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Successful production of monosex female brook trout *Salvelinus fontinalis* using gynogenetic sex reversed males by a combination of methyltestosterone immersion and oral treatments

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ABSTRACT

Recent advances towards the production of all-female populations of brook trout *Salvelinus fontinalis* have shown that methyltestosterone treatments applied after first feeding were not efficient in inducing masculinisation in neomales. A process to create all-female populations of brook trout using methyltestosterone was determined from 4 experiments. Different protocols of masculinisation by oral and/or hormonal immersion treatments were tested and the sex ratio of progeny produced from the cross of sex-reversed gynogenetic males with normal females was checked.

No sexual dimorphism for growth was recorded, at least until two years of age. Masculinisation trials using oral treatments failed to produce any reversal of sex. Only the application of four weekly immersions beginning one week before hatching, combined with an oral treatment, provided 100% masculinisation. Gynogenetic sex-reversed males crossed with normal females produced 100% females, demonstrating the efficiency of this protocol in reversing the sex of brook trout.

This result also highlights an earlier and longer period of sex lability, compared with most other Salmonids, and the need to initiate immersion treatments a long time before the initiation of gonadal differentiation.

1. Introduction

Brook trout (*Salvelinus fontinalis*) is a salmonid charr originating from North America where it was initially farmed (in both Canada and the U.S.A.). Brook trout was introduced into Europe in the 19th century, for restocking purposes, and today annual production in France is limited at 200–300 tonnes of fish [slaughtered at portion size (10 to 24 months old) or sold for restocking]. Its precocious maturation (usually one year for the males and two years for the females) induces great variability in flesh quality, as well as high male mortality due to the *Saprolegnia* fungus. According to Dorson et al. (1991), triploid hybrids between brook trout and rainbow trout are resistant to infectious hematopoietic necrosis virus (IHN) and to viral haemorrhagic septicaemia (VHS). However, the triploid male hybrid suffers from a lower growth rate (Quillet et al., 1986) than the sterile female hybrid. Several authors have underlined the potential interest of farming immature all-female or sterile all-female brook trout populations to solve these problems and to achieve bigger sizes (>600 g), more adapted for the fillet market and with a better feed conversion index (Johnstone et al., 1979; Boulanger, 1991; Galbreath and Stocks, 1999; Sacobie and Benfey, 2005). In addition, the farming of sterile fish is also advocated by FAO and several NGOs to prevent genetic interaction from escaped farmed genotypes with wild populations, in the interests of sustainable aquaculture (Komen et al., 2002). According to the EU Directive 96/22/CE (29th April 1996), only the "indirect feminisation" approach is authorised in Europe. It requires steroid-induced functional masculinisation of genetic females into so called "neomales" and their crossing with normal females to produce all-female progenies. The resulting sex reversed male broodfish must be killed and are not allowed to be sold for human consumption.

The brook trout presents a homogametic genetic sex determination system (Galbreath et al., 2003). Despite protocols to produce all-female populations using neomales having been published for most salmonids (see for review Hunter and Donaldson, 1983; Pandian and Sheela, 1995; Devlin and Nagahama, 2002), a poor success rate is reported for brook trout and the rate of female to male sex inversion remains low (Galbreath and Stocks, 1999; Galbreath et al., 2003). More over, recently it has been concluded that the use of methyltestosterone (MT) is not efficient and that only non-aromatizable androgens should be used even if incomplete efficacy of such treatments has been observed. Sacobie and Benfey (2005) determined the initiation of cytological gonad differentiation in this species, between 393 and 464 degree-days (°d) post-hatching. They

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suggested that previous works were performed too early before the initiation of the period of sex differentiation. They also proposed that higher rates of sex inversion, with lower doses and shorter duration, might be achieved by treatments initiated later and during the period of differentiation. All these publications (Galbreath and Stocks, 1999; Galbreath et al., 2003; Sacobie and Benfey, 2005) seem to indicate the lower sensitivity of the brook trout to sex inversion treatments (androgen, concentration, initiation and/or duration of the treatment). They also raised the question of the feasibility of achieving a high rate of sex inversion of neomales in commercial farming using MT. Nevertheless, none of them tested the efficiency of immersion treatments before hatching although success was reported in other salmonids (for reviews see Hunter and Donaldson, 1983; Pandian and Sheela, 1995; Devlin and Nagahama, 2002).

The objectives of the present study were to set up an efficient protocol to masculinize brook trout in order to produce all-female monosex populations for farming and/or restocking. Four experiments were set up to evaluate protocols of sex inversion using different combinations of immersion treatment performed before, during and/or after hatching, and/or oral treatments. The efficiency of the more efficient sex inversion protocol was also checked by evaluation of sex ratios of progenies from a cross between sex-reversed gynogenetic (neomales) and normal females.

2. Materials and methods

2.1. Farming conditions

The experiments were implemented between 1991 and 2002 at the Aqualande commercial hatchery (Sources de l'Avance, Pissos, France). Water was supplied from the Leyre River, which undergoes large variations in water temperature between winter (3–4 °C) and summer (20–21 °C). Progenies used in the sex reversion trials were obtained from the commercial production and always involved a large number of parents (>30 females and 20 males). Broodstock and experimental fish (alevin, fry, sub adult, broodstock) were fed with commercial foods during the experiments (Le Gouessant, Skretting). Broodstock reproduced naturally by hand stripping under a natural photoperiod regime in November and without hormonal stimulation. Progeny were grown on in fibre glass tanks until the fry stage and were then transferred to concrete race-ways for further growth. The water was oxygenated with liquid oxygen to provide at least 80% oxygen saturation. Density during growth did not exceed 50 kg/m^3. During sex inversion trials, fish were reared on ground water and thus the temperature remained almost constant (11.5–12 °C). In these conditions, eggs began to hatch at 420 °d after fertilisation; this was considered as the reference for defining timing of sex inversion treatments.

