

Preliminary Assessment of Sex Inversion of Farmed Atlantic Salmon by Dietary and Immersion Androgen Treatments

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Abstract.—Dietary and immersion treatments with the androgens 17 α -methyltestosterone (MT) and 17 α -methylidihydrotestosterone (MDHT) were assessed for their efficacy in the masculinization of Atlantic salmon *Salmo salar*. Dietary treatments with MT at 1 or 3 mg/kg food or MDHT at 1 mg/kg food for 800 degree-days resulted in 100% masculinization of all female stocks. Single or double immersion treatments of alevins in MDHT at 400 μ g/L for 120 min during the period 0–28 d after median hatch resulted in significant masculinization. Immersion treatments were most effective when conducted more than 14 d after median hatch. Two immersion treatments 7 or 14 d apart resulted in masculinization levels of up to 100%, whereas single immersions yielded levels up to 77%. Immersion treatment resulted in the production of significantly more sex-inverted males with patent sperm ducts than did dietary treatment. The study shows that immersion treatments are simple and effective for androgen-induced masculinization of Atlantic salmon, that they are suitable for commercial-scale use in hatcheries, and that they offer advantages in production efficiency over dietary treatments.

Since the capacity of exogenous steroid hormones to influence sexual development in fish was demonstrated, numerous studies have investigated both the necessary protocols for manipulating development and some of the mechanisms involved (reviewed by Hunter and Donaldson 1983; Pandian and Sheela 1995; Piferrer 2001; Devlin and Nagahama 2002). The studies of Yamamoto (1969) showed that exogenous androgens masculinize sexually differentiating individuals whereas estrogens are feminizing agents, and Yamamoto proposed that these compounds are the natural masculinizing and feminizing agents in fish. The use of these compounds to produce monosex fish populations for aquaculture is now well established and is routine where monosex populations fulfil production needs or environmental constraints (Hunter and Donaldson 1983; Piferrer 2001).

Although both natural and synthetic steroids are effective for sex inversion, they vary considerably

in their efficacy (Piferrer and Donaldson 1991, 1992; Piferrer et al. 1993; Piferrer 2001). The synthetic androgens 17 α -methyltestosterone (MT) and 17 α -methylidihydrotestosterone (MDHT) have been shown to have greater potency than either testosterone or 11-ketotestosterone (Piferrer et al. 1993; Pandian and Sheela 1995) and have been widely used (particularly MT) both in experimental studies (e.g., Baker et al. 1988; Piferrer et al. 1993; Blazquez et al. 2001) and the commercial production (Johnstone and MacLachlan 1994) of masculinized fish. Similarly, ethynylestradiol-17 α , a synthetic estrogen, is known to be significantly more potent than the natural 17 β -estradiol (Yamamoto 1969; Piferrer and Donaldson 1992). Devlin and Nagahama (2002) proposed that differences in potency reflect differences in receptor affinity, the activities of steroid-receptor complexes, and steroid metabolism.

The administration of steroid hormones to manipulate sexual development has been achieved by a number of means (reviewed by Pandian and Sheela 1995). Dietary administration involves the

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application of a solution of the steroid in a volatile solvent to manufactured, pelletized feed that fish are fed over a period of several weeks. Typically, dietary treatments commence at or near the time of first feeding. An alternative is immersion treatment, whereby individuals are placed in an aqueous solution of the steroid for several hours. Unlike dietary treatments, this technique may be applied to individuals before hatching or feeding and the exposure each individual receives can be accurately determined. Immersion treatments are also easier to complete and may entail fewer issues of worker safety (Pandian and Sheela 1995; Piferrer 2001). Regardless of the method used to apply steroids, treatment is optimal during the labile period, during which sex differentiation may be influenced by exogenous factors (Pandian and Sheela 1995; Baroiller et al. 1999; Piferrer 2001; Devlin and Nagahama 2002).

