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The production of hermaphrodites of Atlantic cod (*Gadus morhua*) by masculinization with orally administered 17-α-methyltestosterone, and subsequent production of all-female cod populations

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ABSTRACT

In order to masculinize Atlantic cod (*Gadus morhua*) with the ultimate goal to produce all-female populations, larvae were treated with dietary 17α-methyltestosteron (MT) at high (H: 15 mg/kg diet) and low (L: 5 mg/kg diet) dosages for varying time-periods from either 12 or 16 mm total length (TL) lasting up to 16 mm, 21 mm or 25 mm.

The results determined by macroscopic and histological evaluation (n = 30 per group) showed that MT were successful in producing hermaphrodites at a dose / duration dependent response, with the highest incidence of hermaphrodites (46.7%) in the groups that were treated for the longest time period at the high dose (the 12-25H group). The treatment groups 12-25L, 12-21H and 12-21L also had high incidences of hermaphrodites, with 32.2%, 29% and 24.1%, respectively, while 0% was observed in the 12-16L and 16-21H groups. The remaining groups had only a few hermaphrodites. The control group had a sex ratio of 46.7% males and 53.3% females and no hermaphrodites.

At two years of age, 3 hermaphrodites and 1 control male were selected on the basis of their plasma ratio of 11-ketotestosterone:estradiol-17β (11KT:E2). The sperm was used to fertilize the eggs of normal females with the aim to produce three all-female and one control population. The sex of the F1 generation was determined at 10 months of age (n = 50 pr group), revealing 100% females in the offspring of all three hermaphrodites, whereas the offspring of the control male consisted of 54.2% females and 45.8% males. These results provide evidence of female homogamety in Atlantic cod.

1. Introduction

Atlantic cod (*Gadus morhua*) is a candidate for intensive fish farming, and substantial efforts have been made to develop cod farming into a sustainable industry during the past 10 years. However, the growing cod farming industry is facing major problems connected to early sexual maturation or early puberty that is reached before the fish obtain desired harvest size (Taranger et al., 2006). Close to 100% of both females and males reach sexual maturation at the age of two years (Taranger et al., 2010), and approximately 30% of the males mature precociously after one year. Early sexual maturation results in economic loss due to weight loss and flesh quality reductions in both males and females (Karlsen et al., 2006), and sexually mature cod can also suffer increased mortality and welfare problems in farming (Taranger et al., 2010). Moreover, farmed cod may potentially have a genetic impact on wild cod stocks as they spawn readily in the cages and release fertilized eggs to the environment (Jorstad et al., 2008). One way to solve this latter problem is by the use of all-female populations, which will prevent the release of fertilized eggs from the cages. If combined with triploidy, all-female populations may also be a solution to the other maturation associated problems in cod farming (Trippel et al., 2008), as triploid females normally do not develop large gonads and hence do not suffer the loss of body weight and other spawning associated problems as seen in diploid fish (Piferrer et al., 2009).

All female populations are most commonly produced by using sperm from masculinized females provided that the species in question has a female homogametic sex determining system (Devlin and Nagahama, 2002). The masculinized females, so-called neo-males, are phenotypic males with testes and sperm, but genotypically they are females, and therefore carriers of XX chromosomes. The sperm will only carry X chromosomes, and on fertilization with normal XX females, the progeny will only consist of XX individuals.

Most fish species are labile in their phenotypic sex, and treatment with sex steroids during the time window of sex differentiation can influence the developmental processes overriding the genetic sex determination (Devlin and Nagahama, 2002; Yamamoto, 1969). In order to successfully masculinize a population it is important to

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understand sex differentiation both in terms of its timing, and its genetic basis. Sex differentiation is the process in which the primordial germ cells (PGC's) in the gonad start to rapidly proliferate and differentiate into oogonia in females and spermatogonia in males (Devlin and Nagahama, 2002). In fish, this is also the time window when the phenotypic sex can be manipulated, e.g. by treatment with androgens or aromatase inhibitors (Devlin and Nagahama, 2002). The timing of sex differentiation varies from species to species, as do the body size and/or the time-window when the phenotypic sex can be affected, as in e.g. the Nile Tilapia (*Oreochromis niloticus*) at 7-14 days post hatch (Kwon et al., 2000); European sea bass (*Dicentrarchus labrax*) where the time window is at 96-126 days post fertilization (Blazquez et al., 2001; Navarro-Martin et al., 2009); Atlantic halibut (*Hippoglossus hippoglossus*) at 30 mm fork length and 45 days onwards (Hendry et al., 2002); Golden rabbitfish (*Siganus guttatus*) at approximately 15 mm and 30 days onwards (Komatsu et al., 2006) and Common carp (*Cyprinus carpio*) at 2.6 g and for 40 days (Hulak et al., 2008). Based on histological findings, Chiasson et al. (2008) suggested that sex differentiation in Atlantic cod commenced at 27 mm body length. However, our own unpublished observations suggest that gonadal differentiation of Atlantic cod starts before 16 mm, hence the chosen starting point in the present study.

The objective of the study was to: 1) determine the time window of sex differentiation in Atlantic cod in order to identify the sex steroid sensitive period to produce hermaphrodites and 2) to subsequently use sperm from sex-reversed fish to produce all-female populations of Atlantic cod.

