DEVELOPMENT OF A PROTOCOL FOR CONTROL OF GONADAL DIFFERENTIATION THROUGH SEX STEROID ADMINISTRATION IN THE ARCTIC CHAR (SALVELINUS ALPINUS)

by

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ABSTRACT

Arctic char (Salvelinus alpinus) underwent oral administration of 17α-methyltestosterone(MT) and estradiol-17β(E_2) to induce masculinization or feminization, respectively. Drugs were administered for 600° C days from swim-up. Controls were developed in conjunction with the treatment groups. Histological examination showed a 60:33 male:female ratio in the MT-treated group(n=196) with 7% intersex, and a 36:49 male:female ratio in the E_2 -treated group(n=192) with 15% intersex. Controls(n=194) yielded a 55:45 male:female ratio, with 0% intersex. Steroid-treated fish were smaller than controls but had a higher condition factor. Conclusions were that the labile period of A. char occurs physiologically earlier than swimup, and therefore immersion therapy at an earlier developmental stage might be more efficacious. A non-aromatizable androgen and more potent estrogen could improve the rates of masculinization and feminization.

The second segment consisted of testing 5 experimental variables utilizing 17α-methyldihydrotestosterone(MDHT) and ethynylestradiol-17α(EE_2) in aqueous solutions. Ambient immersion temperatures were 2-3°C. Weekly immersions were initated one week pre-hatch(424 ATUs) and continued until 4 weeks post hatch(529 ATUs). Treatment durations of 3, 6 12, 24 and 48 hours were completed one week post-hatch(466 ATUs). MDHT dosages of 0.5, 1.0 and 10.0 mg/l, and EE_2 at 0.5, 1.0 and 2.0 mg/l were tested. Triple immersions were initiated one week pre-hatch with 2 subsequent weekly treatments. New treatment groups were initiated 0, 1 & 2 weeks post-hatch. Combined immersion/feed treatments completed one week post-hatch for both steroids. Immersions were followed by steroid-treated feed at swim-up. Feed-only groups were developed for both MDHT and EE_2. All treated feed contained steroid at 7mg/kg

Results indicate maximal lability for masculinization at one week, and for feminization 3 weeks, post-hatch. Extended durations did not elevate masculinization, but high partial feminizations. Elevated dosage improved masculinization over feminization rates. Triple immersions did not elevate masculinization or feminization rates over single immersions. All immersion-feed and feed treatment groups were lost to hypoxia during rearing. These fish did not accept feed readily, which was attributed to hypoxic stress. Conclusions from this study are that low water temperatures significantly reduced rates of masculinization and feminization, that optimum rates of masculinization occur one week post-hatch in response to elevated dosages while optimum feminization occurs 3 weeks post-hatch with extended treatment durations. Multiple immersions do not improve the efficacy of sex control procedures.

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INTRODUCTION:

The control of gonadal differentiation in fish through the administration of sex steroids has been of interest to researchers for several decades. One of the major factors behind these studies is the economic benefits to the aquaculture industry which can be realized through sex control when one sex consistently exhibits superior performance in an aquaculture setting. Due to the relatively high commercial value of salmonids, much of the recent work in this area has focused on this family. The objective of developmental sex control techniques is to override the endogenous sex-determining mechanisms of the fish and thereby redirect the developmental pathway of the gonad. The most effective methods of directly achieving this objective have proven to be through the application of androgens to produce monosex male populations, and estrogens to produce similar populations of females.

Studies regarding the effects of steroid application to fish were initiated in the 1930's (Padoa, 1937;1939) and a large body of research over the next 3 decades indicated that effective sex control could be accomplished using these techniques. The initial research using salmonids was carried out by Ashby (1957). Yamamoto (1969) conducted a thorough review of these studies which has subsequently become the basis for much of the work in this area of research. In this report he concluded that androgens and estrogens were the previously undetermined factors which established the pathway of gonadal differentiation in fish. The study also discussed the genetic basis of sex determination, the "XX-XY" homogametic/heterogametic system, and the parameters of treatments necessary for successful control of gonadal differentiation in teleosts.

I. LABILE PERIOD:

The effective control of gonadal differentiation involves the application of the hormones during development, previous to the time of physiological differentiation of the gonad into testis or ovary (Hunter & Donaldson, 1983). The timing of the steroid application relative to the normal development of the species has proven to be a major factor in the efficacy of treatment outcome. In the course of their development each species appears to have a window of time or "labile period" during which they are maximally sensitive to the effects of sex hormones on gonad differentiation. Goetz et al. (1979) found that in coho salmon (Oncorhynchus kisutch) the androgen 17-α-methyltestosterone (MT) administered orally through the feed at a concentration of 20 mg/kg for 70 days post swim-up induced increased incidence of testis development, and oocytes were present in only 24% of the fish. Similarly, estradiol-17-ß (E2) fed at a concentration of 10 mg/kg over the same time frame resulted in 54% fish with gonads similar to control females, 18% similar to male controls, and 25% intersex fish. These results would appear to conflict with those from rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar) (Johnstone et al., 1978), where E2 administered through the feed for 21 or 30 days respectively resulted in 100% monosex female populations in both groups. The comparison of these two studies gives an indication of the importance of the differences in the timing of effective treatments for these species. Subsequent studies have confirmed that sex control in coho salmon could not be achieved through dietary supplement alone (Hunter & Donaldson, 1982). Hunter et al. (1986) treated coho and chinook salmon (Oncorhynchus tsawytscha) with a range of E2 concentration immersions of newly-hatched alevins to show that

the timing of treatments was of greater importance than dose level in obtaining a high percentage of females. In a very thorough investigation of the labile period of coho salmon, Piferrer & Donaldson (1989), using 4.0 mg/l of either MT or E2, established the period of maximum effectiveness of the steroid immersions. The respective experimental groups were given identical treatments 7 days apart beginning 15 days before hatch and continuing until 48 days after hatch. The results of this study indicate clearly that the period of greatest sensitivity to androgens, as well as estrogens, coincides very closely with the time of hatch in this species. Similar immersion experiments, such as those carried out by Nakamura (1984) using E2 to affect sex differentiation in masu salmon (Oncorhynchus masou) and chum salmon (Oncorhynchus keta) utilized continous immersions which ranged from 18 to 29 days. Although these trials were successful, they do not deliniate the labile period as accurately as Piferrer and Donaldson's study due to the extended duration of the immersions.

Fitzpatrick et al. (1987) have measured a peak of endogenous androgen and estrogen which appears to occur concurrently with the period of greatest sensitivity to exogenous steroid application. Piferrer and Donaldson (1989) conclude from this that critical physiological and morphological changes which occur during this stage of development account for the maximimal sensitivity to the exogenous steroids. As this critical period of development is likely to vary between species, studies which determine the relative efficacy of steroid immersion at specific times throughout the course of development would be needed to maximize control of sexual differentiation.

II. DURATION OF IMMERSIONS:

The duration of steroid immersion has also proven to be a significant factor determining the success of the treatments. A limited number of studies exist which have used a single immersion to influence gonadal development in a manner which would allow the minimum period of treatment required to be determined. In a unique experiment utilizing monosex female populations, Baker et al. (1988) successfully produced 100% male populations of chinook salmon through 2 hour immersions in MT at concentrations of 0.2 mg/l When the duration of the immersion was reduced to 30 minutes only 84.0% of the resultant population was determined to be male. Alternatively, feminization of chinook salmon to the 100% level required an 8 hour immersion in E2 at a concentration of 0.4 mg/l when applied 3 days post-hatch (Piferrer & Donaldson, 1992). A reduction of the immersion duration from 8 to 2 hours reduced the female proportion to 72.2%. It is interesting to note that Hunter et al. (1986) were able to achieve an 81.8% female population with chinook salmon under the same experimental conditions but utilizing two 2 hour immersions administered at 2 and 9 days post-hatch. This evidence indicates that, for a given androgen or estrogen, a minimum combination of steroid concentration and immersion period exists which will result in complete masculinization or feminization of the population. The difficulty which arises in any attempt to assess the double immersion experiments resides in whether one or both of the treatments occur within the effective limits of the labile period. As it has been shown that labile period is of significant importance in achieving control of gonadal differentiation, one of the two immersions may have reduced effect if it is administered outside of the effective range of the labile period.

III. NATURAL vs. SYNTHETIC STEROIDS:

The efficacy of natural steroids relative to their synthetic counterparts is also an important consideration. Comparisons of natural and synthetic androgens were carried out by Piferrer et al. (1993). The synthetic steroids MT and 17-α-methyldihydrotestosterone (MDHT) were much more effective in producing 100% male populations from genotypically female chinook salmon stocks after a 2 hour immersion than were the natural androgens testosterone (T) and 11-ketotestosterone (11-KT). Both MT and MDHT concentrations of 0.4 mg/l proved effective in eliciting 100% male populations as did a MDHT concentration of 2.0 mg/l An 11-KT concentration of 10.0 mg/l elicited a maximum 93.6% male population whereas no T concentration produced more than 1.1% males. These results are somewhat contradictory to another study performed by Piferrer & Donaldson (1991) wherein the same concentrations of MT and MDHT under similar experimental conditions proved somewhat less effective in eliciting masculinization in coho salmon.

Similar to synthetic androgens, synthetic estrogens appear to be more effective inductors of feminization than naturally occurring estrogens. Ethynylestradiol-17 (EE2) at 4.0 mg/l effectively elicited a 100% female population in chinook salmon with a 2 hour immersion whereas the natural estrogen E2 at an equivalent dosage required 8 hours to achieve the same level of success (Piferrer & Donaldson, 1992). In this study immersions in the synthetic hormone for longer than 2 hours reduced the number of females produced. The reasons for this reduction are not understood at this time. However, overall, the synthetic steroid appears superior in eliciting feminization using shorter treatment durations than the natural steroid.

IV. DOSAGES:

Dosage dependent effects vary widely and are difficult to assess from the literature due to differences in immersion times, and other experimental variables. Baker et al. (1988) obtained complete masculinization of a monosex female population of chinook salmon using a 0.2 mg/l MT solution over a 2 hour period. Reducing the dosage to 0.02 mg/l resulted in 11.5% males being produced but 34.6% intersex fish. In Piferrer & Donaldson's (1993) study similar results were achieved, but a 10.0 mg/l dosage of MT produced 8.8% intersex fish and 11.8% females. The production of females at this high dosage of MT can be attributed to paradoxical feminization. This effect is attributed to the enzyme aromatase, present in the livers of the fish, which converts the MT to estrogen and hence paradoxically reverses the intended effect of the androgen. MDHT at these dosages did not produce intersex fish or paradoxical feminization. Successful feminizing dosages of estrogens have been reported from a low of 0.5 µg/l immersion in E2 for 17 days beginning 5 days after hatch in the masu salmon (Nakamura, 1984) and range up to 1.6 mg/l for 2 hours using chinook salmon which resulted in 91.1% feminization, and 9.1% intersex individuals (Hunter et al., 1986). These studies indicate that an optimum range of dosages exists which will give satisfactory results. Concentrations which fall below this range result in intersex individuals and if aromatizable androgens are used, exceeding the dosage range may result in paradoxical feminization. Piferrer & Donaldson (1993) found that high doses of MT produced paradoxical feminization but MDHT did not. This was attributed to the presence of a 5-α-reduction on the MDHT molecule which provides a resistance to aromatase enzyme activity. Aromatization is the process which converts androgen to estrogen, and therefore the MT was converted to female hormone resulting in the differentiation of ovaries in 11.8% of the fish. Although 0.4 mg/l has been the standard dose

reported in the literature it appears that if MDHT is used to achieve masculinization higher dosages can be used without incurring the danger of paradoxical feminization. Also, MDHT does not produce intersex fish at higher dosages (Piferrer et al., 1993).

V. INDUCTION OF STERILITY:

Sterile fish have been produced with MT administered orally, or in conjunction with immersions. Johnstone et al. (1979a) reported that MT administered to rainbow trout through the diet at 3 mg/kg for 90 days after first feeding resulted in deformities of the reproductive tract which inhibited the normal expression of milt. Yamazaki (1976) fed a variant of rainbow trout 50 mg/kg of MT for 5 months starting shortly after hatch and found sterile fish with no testicular differentiation. Solar et al. (1984) using rainbow trout also reported that MT doses of 1, 3 and 9 mg/kg fed for 60 days after swim-up resulted in 100% males, but that sterile fish (79%-90%) were produced only when the dietary dosage was 25, 50 or 100 mg/kg for 120 days post swim-up. This study also noted a paradoxical feminizing effect at the two highest doses of MT regardless of the treatment length. In a combination immersion-feeding experiment utilizing MT 2 groups of coho salmon at the eyed egg stage were immersed for 2 hours at either 0.1 or 0.4 mg/l and this was subsequently followed for 90 days after swim-up by a diet containing 10 mg/kg of the same steroid. The fish from the 0.1 mg/l group showed 70% sterility whereas 94% of the 400 µg/l group were sterile. In a similarly treated E2 group which differed only in being fed at the lower concentration of 5 mg/kg of the steroid, sterility never exceeded 4%. These results clearly indicate that an excess of MT administered through the diet can result in sterility rather than masculinization of the undifferentiated gonad. This may be due to a prolonged high level of MT in the gonadal tissues of the fish having an

inhibitory effect on gonadal development which does not occur when it is exposed to the shorter duration immersion treatments.

