, which were then mounted on slides and stained with Instant Haematoxylin and counterstained with Eosin. Early stages of gonadal development, including formation of primodial gonads and differentiation of germ and somatic cells in presumptive testes and ovaries, were compared to published descriptions (Morrison, 1990).

2.3. Hormonal treatments

Stock solutions (1.25 g/L) of E2 (Sigma Inc., MO, USA) and MDHT (Steraloids Inc., RI, USA) were made in 100% ethanol. The required amount of stock solution was mixed into the microdiets to attain the target treatment concentrations and sufficient 95% ethanol was then added to completely immerse the diets. The saturated feed was stirred for 30 min and then air dried until all ethanol had evaporated. Control diets were prepared in the same way, but without the addition of steroid.

One day prior to the introduction of hormone-treated diets, fish were randomly chosen from the larval tanks where fish size was most homogeneous and evenly distributed among 450 L tanks at a density of 500/tank (Experiment 1; 21 tanks) or 1500/tank (Experiment 2; 18 tanks). For Experiment 1, this was done when the fish had reached 17 ± 2 mm SL (74 dph). Triplicate dietary treatments of 0 (control), 5, 10 and 20 mg/kg E2, and of 0.67, 2 and 6 mg/kg MDHT, were then fed to satiation over a 43-day period, by which time fish were 43 ± 4 mm SL. For Experiment 2, hormonal treatments were initiated when fish were 7.8 ± 1.2 mm SL (26 dph) and ended at 45 ± 2 mm SL (91 dph), with duplicate treatments of 0 (control), 20, 40, 80 and 100 mg/kg E2, and of 3, 6, 12 and 18 mg/kg MDHT. Daily records of mortality, oxygen concentration and water temperature were taken in both experiments.

The in-feed steroid concentration was estimated by measuring E2 concentration in 4 sub-samples from each of the E2 diets prepared for Experiment 2. Steroid was extracted from the diets using diethyl ether and E2 concentration was measured by radioimmunoassay using the methods in McMaster et al. (1992), with the E2 antibody obtained from MP Biomedicals (Solon, OH, USA). Intra-assay variance for E2 measurement was 2.2% ($n = 6$). The measured E2 concentration in the diets averaged 86% of the target concentration (range: 81–89%).

2.4. Sex determination

After the hormonal treatments were completed, fish were fed normal diets (Europa, Skretting) and reared to an average size of 175–180 mm SL, at which time approximately 60 (Experiment 1) or 50 (Experiment 2) fish were randomly selected from each tank, killed, and sexed by macroscopic observation of their dissected gonads. Testes were distinguished by their long, thin shape and translucent appearance, while ovaries presented as an enlarged lump in the basal section of the gonad close to the urinary bladder. To confirm the accuracy of sexing by morphological evaluation, all 10 fish ≤140 mm SL and 37 randomly chosen fish between 140 and 180 mm SL were sexed histologically in Experiment 2. This revealed a 98% accuracy of sexing by morphological evaluation, i.e., only a single fish misidentified.

2.5. Statistical analysis

Sex ratios were calculated as female:non-female due to the difficulty of distinguishing sterile fish from males. If there was no statistical difference among replicates in sex ratio or size, the data from replicates were combined. In the case of significant differences among replicates within a treatment (which only occurred in Experiment 1; see below), individual replicates were compared separately to the control. A chi-square test was used to analyze the deviation of sex ratio between each treatment group and its control, as well as between the controls and 1:1. Size differences of fish at each sampling date were evaluated by one-way ANOVA. A significance level of $p < 0.05$ was used for all analyses. Mortality data were not analyzed because it was not possible to account for the loss of larvae from the tanks via internal standpipes. However, the decline in fish numbers within tanks, for whatever reasons, was no greater than normally encountered with current cod culture practices.

3. Results

3.1. Timing of gonadal differentiation

Primordial germ cells (PGCs) tightly surrounded by somatic cells, often referred to as nests of PGCs, were clearly observed in fish at