Effective steroid doses for sex inversion vary between and within species. In dietary studies, Kitano et al. (2000) achieved 100% masculinization of the Japanese flounder (also known as the olive flounder) *Paralichthys olivaceus* using MT at a dose of 10 mg/kg food for 1,260 degree-days, while a similar result was obtained for rainbow trout *Oncorhynchus mykiss* treated at 0.5 mg/kg food for 548 degree-days (Cousin-Gerber et al. 1989). Complete masculinization of all female stocks of Chinook salmon *O. tshawytscha* was achieved by immersion for 120 min in MT at a concentration of 400 µg/L (Piferrer et al. 1993) or 200 µg/L (Baker et al. 1988). Repeated immersion treatments were more effective for the masculinization of Chinook salmon (Baker et al. 1988); however, masculinization rates declined with repeated immersion treatments of rainbow trout (Feist et al. 1995). Within limits, the effects of steroid treatments may be dose dependent (Baker et al. 1988; Piferrer and Donaldson 1991; Piferrer et al. 1993), but high doses have resulted in the production of a significant proportion of sterile individuals (Johnstone et al. 1978; Goetz et al. 1979; Johnstone et al. 1979; Solar et al. 1984). Paradoxical feminization, the phenomenon of having an increased proportion of females after administering high doses of androgens, has also been reported in some species (Goudie et al. 1983; Solar et al. 1984; Piferrer and Donaldson 1991; Piferrer et al. 1993). This is attributed to the aromatization of androgens to biologically active estrogens (Piferrer and Donaldson 1991; Piferrer et al. 1993).

All-female stocks of Atlantic salmon *Salmo salar* are preferred in Tasmania for the production of non-

maturing triploids (Jungalwalla 1991) and because such stocks have a reduced propensity for early maturation following transfer to seawater (Donaldson et al. 1996). Current production methods use the indirect method of feminization, which is reviewed in detail by Piferrer (2001). This method, which is suitable for species having homogametic females, involves the sex inversion of a small proportion of females that subsequently develop as phenotypic males and are used as broodstock to provide milt for crosses with normal females, resulting in the production of unmanipulated, all-female offspring. While early attempts at the masculinization of Atlantic salmon by dietary and immersion treatments were largely unsuccessful (Johnstone et al. 1978), later studies considerably improved treatment efficacy (Johnstone and MacLachlan 1994). Even so, no treatment to date has had the level of success or ease of application as those achieved for *Oncorhynchus* spp. This study aimed to develop more effective dietary and immersion treatment regimes for the masculinization of Atlantic salmon and to apply such treatments to the production of sex-reversed males under commercial conditions. Accordingly, in an initial experiment, a series of immersion and dietary protocols were conducted to better define the labile period for immersion treatment of this species and to ascertain effective dietary treatment levels. Subsequently, the most successful immersion and dietary treatments were repeated on larger groups of fish, which were then grown to maturity for assessment of fertility. While MT was initially used as the established dietary treatment, nonaromatizable MHDT was preferred for the immersion and latter dietary treatments on the expectation that paradoxical feminization would be avoided.

Methods

Stock and Husbandry

Experiments were conducted over 2 years (1991 and 1992) at Saltas Freshwater Operations, Wayatinah, Tasmania, with the progeny of age-2 and age-3 Atlantic salmon broodstock. Prior to transfer to freshwater for final maturation, broodstock had been maintained at Saltas Marine Operations, Dover, Tasmania. Broodstock were reared in a 65-m, polar-circle-style cage and fed a steam-pelletized commercial diet to satiation (Gibsons, Ltd., Cambridge, Tasmania). Sex-inverted males were used to provide milt in both experiments. These stocks had been masculinized by dietary treatment with MT at 3 mg/kg feed following methods similar to

TABLE 1.—Masculinization rate of Atlantic salmon following immersion in 17 α -methylidihydrotestosterone (MDHT) for 120 min at different times and dietary treatments with 17 α -methyltestosterone (MT) for 800 degree-days. Asterisks denote values that are significantly different from that for group A (control); na = not applicable.

Group	Hormonal treatment			
	Dietary MT (mg/kg)	MDHT immersion (days after median hatch) ^b		% male
		First	Second	
A ^a	na	na	na	8
B	1	na	na	100*
C	3	na	na	100*
D	na	0	na	11
E	na	0	7	61*
F	na	0	14	54*
G	na	7	na	58*
H	na	7	14	77*
I	na	7	21	79*
J	na	14	na	61*
K	na	14	21	90*
L	na	14	28	95*

^a Untreated all-female population (control).

^b Zero days after median hatch is approximately equivalent to 450 degree-days postfertilization, 7 to 510 degree-days, 14 to 570 degree-days, 21 to 630 degree-days, and 28 to 690 degree-days.

those of Solar et al. (1984). Fertilized ova were incubated in commercial upwelling incubators at 8°C. Once eyed, eggs were transferred to Heath vertical incubator trays (Maricourse, Inc., Tacoma, Washington) and maintained at 7–10°C until the swim-up stage. Fry from each treatment group were transferred to separate 1-m³ tanks for first feeding and subsequent rearing. Immersion treatments were timed in relation to the day of median hatch (approximately 450 degree-days (°C) postfertilization), and dietary treatments commenced at first feeding. Populations of parr were maintained in indoor 1-m³ tanks receiving water at 10–12°C and under a natural photoperiod until sampled for gonadal examination (approximately 8 months posthatch).