VI. GROWTH EFFECTS.

A wide variance in the effects of androgens and estrogens on growth rate has been reported in the literature. MT immersion has been shown to have had no effect on growth in coho and chinook salmon (Hunter et al., 1986), to increase the rate of growth in chinook salmon (Baker et al., 1988), and to increase growth of coho salmon only at concentrations below 0.1 mg/l (Goetz et al., 1979). Oral administration of MT to rainbow trout increased growth up to a dose of 3 mg/kg but suppressed growth at higher levels, up to 100 mg/kg (Solar et al., 1984). Estrogen application has been associated with similar disparities in growth rates. Immersion in E2 at dosages ranging from 0 to 400 µg/l followed by 10 mg/kg in the feed for 10 weeks produced no effect on growth (Goetz et al., 1979). Similarly , Johnstone et al. (1979b) treated brook trout (Salvelinus fontinalis) with E2 at 20 mg/kg through the diet for a period of 60 days following swim-up. The resultant females were found to have no significant growth rate differences when compared to control females. The same author achieved similar results using Atlantic salmon and rainbow trout (Johnstone et al., 1978). In Piferrer and Donaldson's (1992) study of the effects of two estrogens on chinook salmon, it was found that both E2 and EE2 increased the rate of growth over control fish and that the synthetic, EE2, produced the greater increase in both length and weight. The effect of sex steroids on growth is difficult to assess from the current literature. As with other aspects of controlled sex differentiation control, a number of variables exist which might affect growth rates other than simply the hormone. To assess these effects, a series of studies would need to be carried out for

each species under tightly controlled experimental conditions. A question which was not addressed in any of the studies was how these steroid treatments of eggs and alevins affect growth in later life. For the aquaculturist, growth suppression during the grow-out phase would be of greater concern than any minor, or transient growth differences in juveniles. Therefore a long-term study of the growth patterns of steroid treated fish relative to controls would provide important information to the fish culturist.

VII. HETERO- vs. HOMOGAMETY:

The breeding of first generation feminized or masculinized fish allows the determination of heterogametic and homogametic sex in the XY-XX sex chromosome system. Crossing the homogametic (XX) sex with a homogametic individual which has had its normal developmental pathway reversed (XXxXX) will result in offspring which are all female (Solar et al., 1984). Alternately, crossing a heterogametic (XY) individual with another reversed heterogametic individual (XYxXY)will result in 25% (XX) homogametics, 50% heterogametics, and 25% (YY) homogametics. In the case of salmonids all known species where the male is the heterogametic sex, the latter cross will result in a 3:1 male to female sex ratio. By identifying the YY portion of this stock at maturity through the sex ratio of their progeny these "supermale" individuals can be retained as broodstock to produce all-male populations in the third (F3) generation after the initial feminization. For the homogametic females XX sperm can be identified at the second (F2) generation following masculinization and all-female populations then become available. The development of YY sperm for the Arctic char was the ultimate goal of the procedures initated here.

VIII. CONTROL OF GONADAL DIFFERENTIATION IN ARCTIC CHAR:

A limited number of studies regarding hormonal sex control have been performed in the genus Salvelinus. Wenstrom (1975) fed T to lake trout (Salvelinus namaycush) at 700 mg/kg for 45 degree days. Seven out of 10 fry examined at the end of the treatment were male. Due to the small sample size it is not possible to consider this a significant result. Johnstone et al. (1979b) reported successful feminization of brook trout (Salvelinus fontinalis) treated orally with E2 at 20 mg/kg One population treated for 40 days following swim-up consisted of 67% females, 21% males and 12% intersexuals. When treatment time was extended to 60 days 99% females, 1% males and no intersexuals were found. There are no reports in the literature of hormonal sex control in the arctic char (Salvelinus alpinus), although an abstract from Skarpheoinsson and Finnsdottir (1989), reports that MT treatment of arctic char through the feed in concentrations ranging from 0.5 to 40.0 mg/kg resulted in 100% male populations. They also show that treatment with E2 treatment at 20.0 mg/kg was completely effective in feminizing fry. As these experiments have not been formally published, the details of the protocol used, most notably the length of the treatment is not available at this time.

Based on this information an investigation was undertaken to determine an effective method for sex control in the Arctic char. This study was initiated with oral administration of an androgen and an estrogen.

SECTION I

SEX CONTROL IN THE ARCTIC CHAR (SALVELINUS ALPINUS) THROUGH ORAL ADMINISTRATION OF 17α-METHYLTESTOSTERONE & ESTRADIOL-17 β

INTRODUCTION:

Two routes of steroid administration have proven effective for the induction of masculinization or feminization in salmonids, but treatment efficacy appears to be differentially successful even amongst species which are closely related. Dietary administration of androgens has resulted in complete masculinization of rainbow trout (Oncorhynchus mykiss) (Solar et al., 1984). Feminization has been successfully achieved through oral administration of estrogen in rainbow trout and Atlantic salmon (Salmo salar) (Johnstone et al., 1978) and in the brook trout (Salvelinus fontinalis) (Johnstone et al., 1979b). However, Goetz et al. (1979) have shown that in the coho salmon (Oncorhynchus kisutch) dietary treatment alone is not effective for inducing feminization. These species differences are currently attributed to the timing of the differentiation of the gonads from primordial undifferentiated tissues into testis or ovary. In species where this developmental stage occurs before the initiation of feeding, the effective outcome of oral treatment will be greatly reduced. For species such as the coho salmon, effective control of gonadal differentiation can only be realized by treatment at an earlier stage of development through direct immersion of eggs or alevins in a steroid solution.

Sex steroid treatment allows monosex populations to be developed without al teration of the genome. In the Arctic Char (Salvelinus alpinus) there is some evidence that mature males exhibit superior growth over mature females in an intensive to semi-intensive culture system (Fig.1).The larger size of the males at maturity has also been reported in wild populations of char in Alaska (Daum et al., 1984). The purpose of the present investigation is to induce masculinization and feminization in the Arctic char by applying 17- α-methyltestosterone(MT) or 17ß-estradiol(E_2) respectively, through the feed and to evaluate the

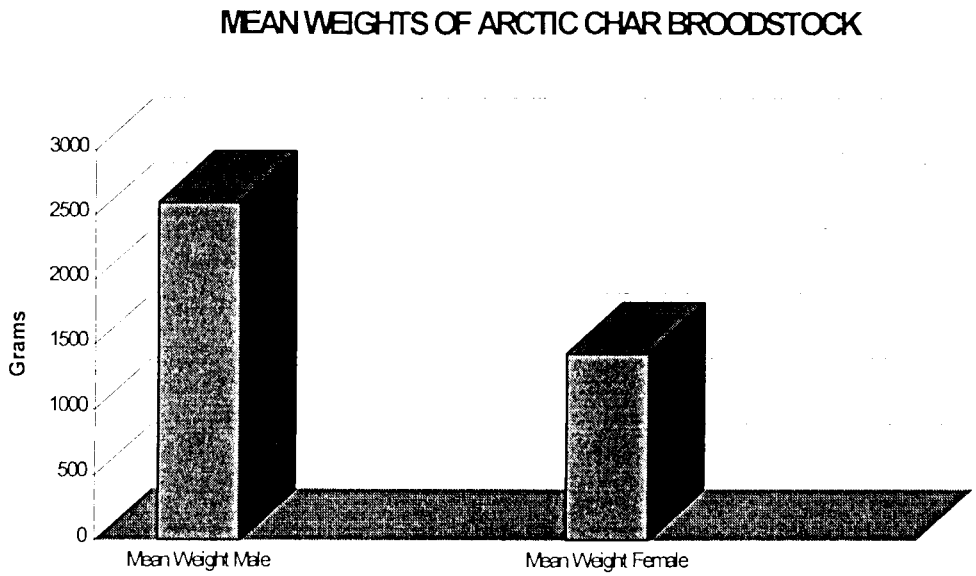

Figure 1: Mean weights of sexually mature Arctic Char of Nauyuk Lake stock; Males(n=43) vs Females(n=244). Differences are significant at P < 0.01.(Data source: Icy Waters Ltd.)

influence of these hormones on the developmental pathway of the gonad. Also, the effects of these steroids on growth will be an important consideration. Feminization will be induced as the first stage of an indirect masculinization procedure, where at maturity XY females will be crossed with XY males in an effort to produce YY "supermales" (see Section VII p.9).

METHODS:

Three groups of fry were subdivided from a sample of a domesticated strain which originated from Nauyuk Lake, Northwest Territories. The fish used in this procedure were spawned at Icy Waters Ltd., Whitehorse, Yukon Territory on September 2, 1992. Hatching was complete on January 16, 1993 591 ATUs from fertilization. After 100% swim-up on March 15, 1993 three groups of 19,500 were separated into 0.25 m^3 tanks. On March 20, 1993 (747 ATUs) feeding was initiated. For the purposes of this procedure the initiation of feeding was considered as 0.0 ATUs and all references to ATUs in the experiment are from this point. The control group (92YG21) began feeding on #1 size starter feed (Bioproducts of Astoria, Washington) which was untreated. A second group (92YG22) was fed the same diet treated with 40mg 17-α-methyltestosterone (MT) (Sigma) per kg of feed. The third group (92YG23) received feed treated with estradiol-17ß (E_2) (Syndel) at 15mg/kg feed. The steroids were dissolved in 95% ethanol, sprayed onto the feed, and mixed while allowing the alcohol to evaporate. The treatments were continued until an accumulated 600 ATUs of feeding were completed. During the treatment period the feed size was progressively increased using mixtures with #2 and #3 feed.

On June 23, 1993 each treatment group was subdivided in half into 25 m^3 tanks to reduce the high fish densities. No other conditions were altered. Sampling was initiated on June 25, 1993 (620 ATU). Twenty five fish were removed from each tank, for a total of 150, and sacrificed using a lethal dose of MS222. They were then blotted dry, weighed to 1.0 mg and fork lengths were taken to 1.0 mm. The fish were placed in 10% buffered formalin for a minimum of 48 hours to ensure fixation of tissues, then packaged and shipped to West Vancouver Laboratory for histological processing and examination. This sampling procedure was carried out every 3 weeks until termination of sampling on September 1, 1993. Sample

numbers vary slightly as in some groups the gonads could not be seen due to damage of the section or lack of clarity, while in other groups extra fish which were included in the sample to accomodate these losses were retained as part of the sample group.

Histological preparation consisted of cross-sectioning through the body of the fish directly behind the pectoral fins and again 0.50-0.75 cm. posterior to this. The cross-sections were then decalcified in a 5% nitric acid/70% ethanol mixture at a 1:20 ratio for 2 hours. Dehydration in an alcohol series, clearing and parrafin embedding was carried out using a TISSUEMATON (Fisher). Air bubbles were removed from the prepared blocks using a 60 °C vacuum oven. Samples were then placed in microtome cassettes in groups of 3 to 6, cooled and sectioned at 8 μm. Three sections were taken from each cassette. The prepared slides were stained using haematoxylin-eosin and examined under a microscope for evidence of gonadal differentiation. Photographs of the gonads in various states were taken with a Zeiss photomicroscope.

Statistical Analysis:

Sex ratio analysis was performed using a Chi-squared test, significance of difference from the expected 1:1 ratio was tested at $P < 0.05$. Growth data was collected using fork length (cm) and mass (g) and converted to condition factor (K) using the formula $K = \text{mass } 10^2 / \text{length}^3$ (Everhart & Youngs, 1989). Significant differences for growth analysis were calculated at $P < 0.05$ with the Student's t-test.

RESULTS:

I: Differentiation of Gonads.

For the assessment of sex ratio differences amongst the groups, fish from the two tanks representing each condition were combined as no other variations in treatment were applied during the procedure or were detected in the results. Identification of male fish was impaired in the first (620 ATU), and to a lesser degree in the second (745 ATU) sample, as clear differentiation of testes had not yet occurred. Ovaries were much more easily identified even at the earliest sampling stage as they were larger and developing oogonia were visible. Therefore, in the first sample, some fish were categorized as male by default if the gonad was small and oocytes were not present (Figure 2). Other fish exhibited more development of the testes even at the first sampling (Figure 3). Identification of testes proved easier during the later sampling periods as the typical triangular shape and spermatogonia became more apparent. No trend reflecting a change in the number of male or female fish in any group was identified over the course of the 4 sampling periods (Table 1). Intersex fish (Figure 4) were identifiable only in the latter 3 samples, but again there was no evidence that their number was increasing over time in either the MT or the E_2 groups.

In the pooled control groups from the 4 sampling periods ($n = 194$) a 55% male:45% female ratio was recorded (Table 1). These values are not significantly different from the expected 1:1 ratio ($P < 0.05$). No intersex fish were identified in any of the control samples. The combined methyltestosterone groups ($n = 196$) were found

Table 1: Cumulative sex ratios of Arctic char resulting from 600ATU oral administration through feed of 40 mg/kg MT (n=196), 15 mg/kg E_2 (n= 192), and control group (n=194), taken from 4 sampling periods.