2.2. Experiment 1

Experiment 1 was designed to evaluate the efficiency of oral treatments alone. Mixed sex (XX and XY) eggs produced in November 1991 were subdivided into five groups (Table 1); a control group (Group A) and four oral-treated groups receiving a diet of 3 or 9 mg of 17α-methyltestosterone (MT)/kg, delivered during 800 or 1065 °d from the first feeding: Group B (3 mg/kg ; 800 °d), Group C (3 mg/kg ; 1065 °d), Group D (9 mg/kg ; 800 °d) and Group E (9 mg/kg; 1065 °d). At 12 months of age, 51 to 64 individuals per group were weighed and

Table 1
Ratio of males (M), females (F) or undifferentiated fish (U) at 12 months of age, or intersex fish (I) at 24 months of age, for brook trout in the treated groups (M:F:I or H) of Experiments 2, 3 and 4 at 1 year old (1994) or 2 years old (1995).

Group	Im − 1	Im − 0	Im + 1	Im + 2	Feed (mg MT per kg feed/degree-days)	Age (months)	*N*	Sex ratio (M:F:U or I)	Males BW (g)	Females BW (g)	Undifferentiated or Intersexed BW (g)
Experiment 1											
Group A (control)						12	51	53:47:0	97.9 ± 34.3§		
Group B					3/800	12	53	51:49:0	55.5 ± 21.4§		
Group C					3/1065	12	52	50:50:0	59.0 ± 16.4§		
Group D					9/800	12	64	41:59:0	42.1 ± 17.0§		
Group E					9/1065	12	53	51:49:0	45.2 ± 22.3§		
Experiment 2											
Group F (control)						12	48	54:46:0	109.2 ± 28.1	124.4 ± 33.2	–
Group G	Im		Im	Im	3/800	12	48	54:33:13	72.3 ± 21.3	70.6 ± 24.7	34.2 ± 20.4
						24	100	56:39:5	367.0 ± 96.0	301.0 ± 102.0	354.0 ± 102.0
Group H	Im		Im		3/800	12	48	54:33:13	81.3 ± 22.3	69.2 ± 22.6	44.5 ± 31.3
						24	100	62:36:2	356.0 ± 113.0	337.0 ± 77.0	422.0
Group I	Im				3/800	12	52	56:38:6	100.8 ± 37.6	94.4 ± 31.6	73.6 ± 72.9
						24	100	47:53:0	371.0 ± 47.0	331.0 ± 53.0	–
Group J	Im			Im	3/800	12	60	27:65:8*	61.8 ± 22.3	63.2 ± 22.3	71.4 ± 14.0
						24	100	43:46:11	358.0 ± 100.0	318 ± 74	370 ± 120
Group K	Im		Im	Im		12	55	47:53:0	69.8 ± 11.9	59.0 ± 20.0	–
						24	100	46:53:1	342 (88)	343 ± 85	345
Experiment 3											
Group L (control)						12	73	40:60:0	33.4 ± 16.8	28.4 ± 10.9	–
						18	100	22:78:0	205.1 ± 65.1	176.6 ± 46.6	–
Group M	Im		Im	Im	3/800	12	100	80:20:0**	205.1 ± 65.1	176.6 ± 46.6	–
						18	100	66:20:14**	234 ± 71.3	254.3 ± 892	229 ± 81.8
Group N	Im	Im	Im	Im	3/800	12	50	74:24:2**	30.7 ± 11.9	36.8 ± 6.6	34.5 ± 0
						18	100	94:2:4**	216.3 ± 86	180 ± 0.0	204 ± 22.6
Group O		Im	Im	Im	3/800	12	50	80:18:2**	29.8 ± 9.6	36.5 ± 4.7	39.1 ± 0
						18	100	77:2:21**	160.5 ± 62.2	162 ± 0.0	179.3 ± 61.5
Group P (sex reversed gynogenic)	Im	Im	Im	Im	3/800	12	26	100:0:0**	36.1 ± 10.1	–	–
						18	100	100:0:0**	285.9 ± 61.2	–	–

The different combinations of hormonal treatments in the experiments by immersion (Im: 400 µg methyltestosterone/L during 4 h with 2000 fries/20 L) and/or by administration in the feed (3/800, 3/1065, 9/800 or 9/1065:3 or 9 mg MT/kg diet during 800° or 1065 °d from the first feeding). Im − 1, Im − 0, Im + 1 and Im + 2 represent immersion treatments performed 1 week before 50% of hatching (Im − 1), the week of hatching (Im − 0), 1 week after 50% of hatching (Im + 1), 2 weeks after 50% of hatching (Im + 2). Age is expressed in months. *n* = number of fish sexed. Mean body weight (BW) of the males, females and undifferentiated or intersexes is expressed as the mean weight ± standard deviation. § = mean weight for the whole population. Significant difference between the sex ratio of control groups and MT treated groups at levels of $P<0.05$ (*) and $P<0.001$ (**).