Experimental Protocols

Experiment 1.—Stock solutions (1.0 mg/mL) of MT and MDHT were prepared in 100% ethanol and refrigerated until required. Feed for dietary treatments was prepared by diluting the MT stock solution in 100% ethanol, mixing it with feed at 200 mL/kg, and leaving it overnight to allow evaporation of the solvent. Feed was prepared with either 1 or 3 mg MT/kg and fed to separate groups of 6,000 fry (treatment groups B and C) for a period of approximately 800 degree-days commencing at the onset of feeding at approximately 850

TABLE 2.—Masculinization rate of Atlantic salmon following immersion in 17 α -methylidihydrotestosterone (MDHT) for 120 min at different times and dietary MDHT treatments for 800 degree-days. See the caption to Table 1 for additional information.

Group	MDHT treatment			
	Dietary (mg/kg)	Immersion (days after median hatch)		% open sperm ducts
		First	Second	
A	na	na	na	2
B	1	na	na	100*
C	na	14	21	100*
D	na	14	28	100*

degree-days postfertilization. Bathing commenced at median hatch (day 0, approximately 450 degree-days postfertilization), baths being undertaken weekly up to 28 d post median hatch (DPMH) or approximately 690 degree-days postfertilization. Treatment groups D–L received either a single bath or two baths 7 or 14 d apart, as shown in Table 1. Bathing was undertaken in Heath trays, the MDHT stock solution being added to water to give a final concentration of 400 μ g/L, which was recirculated through the Heath trays for 120 min at 0.3 L/s. Recirculation ceased at the completion of the immersion period, when flow-through resumed at 0.3 L/s to ensure the dispersal of any remaining steroid. Duplicate trays, each containing 3,000 fry, were used for each immersion treatment group. Owing to restrictions imposed by commercial considerations, only a single population of untreated, all-female fish, derived from the same parental stocks as the experimental fish, was maintained as a control for both the dietary and immersion treatments. Such populations are known to contain a small proportion of genetic males (H. King, unpublished data) resulting from the inclusion of genetic male broodstock in all-female production.

Experiment 2.—To exclude the possibility of paradoxical feminization, MDHT was used for both dietary and immersion treatments in experiment 2. A diet with 1 mg/kg was prepared and fed to a single group of 6,000 fry as in experiment 1. Duplicate Heath trays, each containing 3,000 fry, were immersed for 120 min in a tank containing MDHT at 400 μ g/L. Baths commenced 14 DPMH (approximately 570 degree-days postfertilization), groups of fry receiving a second bath either 21 DPMH (approximately 630 degree-days postfertilization) or 28 DPMH (approximately 690 degree-days postfertilization) (Table 2). As in experiment 1, a single population of untreated, all-

female fish, derived from the same parental stocks as the experimental fish, was maintained as a control for both the dietary and immersion treatments.

Fish that had been grown out at Saltas Marine Operations were returned to freshwater as age-2 and older fish, and phenotypic males were examined for sperm duct morphology.

Gonadal Examination

In both experiments 1 and 2, a sample of approximately 100 fish was collected from each treatment group 9 months posthatch. These fish were euthanized by a blow to the head and dissected for gonadal examination under a dissecting microscope. Individuals having oocytes present within the gonad were scored as female and all others were scored as male; thus, intersex or sterile individuals may have been included with either the male or female fish. In experiment 2, fish remaining after sampling for gonadal examination were retained for a further period of approximately 2 months until they reached a weight of approximately 45 g. Treatment groups were marked by freeze branding, maintained in 1-m³ tanks for a further 4 months until transfer outdoors to 4-m³ tanks, where they remained until smoltification and subsequent transfer to Saltas Marine Operations. Fish were maintained as part of a population of approximately 5,000 fish in a 65-m, polar-circle-style cage and reared under standard Tasmanian commercial conditions from their arrival in September 1993 until they were returned to Wayatinah in February 1995 for assessment of maturity, fertility, and sperm duct patency.

Statistical Analysis

Alterations in sex ratio relative to untreated, all-female (control) groups were tested by means of a contingency chi-square test for each treatment group. In experiment 2, differences in sperm duct patency frequency between treatment groups B, C, and D were assessed with the same test.