ATU	CONTROL		17-α-MT			ESTRADIOL 17-β		
	male	female	male	female	intersex	male	female	intersex
620	16	21	34	19	-	28	24	-
745	31	27	30	17	3	7	32	8
985	29	20	31	12	2	18	19	11
1200	30	20	22	17	9	16	20	9
TOTAL(%)	**55**	**45**	**60**	**33**	**7**	**36**	**49**	**15**

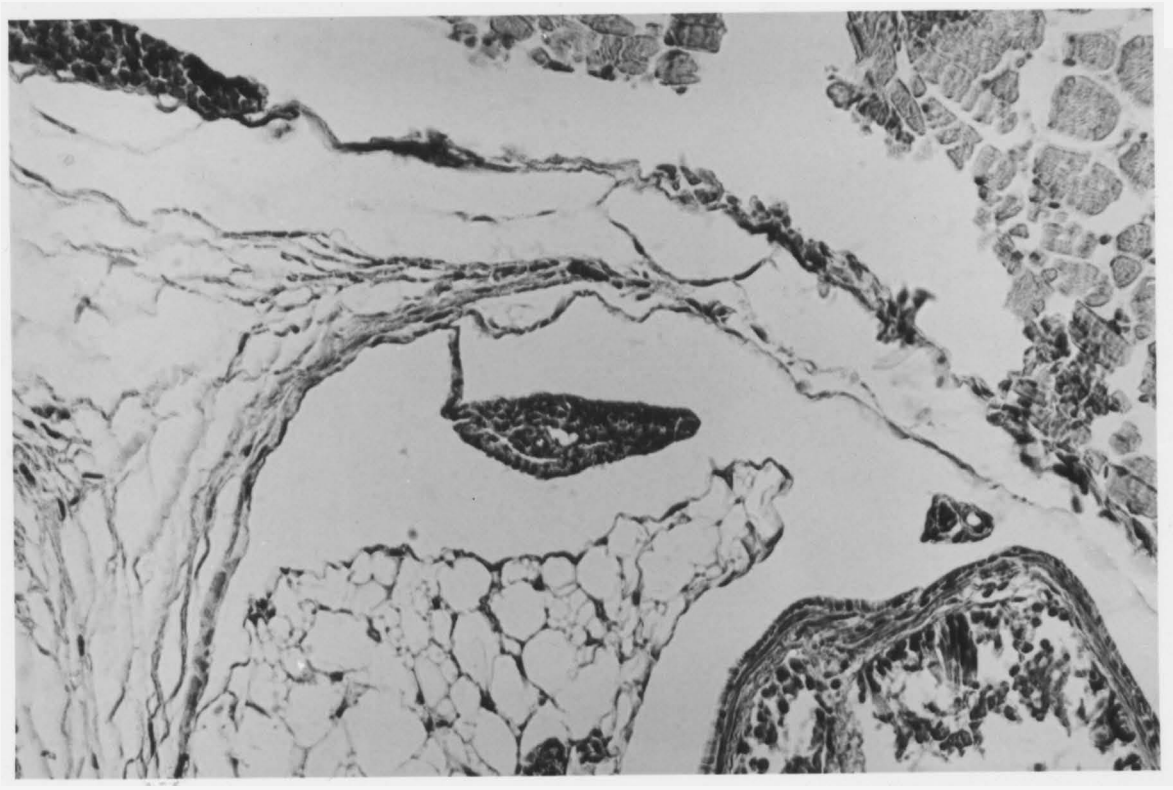

FIGURE 2: Undifferentiated testis from control group 620 ATU sample. As no ovarian characteristics were present these gonads were classed as testes. (Magnification x 40)

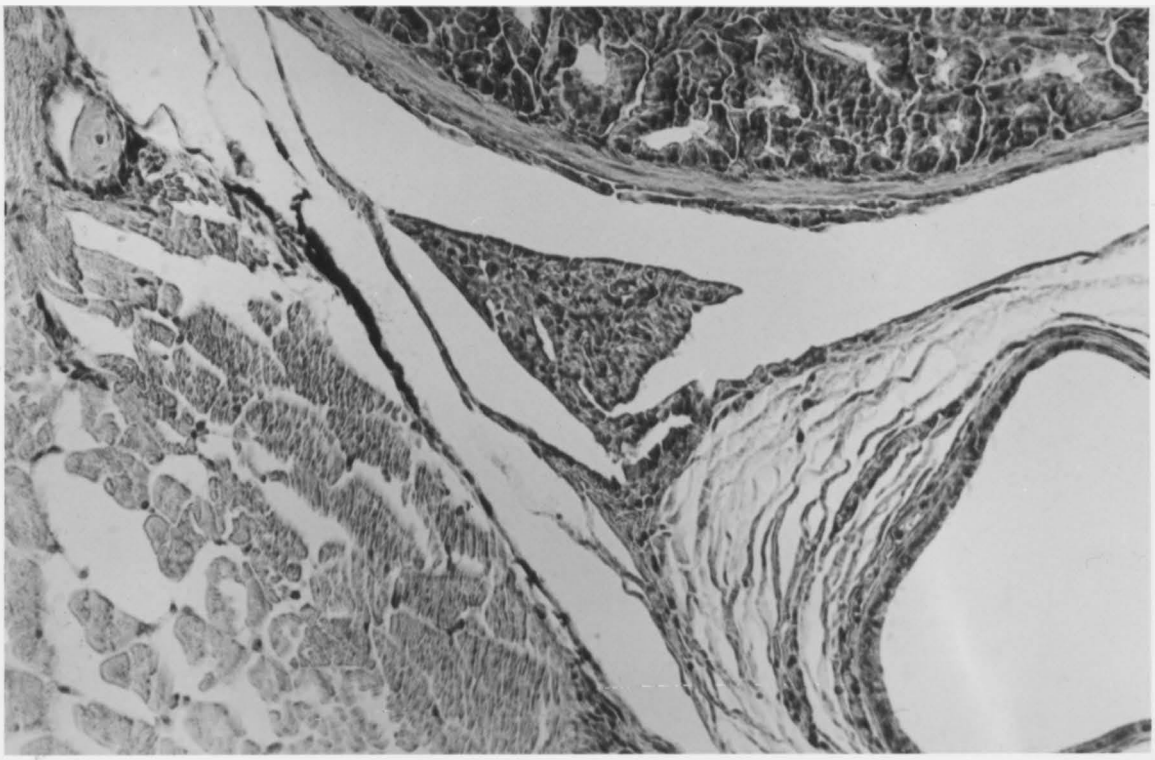

FIGURE 3: Testis from estradiol treated group 620 ATU sample exhibiting characteristics typical of more advanced development. (Magnification x 40)

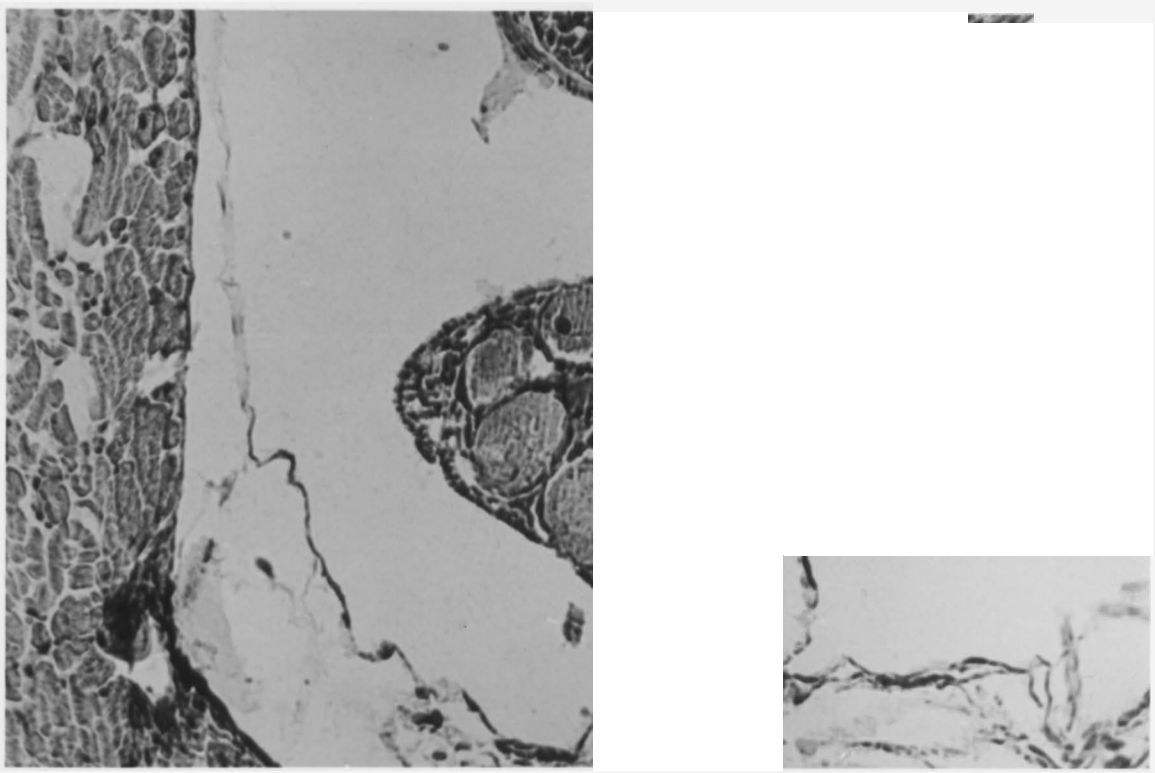

FIGURE 4: Intersex gonad from estradiol 985 ATU sample. Shows typical size and shape of testes but also several underdeveloped oogonia filled with undifferentiated germ cells. (Magnification x 40).

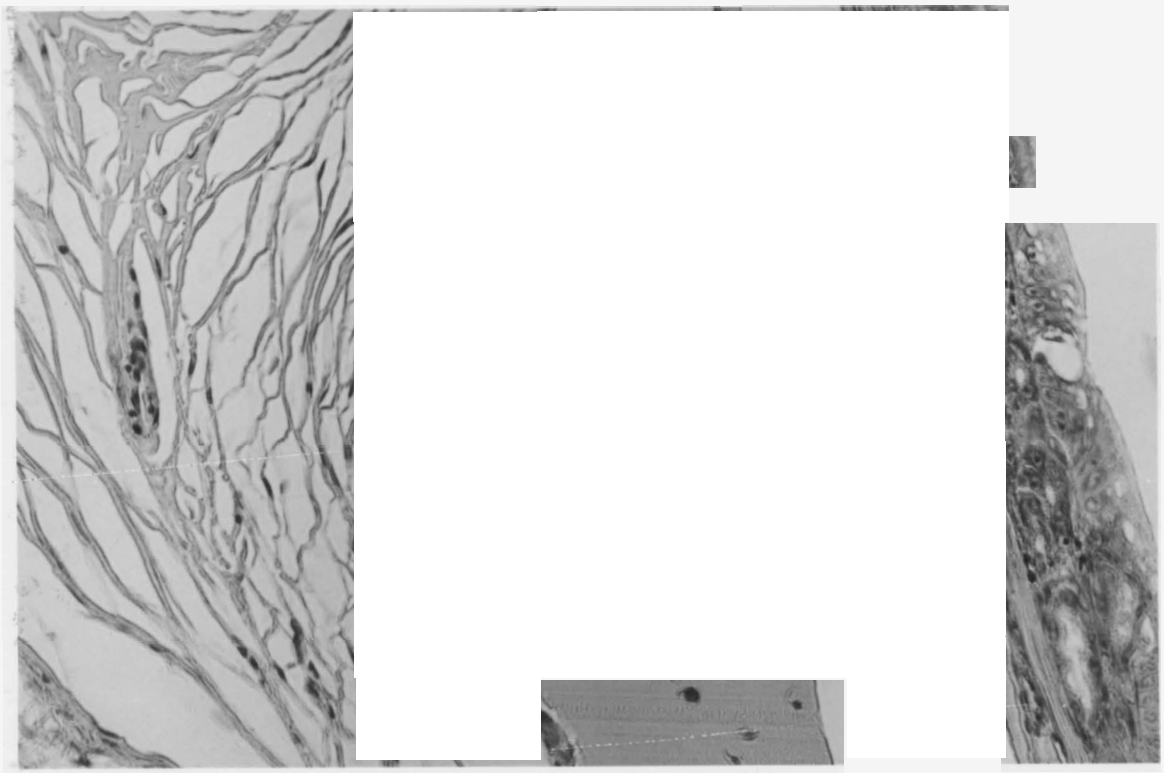

FIGURE 5: Intersex gonad from testosterone group 620 ATU sample. Gonad shows typical shape and undifferentiated germ cells of testes but with 2 oocytes clearly visible. (Magnification x 40).

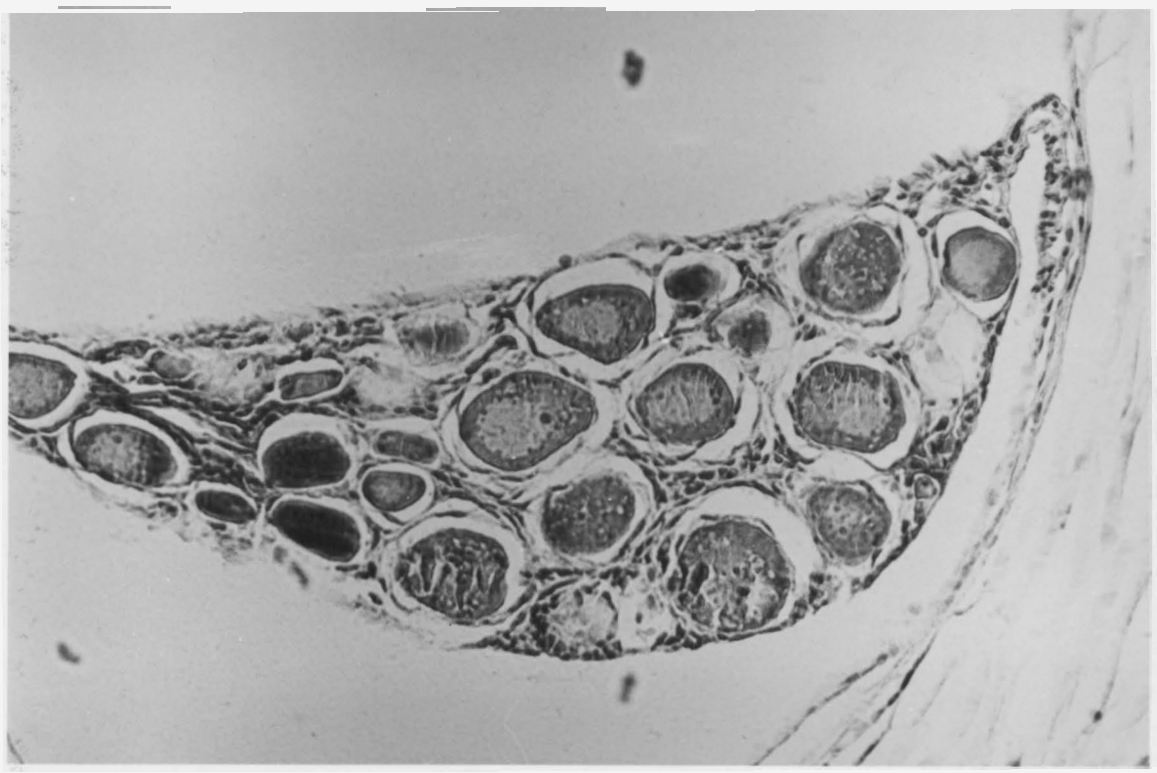

FIGURE 6: Ovary from control group 985 ATU sample. Exhibits larger size, well developed oocytes and typical semi-oval shape of ovaries. (Magnification x 40).