2. Materials and methods

2.1. Fish

Farmed Atlantic cod of Norwegian coastal cod origin were maintained under simulated ambient light conditions in spawning tanks supplied with sea water from 165 m depth (8.2 °C ± 0.3) at Institute of Marine Research (IMR), Austevoll, in Western Norway. Two L of fertilized eggs resulting from natural spawning in indoor spawning tanks were collected and transferred to four 70 L incubators at constant temperature (6.2 °C ± 0.1) and salinity (35‰). The fertilization rate was 87% 24 h after fertilization. At four days posthatch (dph) (118.6 day degrees) the larvae were transferred to 3 start feeding tanks (1 m diameter (Ø)) with water temperature of 8.3 °C. The water temperature was raised gradually to 12 °C over the next 10 days (14 dph). The tanks were supplied with algae paste and rotifers according to IMR's standard protocols on first feeding. At 33 dph the larvae received Artemia sp., and were weaned onto dry feed from 36 dph until 42 dph.

At 42 dph, the juveniles were divided into 36 50 L experimental units with 500 individuals per unit (n = 18 000) assuring an even size distribution amongst the experimental groups.

The temperature and oxygen level was measured daily, and water flow was adjusted accordingly so that the oxygen level was maintained above 80%, preferably over 90%. Mortality was recorded daily in all units in conjugation with the daily cleaning procedure until two weeks after the androgen treatments were completed.

2.2. Experimental diets

The experimental diet was a commercial agglomerated dry pellet (Ewos Aglo norse) with a particle size in the range of 300-500 μm for fish length of approximately 9-14 mm total length (TL), and 400-600 μm for fish lengths of approximately 13-22 mm TL. The diets were prepared as described by Komatsu et al. (2006) with 15 mg/kg diet (high dose) and 5 mg/kg diet (low dose) of 17α-methyltestosterone (MT) (Fluka, Buchs, Switzerland) dissolved in ethanol (60 ml/kg diet) and a control diet treated with ethanol only. The feed was supplied with an automated conveyer belt feeder to ensure continuous feeding 24 h a day, and trapped into a wide necked funnel, extended with a pipe releasing the feed 4 cm from the water surface, avoiding feed spill.

2.3. Experimental design

Preliminary histological studies (Haugen T, et al. in prep) demonstrated that the proliferation of PGC's had started at 16 mm TL in Atlantic cod, and that it was possible to separate the sexes by gonadal morphology and germ cell counts and size at 16-20 mm TL. It was therefore hypothesized that sex differentiation starts before 16 mm TL in Atlantic cod. Based on this information, the chosen starting points for androgen treatments were set to 12 mm and 16 mm TL.

Triplicate tanks were fed diets containing 15 mg/kg diet or 5 mg/kg diet MT from an average total body length of 12 mm to 16, 21 or 25 mm, and from 16 mm to 21 or 25 mm, giving a total of 10 different experimental groups (Fig. 1). The control diet was supplied to 6 replicate tanks. The replicates of the different treatments and the controls were randomly distributed among the 36 experimental units.

The cod juveniles had an average total body length of 12.1 mm (± 1.45) at the start of the experiment (42 dph). Total length of the juveniles was measured weekly, and the timing of the androgen treatments were set accordingly: 12-16 mm: 42-52 dph; 12-21 mm: 42-61 dph; 12-25 mm: 42-68 dph; 16–21 mm: 52–61 dph and 16-25 mm: 52–68 dph.

Two weeks after the MT treatments, triplicates were pooled for each treatment and the groups transferred to separate 1 m Ø tanks. The cod were kept at 12 °C until 7 months of age when the fish had reached a size (average weight of all groups; 63.0 g ± 5.6) which allowed them to be individually PIT tagged. All tagged fish were then pooled in a single 3 m Ø tank and reared until sex could be determined. At 10 months of age 60 fish pr treatment were weighed and 30 fish from each group sacrificed for sex determination and histological examination of the gonads. Thereafter the remaining fish were maintained in the tank until two years of age for the subsequent all-female production trial.

2.4. Sex determination and histological examination

2.4.1. Sex ratio

The fish were netted into a holding tank containing 0.04 g/l SW MS-222 (Tricaine Methanesulfonate, Finquel, Washinton, USA). 30 fish from each treatment were killed by a blow to the head and dissected, the gonads were photographed and sex was macroscopically

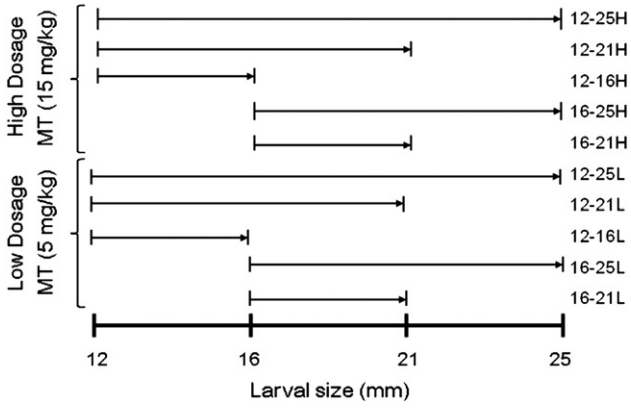

Fig. 1. The experimental setup and its respective group notations. Arrows indicate the length of the different treatments, all carried out in high (15 mg/kg diet) and low (5 mg/kg diet) dosages of added 17α-methyltestosterone. A control group was included in the setup (not shown).