Results

Experiment 1

With the exception of a single immersion treatment on median hatch day (treatment D), all dietary and immersion treatments significantly increased the proportion of phenotypic males (Table 1). Both dietary treatments (B and C) resulted in complete sex inversion of the population. Single immersion treatments 0, 7, or 14 DPMH (treatments D, G, and J) resulted in masculinization levels of 11, 58, and 61%, respectively. Conducting

a second immersion treatment 7 or 14 d after a bath at 0 DPMH (treatments E and F) significantly increased the proportion of masculinization (61% and 54% males, respectively) in comparison with the control population (8% males). Although all other treatments resulted in significant levels of masculinization compared with the control populations, some qualitative differences between treatments were apparent. A second immersion following a bath at 7 DPMH (treatments H and I) resulted in increased levels of masculinization (77% and 79%, respectively), as did a second immersion following a bath at 14 DPMH (treatments K and L; 90% and 95%, respectively) (Table 1).

Experiment 2

All MDHT treatments resulted in the complete masculinization of fish sampled at 9 months posthatch, which was significantly different from the untreated, all-female population (Table 2). The incidence of sperm duct patency was highest in the immersion-treated fish (treatments C and D; 92% and 84%, respectively; Table 2) and differed significantly between treatment groups B (59%), C, and D ($\chi^2 = 14.4$, $df = 2$, $P < 0.01$).

Discussion

The high levels of masculinization achieved by dietary treatments of both MT and MDHT in this study are consistent with, or improvements upon, the levels achieved previously using dietary androgens with Atlantic salmon. Johnstone et al. (1978) achieved 100% masculinization of Atlantic salmon after dietary treatment with MT, but 17% of these fish proved to be sterile with gonads comprised primarily of connective tissue. Johnstone and MacIachlan (1994) reported the same phenomenon at a high level in Scottish commercial hatcheries using a dietary treatment of 3 mg MT/kg feed. Similar effects have been noted in other species treated with high doses of androgens (Goetz et al. 1979; Solar et al. 1984; Piferrer et al. 1994a; Galbreath and Stocks 1999; Blazquez et al. 2001). As neither fertility nor gonad histology were assessed in experiment 1, it is not known whether a similar effect occurred in similarly treated animals here (treatment group B); it is possible that animals with gonads containing primarily connective tissue were present but that, having apparently filiform gonads, they were recorded as males.

The effectiveness of low-dose androgens (1 mg/kg feed) found in this study (experiment 1, treatment C; experiment 2, treatment B) is substantially higher than that found by Johnstone and MacIach-

lan (1994), who reported a masculinization level of 46–56% in mature fish treated with 0.25–1.0 mg MT/kg feed. However, levels in this range may underestimate the actual level of masculinization, as the sex of immature fish was not determined (Johnstone and Youngson 1984). Alternatively, the observed differences between the present study and that of Johnstone and Maclachlan (1994) may reflect the differences in treatment duration (800 degree-days compared with 500) between the studies. The duration of steroid exposure has been previously shown to affect the degree of sex inversion in salmonids (Chevassus and Kreig 1992; Piferrer and Donaldson 1992). Increased duration of both dietary (Chevassus and Kreig 1992) and immersion (Piferrer and Donaldson 1992) steroid treatments resulted in increased levels of sex inversion.

There was no evidence of paradoxical feminization in the present study, with dietary doses of the aromatizable androgen MT ranging from 1 to 3 mg/kg feed for 800 degree-days. Substantially higher doses than those used in the present study (>25 mg/kg feed for 330–1,320 degree-days) caused some degree of paradoxical feminization in rainbow trout (Solar et al. 1984), and high-dose (6.4 mg/L) immersion treatments with MT were associated with the production of an increased proportion of females in coho salmon *O. kisutch* (Piferrer and Donaldson 1991). This effect was not apparent with an equivalent dose of nonaromatizable MDHT (Piferrer and Donaldson 1991), and on this basis it has been proposed that the mechanism of paradoxical feminization is aromatization of androgens to estrogens (Piferrer et al. 1993). While the doses of MT and MDHT used in the present study were sufficient to induce complete sex reversal, there was no evidence of other reported effects, such as the production of intersex or sterile individuals (Johnstone et al. 1978; Johnstone and Maclachlan 1994), that have been attributed to insufficient or excessive doses (Galbreath and Stocks 1999).