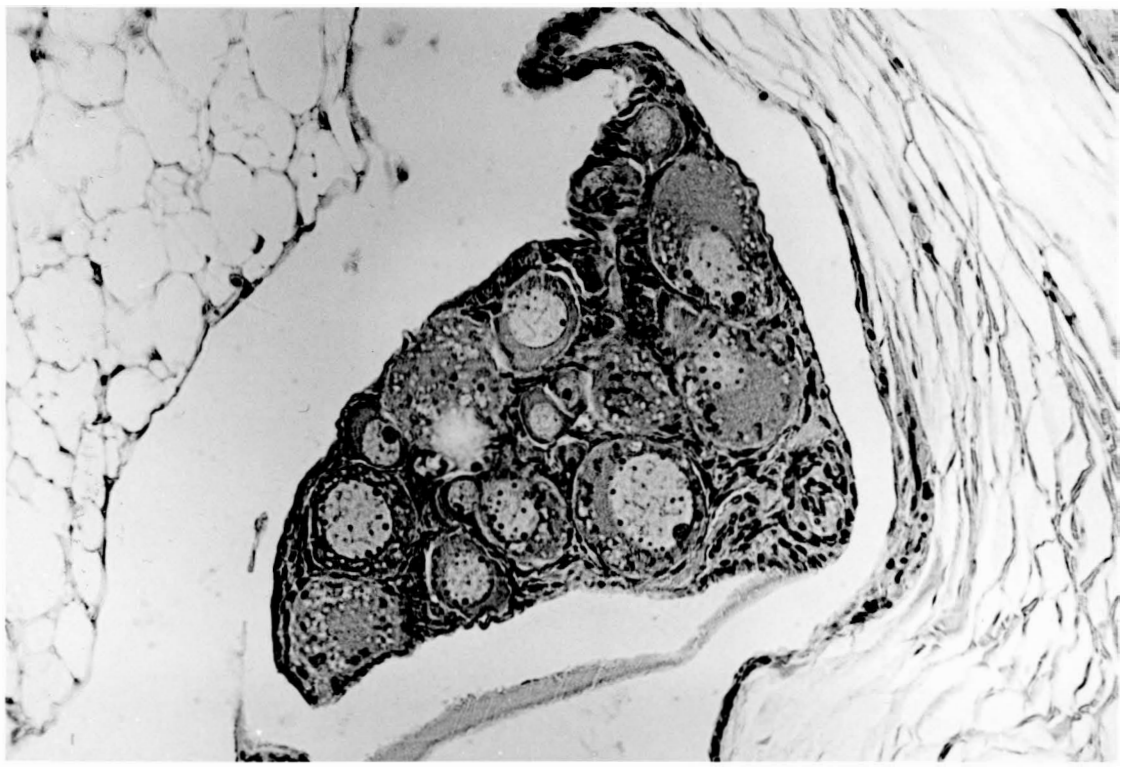

FIGURE 7: Ovary from estradiol treatment 985 ATU sample. This gonad is typical of those exhibiting advanced development with large perinuclear oocytes. (Magnification x 40)

to have 14 intersex fish which contained gonads exhibiting the smaller size, triangular shape and undeveloped germ cells typical of normal control group testes yet perinucleolar oocytes were also present (Figure 5). In the E_2 groups (n = 192) 28 intersex fish were identified by the presence of the previously noted testicular characteristics in conjunction with oocytes. Normal ovaries were much larger than testes and tended toward an oval or semi-oval shape (Figures 6 & 7). For the purposes of sex ratio analysis of the treatment groups all intersex fish were excluded from the sample. As no intersex fish are found in the control groups there is strong evidence that the steroid had a major influence on the pathway of differentiation within the gonad. Whether the intersex fish would ultimately develop as male, female or hemaphrodite could not be determined, and therefore for sex ratio assessment purposes were not classified as male or female.

Upon exclusion of the 7 intersex fish from the combined MT groups, a 62% male:38% female sex ratio resulted (Table 1). This proportion is significantly different from the expected values of unity ($P < 0.05$). In the E_2 pooled groups 28 intersex fish were subtracted from the total for sex ratio analysis. The remaining sample consisted of a 42% male:58% female population. This value is also significantly different from the expected 1:1 ratio ($P < 0.05$). As the only difference between the control and the treatment groups was the application of steroid, these results indicate that the MT had a masculinizing effect while the E_2 induced feminization within the respective treatment groups.

Mortalities in the treatment groups were not significantly different from those of the control group and therefore sex specific mortalities are not likely to have affected the observed sex ratios.

II: Growth Effects.

Comparison of growth rate data between the groups showed that the MT and E_2 treated groups lagged behind the control group during all 4 sampling periods, with respect to both length and weight (Figures 8 & 9). Student's t-test analysis comparing the treatment groups with the controls at each of the samplings showed that all of the differences in mean weight were significant at 95% confidence limits, with the single exception of the estradiol group at the final 1200 ATU sampling. The weight and length data were converted to condition factors which showed an increasing trend over the period of the study for both the control as well as the 2 treatment groups (Figure 10). The estradiol group exhibited a significantly greater condition factor to that of the controls at all 4 sampling periods, whereas the testosterone group was significantly greater only at the 745 and 985 ATU samplings ($P < 0.05$). Interpretation of the condition factor analysis in conjunction with the weights and lengths indicates that both the MT and E_2 groups underwent a suppression of linear growth in terms of spine length increase, but that their increase in body weight has not been affected to the same degree relative to that of the control fish.

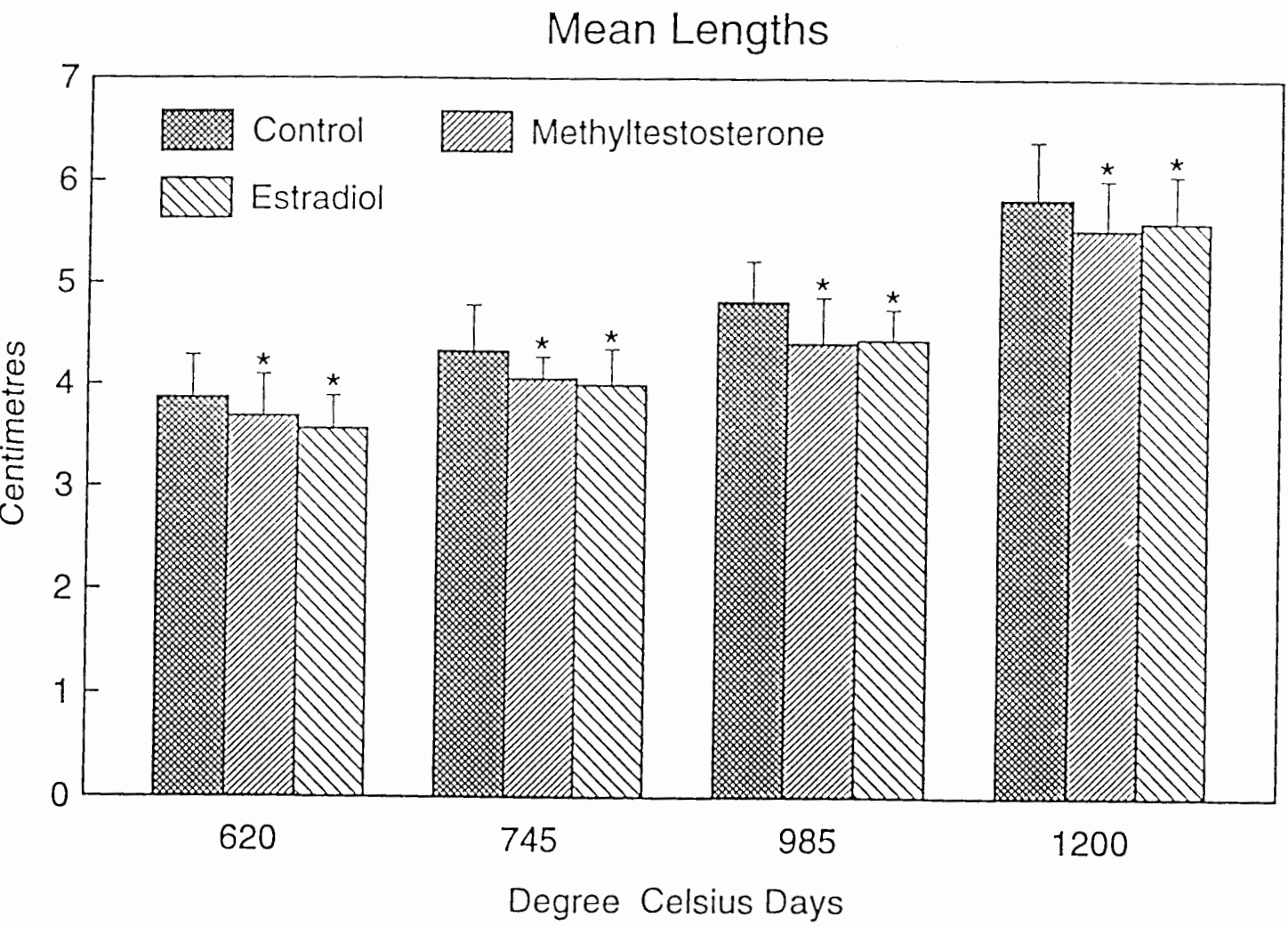

FIGURE 8: Mean lengths for control and treatment groups over 4 sampling periods. (± Standard Deviaton; * denotes significantly different from control value at 95% confidence levels)

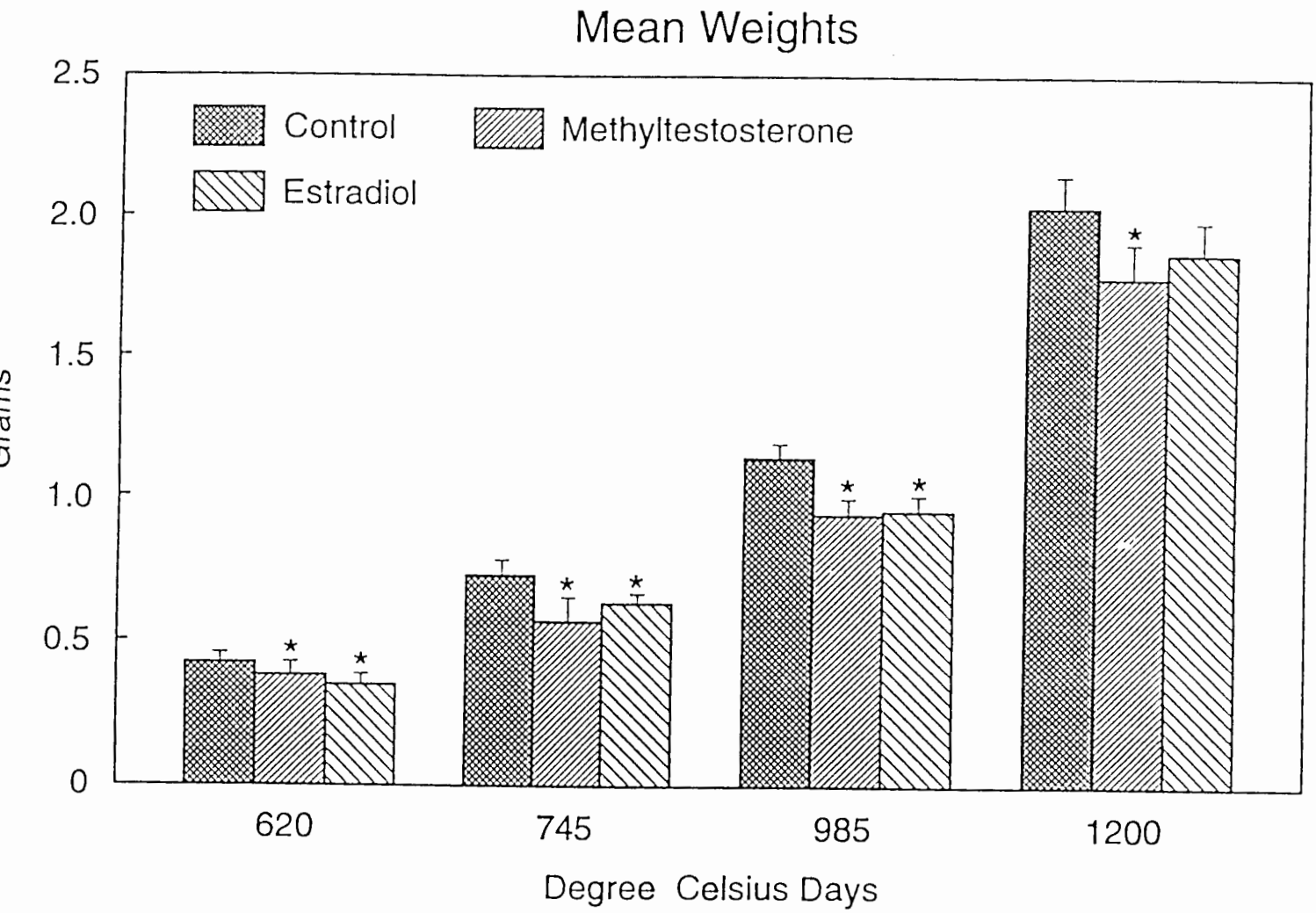

FIGURE 9: Mean weights for control and treatment groups over 4 sampling periods. (± Standard Deviation; * denotes significantly different from control value at 95% confidence levels).

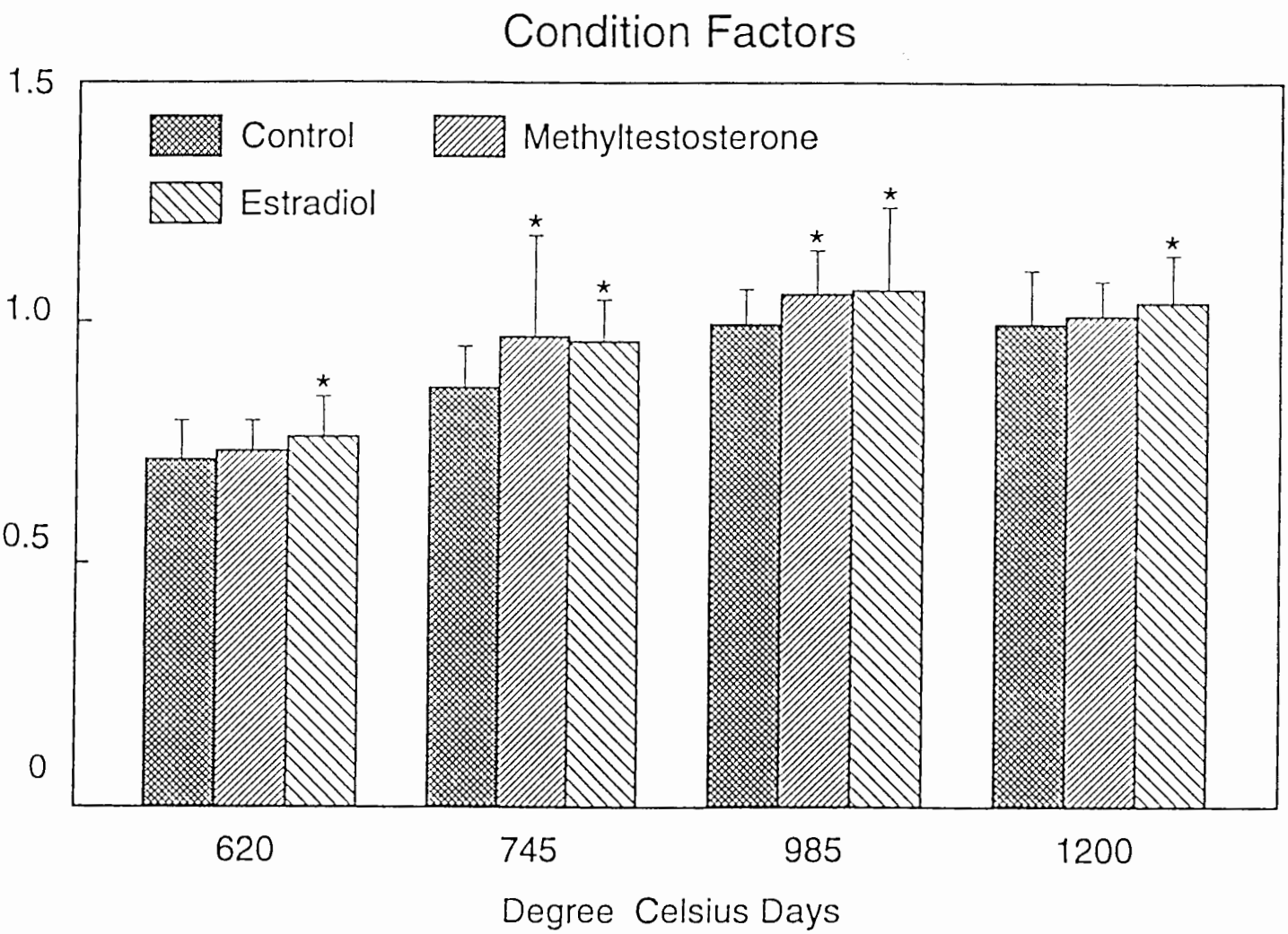

FIGURE 10: Condition factors for control and treament groups over 4 sampling periods. (± Standard Deviation; * denotes significantly different from control value at 95% confidence level).

DISCUSSION

Sex Ratio Analysis:

The sex ratios obtained in the MT and E_2 treatment groups indicate that control of gonadal differentiation through dietary administration of steroid has been partially successful. However, the presence of intersex individuals in both the MT and E_2 groups provides evidence that there is a limiting parameter within the protocol used here.

Treatment with MT at 40 mg/kg of feed for 600 ATUs beginning at first feeding produced a 62% male population. This dosage of androgen may represent the upper limit of steroid concentration which should be used to induce masculinization. In species such as the rainbow trout, levels above 25 mg/kg have been found to induce sterility (Solar et al., 1984). Some or all of the fish which have been categorized as male in the masculinization group may in fact be sterile due to the high dosage of MT which was applied. The results obtained here indicate the development of the testis was slow relative to that of the ovary, and that a large range of variation in development existed within the treatment groups. Some testes were small with no signs of differentiated tissue whereas others were larger and the blood vessels as well as the sperm duct were visible. Although, at the time of sampling, the testes of the MT-treated group did not appear different from those of the controls, there is no conclusive evidence that the testes of the MT treated fish would ultimately proceed through the normal stages of development.

It is unlikely that incomplete masculinization within the MT group occurred due to a low dosage of the androgen. Paradoxical feminization, the production of female fish which

sometimes occurs at high levels of aromatizable androgens (Piferrer et al., 1993), such as MT and although it was not apparent here, it cannot be ruled out.

Feminization was also partially successful, with a 58% female population resulting from the 15 mg/kg E_2 treament for 600 ATUs. This dosage of steroid is slightly lower than that reported in the literature to induce feminization in other salmonids. Goetz et al., (1979) fed coho salmon E_2 at 10 mg/kg for 70 days resulting in sex ratios which did not differ significantly from unity. Treatment with E_2 at 20 mg/kg for 60 days following swim-up resulted in a 99% female population (1% intersex) in the brook trout (Johnstone et al., 1979b) and 68% females (17% intersex) in the Atlantic salmon (Salmo salar) (Sower et al., 1984). As the length of treatment used in this procedure is well in excess of the 60 days reported in the two successful studies it is unlikely that length of treatment is a factor limiting the degree of feminization seen in the E_2 group, although a low dosage level cannot be ruled out.

It is important to note that in discussing applied dosage, the assumption has been made that the total exposure of the fish to the steroid concentration in the feed is equivalent to the exposure at the gonadal primordia, the target tissue. This may in fact not be the case, and if only a portion of the steroid in the feed is reaching the target tissue, underdosing would become an important consideration in the efficacy of the treatments.

It is now well established that effective control of gonadal differentiation involves the application of the appropriate hormone during development previous to the time of differentiation into testis or ovary (Hunter & Donaldson, 1983; Piferrer & Donaldson, 1989). Each species appears to have a window or "labile period" during which they are maximally sensitive to the effects of steroids on gonad differentiation. These differences may even extend to the level of sub-species or strains (Goetz et al., 1979). If the sensitivity to the hormones

exists at this level, it would explain why different results were obtained in this procedure when similar treatment in the closely related brook trout was much more successful. A subspecies difference in sensitivity may account for the conflicting results obtained here compared with those reported by Skarphéòinsson & Finnsdòttir (1989) where 100% masculinization and feminization was reported. A study comparing the efficacy of oral administration of hormone between the N.W.T., Labrador and Norwegian strains of Arctic Char would be useful in determining if these differences do exist and how pronounced they are.

If the labile period in the Nauyuk Lake Arctic char occurs at an earlier developmental stage than in other strains, oral administration of hormone may be an inappropriate technique to induce masculinization or feminization. As no studies have been carried out to determine the timing of gonadal differentiation in the Arctic char, it is difficult to speculate when this process occurs during development and therefore when hormone treatment would be maximally effective. The closest species in which gonadal differentiation has been reported is the whitespotted char (Salvelinus leucomanis). Gonads had visibly differentiated 131 days after hatch at 1-6^{O}C. (Nakamura, 1982). These fish were held at colder temperatures than used here and subsequently their development would be slower. The true or "physiological" differentiation of the gonads occurs before the time at which these differences become apparent under the microsope (Nakamura & Takahashi, 1973). It is this physiological differentiation which defines the critical time before which steroids must be administered to effect successful control of gonad differentiation (Nakamura, 1984). If first feeding is initiated at the boundary of this labile period of physiological differentiation in the Arctic char, oral steroid administration would show reduced success. In the coho salmon sex control cannot be achieved through dietary supplement alone (Hunter & Donaldson, 1982). It was subsequently

shown that coho salmon are maximally sensitive to steroids when administered 8 days pre-hatch to 13 days post-hatch, before the initiation of feeding (Piferrer & Donaldson, 1989). Fitzpatrick et al. (1987) have measured a peak of endogenous androgen and estrogen which appears to occur concurrently with the period of maximum sensitivity to exogenous steroid application. Piferrer & Donaldson conclude from this that critical physiological and morphological changes which occur during this stage account for the level of sensitivity to the application of exogenous steroids which serve to override the action of endogenous steroid and concomitantly the pathway of gonad differentiation.

Steroid application previous to the initiation of feeding is achieved through immersion treatments. The hormones are dissolved directly into the water at concentrations from 0.4 - 2.0 mg/l over a 2 - 4 hour period. If the effective range of steroid application relative to labile period occurs previous to first feeding, immersion will prove to be much more successful.

Growth Effects:

Wide variance of the effects of androgens and estrogens on growth rates has been reported in the literature. Both MT and E_2 have been shown to increase, decrease or have no effect on growth in Pacific salmon (Goetz et al., 1979; Solar, Donaldson & Hunter, 1984; Hunter et al., 1986; Baker et al., 1988). In this study the application of either MT or E_2 was found to significantly suppress ($P < 0.05$) both length and weight of the fish relative to the controls at all sampling periods (Figure 8 & 9). However, condition factors were higher for the steroid-treated fish. (Figure 10).

With the exception of the MT groups at the first (620 ATUs) and last (1200 ATUs) sampling dates the treatment groups showed a significantly greater condition factor ($P < 0.05$) relative to that of the controls. Assimilation of the length and weight data with the condition

factors indicates that the treatment groups exhibit inferior linear growth but that their weights are not suppressed by the same margin relative to their lengths. Growth effects are often difficult to assess due to differences in rearing conditions, however in this instance these factors should be minimal. Methyltestosterone is reported to have an anabolic effect when administered orally (Higgs, 1977; Solar et al., 1984), but can suppress growth when administered at high dosages in immersion treatment (Goetz et al., 1979). In this study the steroid appeared to suppress growth rates. What is more important is the long term effect of steroids on growth. Elimination of steroid from the carcass of a fish is relatively rapid. In a study using rainbow trout it was shown that MT levels fell to less than 1% of their initial levels after 100 hours (Johnstone et al., 1983). It therefore seems unlikely that residual hormone caused the growth suppression in the MT-treated fish which persisted even until the final (1200 ATU) sampling period. Monitoring weights and lengths of treatment versus control fish over their growout period would provide important information on the long term effects of steroids on growth.

CONCLUSIONS & RECOMMENDATIONS:

The altered sex ratios seen in the MT and E_2 treatment groups relative to those found in the control groups, as well as the differences in growth rate, indicate that the steroids were successfully delivered through the feed. As only partial masculinization and feminization occurred when using oral administration of MT and E_2 at 40 mg/kg and 15 mg/kg respectively, it is apparent that a more "efficient" protocol should be established to improve success. The parameters which must be addressed in this regard are dosage levels, timing of the treatment, route of administration, and the chemical properties of the steroids used.

One of the major factors determining the outcome of sex control procedures is the timing of treatments relative to the labile period of gonad differentiation. Based on the results obtained in this procedure it is very likely that the peak of the labile period occurred previous to first feeding, in this strain of Arctic char. Oral administration of hormone exhibits reduced efficacy because of this. Therefore, it is recommended that steroid treatment be initiated at an earlier stage of development. This can be achieved through immersion of eggs or alevins in an aqueous solution of steroid. Immersion treatment has proven to be much more effective for the purposes of sex control in species where oral administration has had limited success. In the coho the most effective timing for immersions has proven to coincide closely with hatch (Piferrer & Donaldson, 1989). To deliniate the period of maximum sensitivity in the Arctic char, the most effective method would consist of a series of immersions spaced approximately one week apart. Separate treatment groups would be immersed in hormone for 2 to 8 hours at concentrations ranging from 0.5 - 2.0 mg/l. These experimental parameters have proven to be effective in other studies (Piferrer & Donaldson, 1989; 1991). Treatments would be initiated several weeks before hatch and continue for several weeks after. A comparison of the sex ratios achieved with altered treatment timing would indicate the period of maximum sensitivity to the steroid. Variation of steroid concentration would be carried out concurrently with these treatments. Replicate immersion treatments at higher doses of steroid carried out simultaneously would indicate the optimum dosage of steroid for control of gonad differentiation.