The results of the present study suggest that treatment by immersion is most effective in the period 14–28 DPMH, suggesting that the labile period for masculinization of Atlantic salmon commences during this time. This result is consistent with other data for Atlantic salmon, for which immersion was most effective from 650 to 900 degree-days postfertilization (Johnstone and Maclachlan 1994); this is equivalent to 16–45 DPMH under the conditions in this study. However, these findings contrast with those of Baker et al. (1988), Piferrer and Donaldson (1989), Pi-

ferer et al. (1993), and Feist et al. (1995), who showed that in Chinook salmon, coho salmon, and rainbow trout the labile period for androgen immersion treatment commenced at or before hatching but could extend to 13 DPMH. Variations in the labile period among different taxa are well recognized (Hunter and Donaldson 1983; Pandian and Sheela 1995; Piferrer 2001), probably reflecting interspecific differences in developmental rates (Devlin and Nagahama 2002). In Atlantic salmon, gonadal primordia appear at 60 degree-days pre-hatch while gonadal differentiation continues to 600 degree-days posthatch (Laird et al. 1978). In contrast, in coho salmon gonadal primordia were detected at 10 degree-days pre-hatch, differentiation commenced at 270 degree-days posthatch (Piferrer and Donaldson 1989), and differentiated gonads were discernible at 490 degree-days posthatch (Goetz et al. 1979). This suggests that observed differences in the labile period between Atlantic and Pacific salmon arise from differences in the timing and duration of gonadal differentiation.

The qualitative increase in masculinization following repeated immersion suggests that under the rearing conditions used double treatments may be more effective than single ones. Repeated immersion in MT has been used to produce a high masculinization rate for Atlantic salmon (Johnstone and Maclachlan 1994); however, that study did not compare the treatment with a single immersion. Other studies investigating the effects of multiple immersion treatments on sex inversion in salmonids have had varying results. Baker et al. (1988) found increased levels of masculinization in Chinook salmon following a second immersion treatment; however, a decrease in masculinization was seen with additional immersion treatments of rainbow trout (Feist et al. 1995). Single immersion treatments of coho and Chinook salmon have resulted in masculinization rates of 70–100% (Piferrer and Donaldson 1989, 1991; Piferrer et al. 1993, 1994b). It is likely that the increased effectiveness of a second immersion treatment is due to the maintenance of increased tissue loading of androgen, thereby allowing the increased exposure of susceptible individuals over time. The results of the present study are therefore consistent with a requirement by Atlantic salmon for higher levels of androgens (or elevated levels of androgens for a longer period) for masculinization to occur than is the case for Pacific salmon.

The results of experiment 2 showed that both immersion and dietary treatment of Atlantic salm-

on with MDHT are successful means of sex inversion. This is the first time that MDHT has been reported to masculinize Atlantic salmon, as well as the first successful use of MDHT by dietary treatment. MDHT has been successfully used in immersion treatments of coho and Chinook salmon (Piferrer and Donaldson 1991; Piferrer et al. 1993) and rainbow trout (Krisfalusi and Cloud 1999), although dietary treatment of channel catfish *Ictalurus punctatus* with MDHT was unsuccessful (Davis et al. 1990).

The increased incidence of patent sperm ducts in immersion-treated fish (relative to that in dietary-treated fish) is consistent with the results of other studies. The association between sex inversion by dietary administration of androgens and malformations of the gonad has been noted by several authors (Johnstone et al. 1979; Johnstone and Youngson 1984; Cousin-Gerber et al. 1989; Feist et al. 1995), while studies involving immersion treatments only report few malformations. In a study directly comparing the effects of immersion and dietary androgen treatments in rainbow trout, Feist et al. (1995) found a higher incidence of patent ducts in immersion-treated fish. While the dose and duration of dietary androgen treatment can affect the incidence of sperm duct patency (Cousin-Gerber et al. 1989), the phenomenon of impaired development of the testes in dietary-treated animals may be a function of the developmental timing of dietary treatment, gonads at a later stage of development being less plastic in their growth response.

The current reliance of hatcheries on gonad morphology to distinguish sex-inverted male Atlantic salmon from genetic males means that malformations of the sperm duct and other traits typical of dietary-treated animals are necessary for the recognition of sex-inverted broodstock—although their presence restricts the use of such animals to a single season (Johnstone and MacLachlan 1994). Furthermore, the recognition of sex-inverted males by morphological features may still be subject to error, as the presence of a small proportion of males in the all-female lines used in this study indicates. The absence of a determinant of genotypic sex therefore not only places a restriction on the commercial use of immersion treatments for the sex inversion of Atlantic salmon but also affects the quality of the stock produced from broodstock that have been sex-inverted by dietary treatments.

The present study has demonstrated the effectiveness of both dietary and immersion androgen

treatments for the masculinization of Atlantic salmon and that masculinization by immersion results in a high proportion of sex-inverted males with patent sperm ducts. Furthermore, the study has demonstrated the successful application of these treatments on a commercial scale. This has significant implications for the Atlantic salmon farming industry in Tasmania, which is increasingly reliant on all-female stocks for year-round production.

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