Synthetic estrogens and non-aromatizable androgens have proven to be much more effective in eliciting both feminization and masculinization of chinook salmon (Piferrer & Donaldson, 1992; 1993). In the case of estrogens, ethynylestradiol (EE_2) has proven to be 100% effective over a short-duration (2 hour) immersion whereas with E_2 an 8 hour

immersion was required to achieve the same results. The non-aromatizable synthetic 17 α-methyldihydrotestosterone (MDHT) elicits consistent masculinization with 2 hour immersions. The use of MDHT has a significant advantage over MT as it cannot be aromatized and therefore, cannot produce the paradoxical feminization witnessed with MT when administered through immersions at concentrations greater than 0.4 mg/l. It is therefore recommended that the synthetic estrogen EE_2 and the non-aromatizable androgen MDHT be substituted for E_2 and MT in future sex control procedures. Due to the known superior performance of MDHT and EE_2 for the purposes of gonad differentiation control the feed experiment carried out here should be repeated substituting these steroids for MT and E_2 to determine if improved success can be achieved with oral administration of hormone.

Growth effects of the steroids are also important as these may indicate other physiological changes which have occurred. A long-term monitoring of growth rates in the treated groups should be undertaken. When these data are combined with those obtained in the earlier stages recorded here, a growth profile can be obtained comparing the treated fish with their untreated counterparts. Continuing study of the MT treated fish through to maturity would indicate whether sterility has been induced by these treatments. Sex ratio analysis of the offspring from the E_2 treated groups will be necessary to determine which of the females possess the "XY" genotype, and are capable of producing "YY" supermales when crossed with normal males.

A study of the normal differentiation of the gonads in the Arctic char would be very useful in determining the most effective timing of treatment and as this study has not yet been completed it would also provide valuable information on the development of this species. This should be undertaken by histological examination of the gonads from the egg stage continuing

through until gonad differentiation is complete. This study could be completed in conjunction with the proposed immersion treatments, using samples from the control group.

In conclusion, the sex control procedures carried out here have been partially successful with MT inducing masculinization and E_2 feminization. Both groups contained a small proportion of intersex individuals. Growth was suppressed in the treatment groups but condition factors were higher than those of the controls. It is recommended that immersion treatments with a non-aromatizable androgen and a synthetic estrogen be used to increase the efficacy of the sex control procedures. Studies of normal gonad differentiation, and long-term of growth patterns in response to steroid treatment would also be beneficial.

SECTION II

SEX CONTROL IN THE ARCTIC CHAR (*SALVELINUS ALPINUS*) THROUGH IMMERSION IN 17α-METHYLDIHYDROTESTOSTERONE AND ETHYNYLESTRADIOL-17α

INTRODUCTION:

Based on the information currently available, an effective protocol for sex inversion in the Arctic char can likely be developed. The most important factor is labile period, as this will determine the appropriate timing and route of steroid administration. If the effective range of the labile period occurs before first feeding in this species, oral administration of the hormone after the labile period would show reduced efficacy relative to immersion of the eggs or alevins during the labile period.

A study was carried out at Icy Waters Ltd., Whitehorse, Y.T. using a strain of Arctic char originating from Nauyuk Lake, N.W.T.(see Section I). The experimental procedure involved treatment through the feed using the androgen 17α-methyltestosterone (MT) and 17ß-estradiol (E2) to induce masculinization and feminization respectively. The results of this experiment were a partial success in both areas, with sex ratios skewed from the normal 1:1 male:female sex ratios of the control group. Masculinization through the feed produced 62% males while feminization produced 58% females, percentages which were significantly different ($P < 0.05$) from those expected. Within both treatment groups a small percentage of intersex individuals were found whose gonads exhibited both testicular and ovarian characteristics. It is also important to note that the gonads, especially the ovaries, were well developed even before the time at which oral hormone treatments were completed.

The information obtained during this investigation has indicated that oral administration of steroid is probably not an effective route of sex steroid application for the Arctic char. The inefficiency of this treatment protocol, in conjunction with the developmental stage of the gonads at the time of sampling provide evidence that differentiation, and therefore the labile period of the gonad occurs at a relatively early stage during development. As immersion

treatments are able to influence gonadal differentiation at an earlier stage of development than oral administration, they are indicated as a more effective method of steroid application.

The following procedures are aimed at elucidating an effective set of parameters for consistently obtaining successful sex inversion in the Arctic char by focusing on immersion treatments. Using Piferrer & Donaldson's (1989) study as a guideline, the treatments most likely to produce these results will be tested.

PROTOCOL DEVELOPMENT FOR SEX-CONTROL TREATMENTS:

As it appears the labile period for sex inversion in the Arctic char occurs at approximately the same stage of development as that of coho salmon, hatching was considered the target for timing of treatment. To ensure that treatment was applied at the stage of development where hormone sensitivity was maximal, different treatment groups were treated through immersion in water containing the appropriate steroid. These treatments were begun one week before hatch and were continued on a weekly basis for 4 weeks afterward. The procedures were undertaken for both the masculinization group using androgen, and for the feminization group using estrogen.

This approach is very useful in determining the timing of the labile period for steroid treatment. By comparing the ratios of fish within each treatment group which have undergone effective sex control using steroid application at a particular stage of development, the precise timing of maximum sensitivity to steroid treatment can be effectively ascertained. A more precise method of determining labile period would involve histological and endocrinological examination of the gonadal tissues through the stages of physiological development to ascertain

the natural labile period. However, for the purposes of sex control the procedures carried out here are sufficient.

Another parameter which must be addressed in this study is the effective dosage of steroid to be used. This, in turn, is dependent on the chemical nature of the particular drug applied. It has been shown that synthetic estrogens are more effective for controlling gonad differentiation in salmonids than is their natural counterpart at equivalent dosage, and give more consistent results. A comparison of the natural estrogen, estradiol-17ß(E_2) with the synthetic ethynylestradiol-17α(EE_2) via immersion treatments in the chinook salmon (Oncorhynchus tsawytscha) shortly after hatch, showed that at equal concentrations of 0.4mg/l, the synthetic gave 100% female populations after a 2 hour immersion whereas with E_2 an 8 hour treatment was required to obtain the same result (Piferrer & Donaldson, 1992).

A problem encountered during masculinization procedures at high concentrations of some androgens is that of paradoxical feminization. This occurs when the dosage of applied androgen administered is large, either due to high concentration or extended length of immersion. The end result is that feminization occurs rather than masculinization in response to the androgen treatment. The currently favoured, and most likely explanation for this phenomenon is that through a process of aromatization, the androgen is converted to estrogen. This occurs as a response to the enzyme aromatase which is present in the liver of many salmonids. To prevent paradoxical feminization from occuring a non-aromatizable androgen must be applied. In a comparison of aromatizable and non-aromatizable androgens for the purpose of inducing masculinization in the chinook salmon, Piferrer et al., (1993) found that the non-aromatizable 17α-methyldihydrotestosterone (MDHT) resulted in complete

masculinization at all doses greater than 0.4 mg/l whereas equivalent high dosages of the aromatizable androgen methyltestosterone (MT) resulted in some paradoxical feminization.

Based on the results of these studies the synthetic estrogen EE_2 was selected for feminization procedures to increase the success rate while minimizing the necessary immersion time. Also, the non-aromatizable androgen, MDHT was selected to prevent paradoxical feminization. These steroids have been proven to yield optimum results in species of Pacific salmonids such as coho and chinook.

Dosage levels are another important consideration for the regulation of sex differentiation. Very few references exist with regard to the relative efficacy of the synthetic estrogen EE2 compared to that of the natural E_2 in salmonids. EE_2 is known to be approximately 3.5 times more efficient than E_2 for inducing feminization in the medaka (Oryzias latipes) (Yamamoto, 1969). Based on the previously mentioned work of Piferrer & Donaldson (1992) using 2 hour immersions at 0.4 mg/l, it seems reasonable to use this dosage range as a starting point. A major factor which must be addressed with immersion treatments of Arctic char is the cold ambient water temperatures which are encountered during the late winter when immersions would take place. Colder water temperatures cause a concommitant decline in the rate of metabolism of all poikilotherms including teleosts, and consequently the effects of the steroids may be lessened for an equivalent duration of immersion. As most of the immersion experiments involving salmonids have been carried out at ambient temperatures of 10°C or higher, accomodations must be made for the temperature range from 2-4°C which will be present during this investigation. To ensure that underdosing due to temperature differences does not occur, a known safe dosage of 1.0 mg/l will be used as a standard. To assess the efficacy of this dosage level, a higher and a lower dosage group will also be run. In the case of

MDHT the higher dosage will be at 10.0 mg/l and the lower at 0.5 mg/l Androgens are known to be less effective for control of gonad differentiation than are estrogens at equivalent dosages (Piferrer & Donaldson, 1989;1991; 1992). In the case of MDHT, immersion dosages of 2.0 and 10.0 mg/l have proven safe and effective in eliciting 100% masculinization in the chinook salmon over shorter immersion times (Piferrer, Baker & Donaldson, 1993). In an attempt to ensure successful masculinization, dosages known to be effective yet not deleterious to the fish, will be used for the Arctic char.

A similar dosage experiment was carried out simultaneously using EE_2. The only difference being that the higher dosage was reduced to 2.0 mg/l and all immersion durations were half those used with MDHT.

Immersion treatments for both masculinization and feminization carried out over 5 weeks using three different dosages of steroid represents a large number of treatment groups. To minimize this number and still complete a study which yields useful data regarding the optimum treatment protocol, the higher and lower dosages will be applied during the week following hatch while the intermediate dosage will be given over the entire length of the study for both groups. Based on the data obtained in other immersion studies the week after hatch represents the time period during which the fish are most likely to be at maximum sensitivity to androgen or estrogen application (Piferrer & Donaldson, 1989). This compromise will greatly reduce the number of fish and other resources which must be committed to the study while not curtailing the usefulness of the results for the purposes of identifying optimum treatment regime with regard to labile period and dosages.

Multiple immersions have been proven to be an effective treatment method for the purposes of attaining high rates of masculinization and feminization (Hunter et al., 1986; Baker

et al., 1988). Therefore in these procedures, along with using dosages that represent the upper known safe concentration limits of steroids in the immersions, other treatment groups were developed which underwent triple immersions spaced 7 days apart utilizing the intermediate dosage, but half the duration of immersion of the weekly groups. Ultimately these triple immersion groups will receive 1.5 times the steroid exposure of the intermediate dosage regular treatment groups. Multiple immersions should compensate for any effects attributable to the low water temperature. Also, if there is an inductive effect caused by the initial immersion treatment which causes the subsequent immersions to be more effective, this would be indicated in the sex ratios of these multiple immersion groups. A total of 4 multiple immersion groups will be produced for both MDHT and EE2. The first immersion will occur the week before hatch, the second at hatch, the third 1 week post-hatch and the last 2, weeks post-hatch.

An experimental group which represents a combination of treatments, via immersion as well as through the feed, will be produced for both the androgen and the estrogen. These 2 groups will ensure that if it is not possible to redirect gonadal differentiation via either immersion or feed administration alone, feminized and masculinized fish should be available at the end of this procedure. These groups will be produced by administering the intermediate dosage of EE2 or MDHT through immersion during the week following hatch. Continuing steroid treatments, through the feed at 7 mg/kg for 600 ATUs after the initiation of feeding would be carried out in these groups.

The final treatment group will be similar to the initial experiment carried out between May and October 1993 (see Section I), but will use MDHT and EE_2 instead. Steroid would be applied through oral administration at 7.0 mg/kg only, for 600 ATUs in the feed. Data obtained from this procedure would indicate if EE_2 and MDHT are more effective for the purposes of

feminization and masculinization than were Estradiol-17ß(E_2) and 17α-methyltestosterone. Any increase in success using $EE_2$and MDHT will also indicate whether the choice of steroids rather than treatment timing alone limited success in the initial experiment.

Therefore, a total of 20 estrogen-treated groups and 20 androgen-treated groups will be produced over the course of this study. For the purposes of sex ratio analysis 100 fish would normally be sacrificed from each group for a total sampling of 4,000 fish. To reduce this number 50 fish will be sampled from each of the estrogen and androgen treatments which will produce an error of 2.0% in the sex ratio. Appropriate control groups will be produced for the treatments. One control group will undergo no immersion treament. Other control groups will undergo sham immersions which will reproduce the treatments in every way except for the application of the steroid.

The design of these procedures are such that the maximum amount of information may be gleaned from the data regarding the efficacy of the treatments with regard to both dosage levels and the timing of the treatments and their coincidence with the period of maximal sensitivity of Arctic char to the steroids. The dosages, lengths of immersion and treatment timing used in these procedures have all been chosen due to their proven success in other closely related species of salmonids. The one factor in this procedure which has not been studied adequately is the effect of the very low incubation temperatures which are utilized in Arctic char culture. However, by using maximal dosages and immersion times in conjunction with double immersions spaced 7 days apart any problems regarding reduced sensitivity to the steroids at low ambient temperatures should be avoided.

METHODS:

The eggs used in these procedures were spawned at Icy Waters Ltd. in Whitehorse, Yukon on September 6, 1993. After fertilization the eggs were incubated in Heath tray incubators at an initial water temperature of 5.5°C. The temperature had decreased to 3.0°C. by December 29, 1993 when the fish had reached a stage of physiological development appropriate for the treatments.

The time of mean hatch for this strain of Arctic char was determined by observation to be 445°C-days post- fertilization. As hatch does not represent a true physiological stage of development (Piferrer & Donaldson, 1989) it was necessary to use ATUs to define a more objective measure on which to base the timing of treatments. A total of 20,000 eggs were utilized in the project. These were subsequently divided into 36 treatment groups of 500 fish, and 14 control groups of approximately 50 fish each.

Immersion treatments were conducted in 10 l plastic Heath trays, utilizing static baths. All treatments were carried out in an environmental chamber where air temperatures were regulated to maintain the water temperatures in the trays at 2-3°C, which is similar to the normal incubation temperature. All immersions of eggs were carried out in darkness except for short periods when eggs were added to or removed from the baths, or for short periods to allow oxygen to be bubbled into the trays for a 30 minute period every 6 hours.

Stock solutions of MDHT and EE_2 were prepared by dissolution of the steroids in 100% ethanol to a final concentration of 1 mg/ml. Stock solutions were immediately refrigerated until used. At the initiation of treatments an appropriate aliquot of the stock solution was measured out, placed in the egg tray with 10 l water, and stirred to ensure even distribution of steroid. The eggs were placed in a plastic container with a 1mm mesh bottom which was then placed

into the egg tray. Upon completion of the treatments, the eggs were returned to the stacks in the incubation room at the lowest point in the stack to ensure that no other treatment groups were contaminated by steroid residues.

Weekly Immersions:

These treatments were initiated one week pre-hatch, which, at a mean water temperature of 3^{o}C occurred at 424 ATUs post-fertilization. Each weekly immersion group underwent a single immersion. MDHT groups were treated for 24 hours at a steroid concentration of 1mg/l whereas EE_2 groups were treated for 12 hours, also at a concentration of 1mg/l Both masculinization and feminization treatment groups were prepared to investigate respective steroid efficacies beginning one week pre-hatch (424 ATUs) and continuing weekly until 4 weeks post hatch (529 ATUs). Control groups for weekly immersions for week -1 through week 4 underwent simultaneous 24 hour immersions in ethanol only at concentrations of 0.1%, a level equal to that found in the steroid treatments completed at concentrations of 1 mg/l (Figures 1&2).

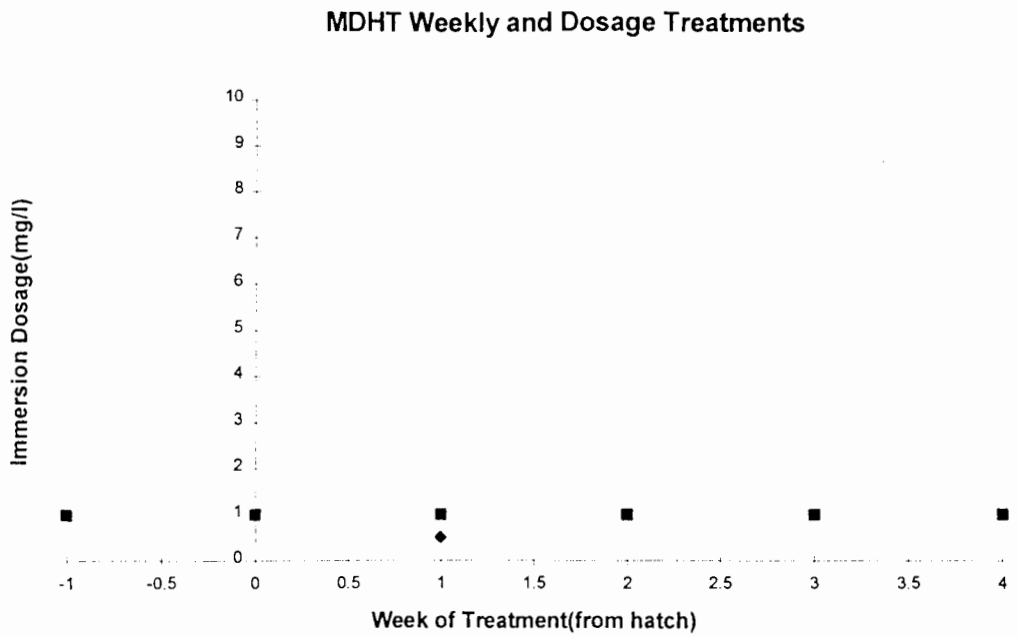

Figure 1: Physiological timing and dosage levels of single 24 hour MDHT immersions completed to identify labile period and dosage effects on rates of masculinization.

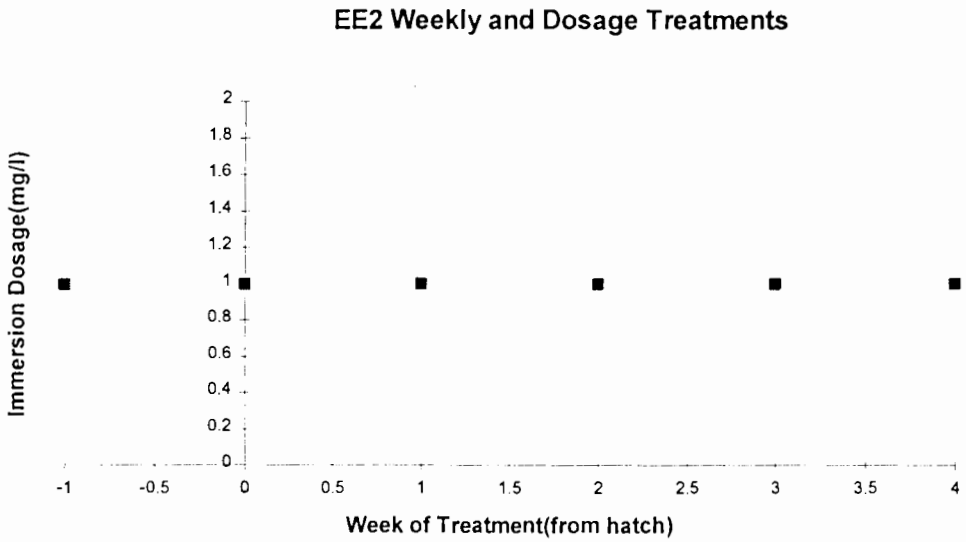

Figure 2: Physiological timing and dosage of single 12 hour EE2 immersions completed to determine effects of treatment timing and dosage levels on rates of feminization.

Immersion Duration:

All immersion duration treatments were carried out one week post-hatch 466 ATUs (Figure 3). Groups were treated for durations of 3, 6, 12, 24 and 48 hours in either MDHT or EE_2. All immersion baths in the duration study were at a concentration of 1mg/l Control groups were immersed simultaneously in 0.1% ethanol for 12, 24 or 48 hours. Control groups for 3 and 6 hours of ethanol exposure were not developed as it was believed that any ethanol effects occurring in these groups would also be present with longer immersions.

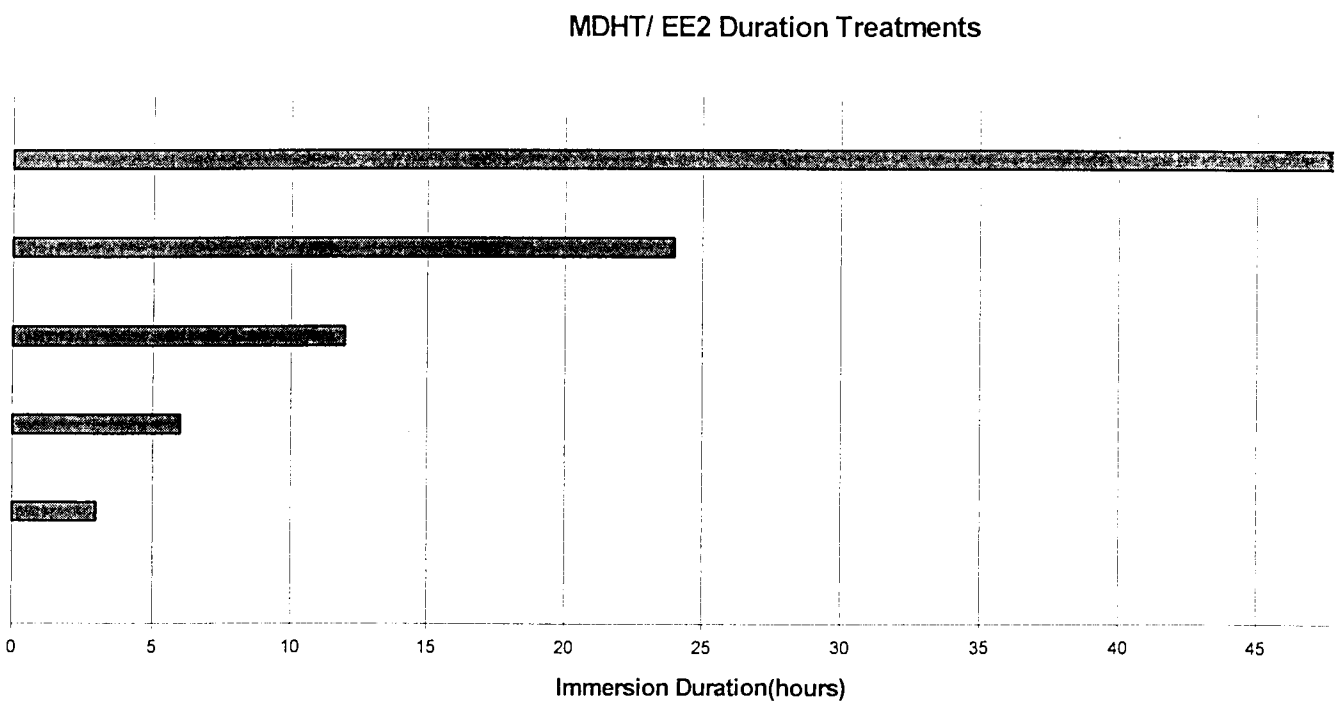

Figure 3: Durations of single MDHT and EE2 immersions completed one week post-hatch at 1 mg/l dosage to determine effects of extended immersions on rates of masculinization and feminization.

Immersion Dosage:

Dosage treatments were carried out one week post-hatch 466 ATUs. In both the masculinization as well as the feminization groups the low dosage was 0.5 mg/l and the medium dosage 1.0 mg/l. For MDHT the high dosage was 10.0 mg/l while for EE_2 the high dosage was 2.0 mg/l Treatment durations were 24 hours for MDHT immersions and 12 hours for EE_2 immersions. Control groups underwent simultaneous immersion for 24 hours in aqueous solutions of ethanol at concentrations of 0.05, 0.1 and 1.0% (Figures 1&2).

Triple Immersions:

A total of 4 triple immersion groups were developed for both MDHT and EE_2 (Figure 4). Triple immersions were initiated one week pre-hatch and continued through 2 weeks post-hatch with the final treatment occuring four weeks post-hatch. In the masculinization groups all treatments were for 12 hours duration and for the EE_2 feminization groups the immersions lasted 6 hours. Although the duration of the immersions for the triple immersion groups was half that of other groups, outside of the duration trials, total steroid exposure over the 3 treatments was 1.5 times greater. Control groups underwent triple ethanol immersions for 12 hours per treatment at a concentration of 0.1% ethanol.

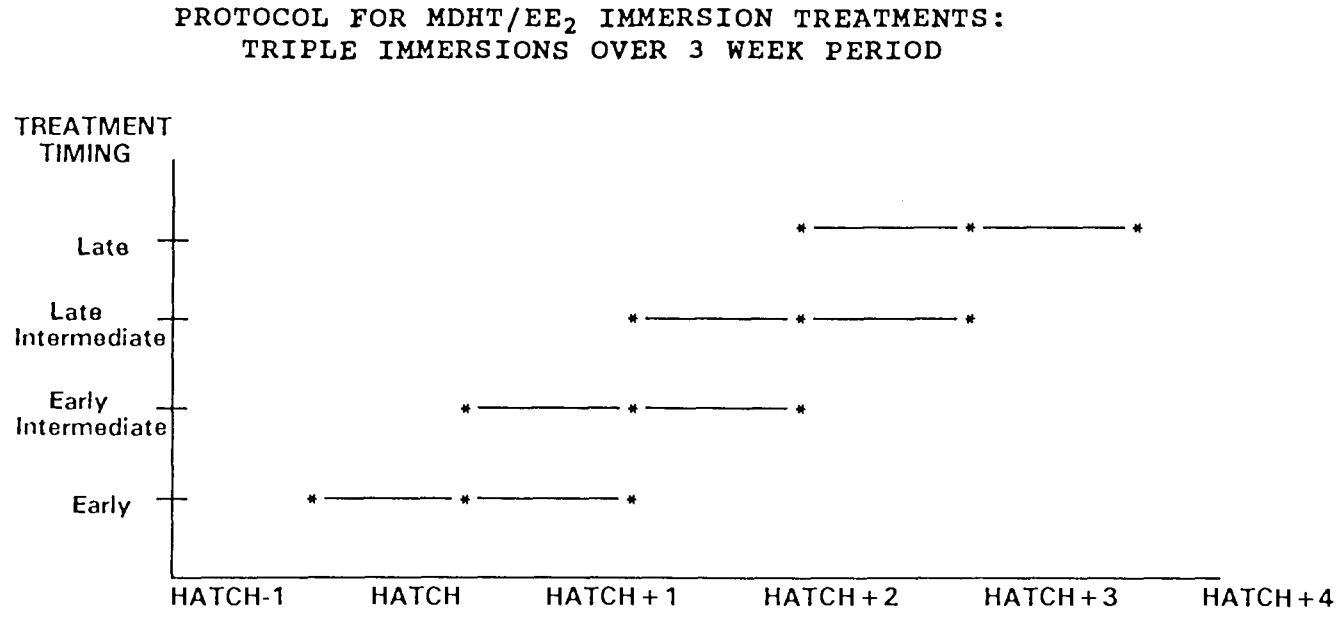

Figure 4: Treatment protocol for triple immersion treatments of Arctic Char using MDHT or EE_2. MDHT treatments consist of 3 immersions of 12 hours and those of EE_2 3 immersions of 6 hours duration. All treatments were separated by a one week period.

Feed Treatments:

A total of 4 treatment groups received oral treatments, i.e. 2 groups per steroid. The combined immersion and feed treatment groups underwent immersion one week post-hatch. The MDHT immersion was for 24 hours and the EE_2 immersion was for 12 hours. The concentration was 1.0 mg/l in both instances. In the feed only treatments the MDHT and EE_2 groups were fed beginning at swim-up on March 17, 1993 with a 735 ATU. Feed for both groups was prepared by dissolving the steroid in ethanol at a concentration of 1 mg/ml and spraying this mixture onto feed to a concentration of 7mg/kg, mixed thoroughly and exposed to the air to allow ethanol evaporation. All prepared feed was stored frozen at -20^{o}C until

used. The control group for the feed treatments underwent a simulaneous immersion in ethanol at 0.1% and was fed the same diet treated with ethanol at 7 ml/kg, then dried and frozen in an identical manner, but without the addition of steroid.

The alevins were ponded on March 17, 1994 into 5 l plastic buckets which had holes cut into the sides and bottom covered with 1mm plastic screen to allow flow through. The buckets were floated in 0.5 m^3 tanks. Feeding was carried out by hand, initially utilizing Bioproducts #1 starter, and feed size was increased through #2 and #3 as necessary. Buckets were cleaned by removing all fish, scrubbing the screen and disinfecting in iodine solution. Water temperature at the time of ponding was 3°C, and subsequent temperatures ranged from 3°C up to 10°C over the course of the rearing period.

Sampling was initiated on July 21,1994. Random samples of fish were removed from the tanks and given a lethal dose of MS222. They were then weighed to the nearest 0.01 g, had fork lengths taken to 1.0 mm and, were preserved in 10% formalin

Histological preparation began with sectioning of the fish at a point directly behind the pectoral fins and again at a point 0.5-0.75 cm. caudally to this. The sections underwent decalcified in a solution of 70% ethanol containing 5% nitric acid, dehydration by ethanol series, and paraffin embedding. Five serial 8 µm sections of each fish were floated onto a microscope slide and attached with egg albumin. The sections were stained in Harris haematoxylin/eosin and a coverslip attached with Permount. Sex identification by evidence of gonadal differentiation was carried out under a microscope.

Statistical Analysis:

Sex ratio analysis was performed using the Chi-square test. Significant difference from the expected ratio was tested at $P < 0.05$. For the purposes of sex ratio analysis intersex fish were combined with males in the MDHT treated groups and with females in the EE_2 treated groups.

RESULTS:

Classification of Sex by Gonadal Differentiation:

Several distinguishing features of the gonad were used to classify the fish into the categories of male, female, intersex or sterile. Male fish were positively identified by the presence of gonads of small cross-sectional area which were filled with spermatogonia (Fig. 3, Section I). In addition to spermatogonia, a vascular system consisting of a gonadal artery, gonadal vein and sperm duct was visible in many cases. Female fish were positively identified by the presence large gonads filled with normal oogonia and/or oocytes (Figs. 6 & 7, Section I). Classification to intersex condition represented a range of appearance from abnormal or reduced numbers of oocytes or oogonia (Fig. 4, Section I), through to the presence of both spermatogonia and oocytes in a single gonad (Fig. 5, Section I). Sterility was indicated by gonads which were devoid of any germinal elements, consisting strictly of non-germinal tissues (Solar et al., 1984).

EFFECTS OF STEROID APPLICATION ON GONADAL DIFFERENTIATION:

Control Groups:

Gonads of fish examined from the control groups were easily categorized as either male or female and lacked any of the ambiguities observed in the intersex individuals from the steroid treated groups. Male fish exhibited small gonads with densely packed spermatogonia. Female fish contained larger oval gonads which contained oocytes and/or oogonia with clearly defined nuclei and regular cell borders.

Sex ratios in the control groups showed, overall, significantly less variation from the expected sex ratios than did the steroid-treated groups. The most extreme sex ratio witnessed amongst the control groups was a ratio of 37.7% female and 62.3% male. When all control group fish were pooled (n=417) the resulting sex ratio was 54.5% male and 45.5% female. This is exactly the same control group sex ratio witnessed in the previous feed treatment trials (see Section I). Because of this observation, sex ratios in the treatment groups were compared to the 55% ratio observed in the control groups, which was considered as the expected ratio of males, and an expected ratio for females was defined as 45%. Although the lower densities of the control groups made them less susceptible to hypoxias they also contained far fewer individuals than the treatment groups, and therefore were more prone to total mortalities if hypoxia occurred. Because of this disparity 5 of the control groups were completely lost during the rearing stage.

No Treatment:

Sex ratios in the control group which did not undergo exposure to ethanol produced sex ratios which were not significantly different from the expected ratios($P < 0.05$).

TABLE 1: Sex ratios of control fish which underwent no treatment.

	MALES(%)	FEMALES(%)
(n = 38)	50.0	50.0

Weekly Immersions:

The control group treated during the week of hatch was the only treatment in this series in which the sex ratio of the group was significantly different from the expected 55:45 male:female sex ratio. This group produced a sex ratio of 45.1% male and 54.8% female, which is significantly different from the expected ratios ($P<0.05$). Although there does appear to be a trend in the sex ratios produced over time, with only one group showing sex ratios significantly different from those expected it is difficult to establish this trend as being attributable to the effects of ethanol exposure (Table 2).

TABLE 2: Sex ratios of control fish treated in 0.1% ethanol solution for 24 hours to test effects of timing of ethanol immersion. The groups treated during week hatch - 1 and hatch + 3 were lost to hypoxia during rearing; * denotes significantly different from expected sex ratio ($P < 0.05$).

	MALES(%)	FEMALES(%)
WEEK		
HATCH - 1		
HATCH (n = 31)*	45.1	54.8
HATCH + 1 (n = 53)	62.3	37.7
HATCH + 2 (n = 63)	57.1	42.9
HATCH + 3		
HATCH +4 (n = 41)	53.6	46.3

Immersion Duration:

Of the 3 duration of treatment groups tested for the effects of ethanol exposure, none showed sex ratios which were significantly different from those which were expected ($P > 0.05$). There were no clear effects on sex ratio which could be related to the duration of ethanol exposure (Table 3).

TABLE 3: Sex ratios of control fish treated in 0.1% ethanol solution at one week post hatch to test effects of duration of ethanol immersion.

	MALES(%)	FEMALES(%)
DURATION(HOURS)		
12.0 (n = 36)	57.1	42.9
24.0 (n = 53)	62.3	37.7
48.0 (n = 66)	56.1	43.9

Immersion Dosage:

None of the control groups used to compare the effects of treatment dosage of ethanol contained sex ratios which were significantly different from the expected ratios ($P > 0.05$). There were no apparent trends in the data correlating to the dosage of ethanol (Table 4).

TABLE 4: Sex ratios of control fish treated in ethanol solution for 24 hours one week post-hatch to test effects of ethanol concentration on sex ratios.The group treated at 0.05% concentration was lost to hypoxia during rearing.

	MALES(%)	FEMALES(%)
ETHANOL CONC'N(%)		
0.05		
0.1 (n = 53)	62.3	37.7
1.0 (n = 52)	48.1	51.9

Triple Immersions:

Although only 2 of the 4 control groups used to test the effects of triple immersions in ethanol survived through to the time of sampling, neither of these produced sex ratios which were significantly different from the expected ratios ($P > 0.05$).

TABLE 5: Sex ratios of control fish undergoing 3 immersions in 0.1% ethanol solution for 12 hours per treatment at one week intervals to test effects of multiple ethanol immersions on sex ratios. Groups treated over weeks -1 - 1 and 0 - 2 were lost to hypoxia during the rearing stage.

	MALES(%)	FEMALES(%)
WEEKS OF TREATMENT		
-1 - 1		
0 - 2		
1 - 3 (n = 17)	47.1	52.9
2 - 4 (n = 20)	60.0	40.0

MASCULINIZATION/FEMINIZATION TREATMENTS:

Weekly Immersion Effects:

A clear trend was observed in the number of males present within these groups treated with MDHT immersions over the course of the 6 treatment weeks (Table 6; Fig.5). The number of males increased over the first 3 treatment weeks from 60.0% one week pre-hatch, to the highest percentage of males (69.1%) in the group treated one week post-hatch. For the following weeks the number of males decreased, and during the last 2 treatment weeks these numbers were at or below the expected sex ratios.

In the feminization groups treated with EE_2 the best response to the steroid occurred during weeks 2 through 4 post-hatch.Maximum feminization occurred 3 weeks post hatch when 70.1% of the sample were female (Table 6a; Fig.6)

TABLE 6: Sex of fish in masculinization groups treated by week through MDHT immersion at 1 mg/l concentration for 24 hours. * Indicates significantly different from expected ($P<0.05$).

	MALES(%)	FEMALES(%)	INTERSEX(%)	STERILE(%)
Hatch-1 (n=55)*	33(60.0)	13(23.6)	7(12.7)	2(3.6)
Hatch (n=59)	36(61.0)	20(33.9)	3(5.1)	0(0)
Hatch+1 (n=55)*	38(69.1)	16(29.1)	1(1.8)	0(0)
Hatch+2 (n=52)*	34(65.3)	17(32.7)	1(1.9)	0(0)
Hatch+3 (n=53)	27(51.9)	21(40.4)	4(7.7)	0(0)
Hatch+4 (n=52)	29(55.8)	23(44.2)	0(0)	0(0)

TABLE 6a: Sex of fish in feminization groups treated by week through EE_2 immersion at 1mg/l concentration for 12 hours. * Indicates significantly different from expected ($P < 0.05$).

	FEMALE(%)	MALE(%)	INTERSEX(%)
Hatch-1 (n=56)*	34(60.1)	18(32.1)	4(7.1)
Hatch (n=50)*	24(48.0)	17(34.0)	9(18.0)
Hatch+1 (n=49)	23(46.9)	21(42.9)	5(10.2)
Hatch+2(n=50)*	32(64.0)	7(14.0)	11(22.0)
Hatch+3(n=57)*	40(70.1)	9(15.8)	8(14.0)
Hatch+4(n=49)*	32(65.3)	7(14.3)	10(20.4)

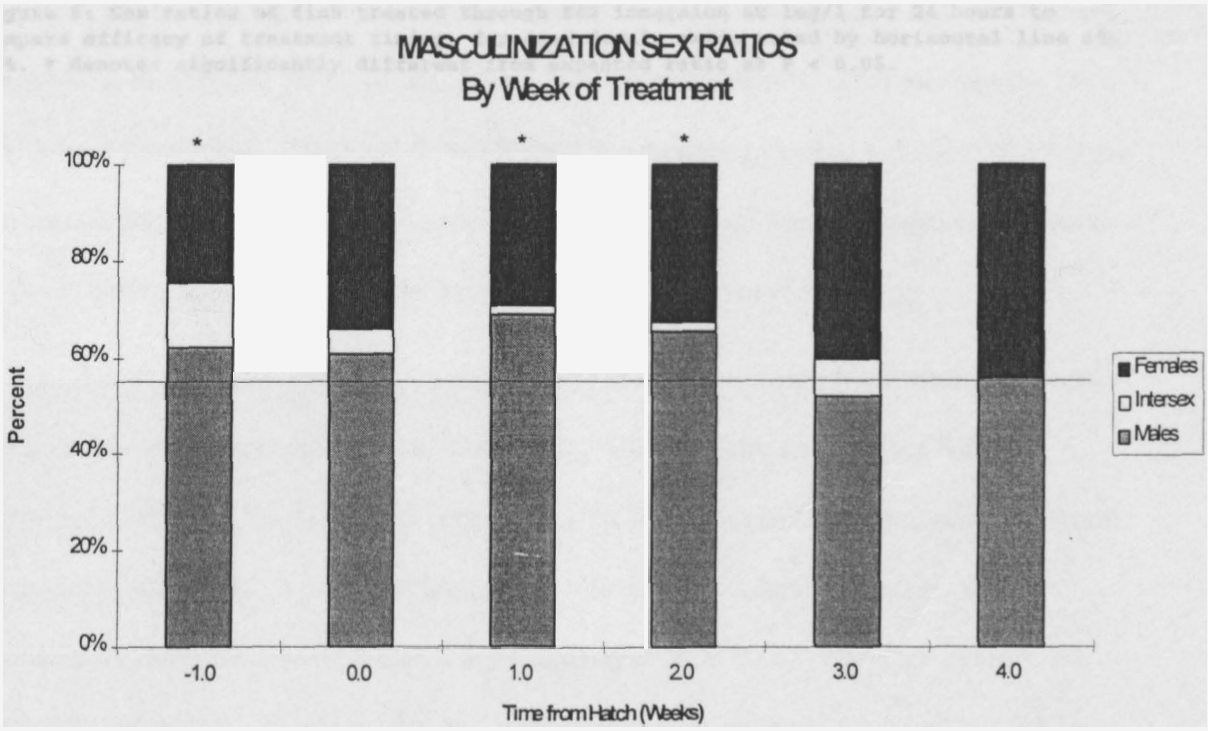

Figure 5: Sex ratios of fish treated through MDHT immersion at 1mg/l for 24 hours to compare efficacy of treatment timing. Horizontal line at 55% denotes expected male ratio * indicates significantly different at P < 0.05. Steriles not included.

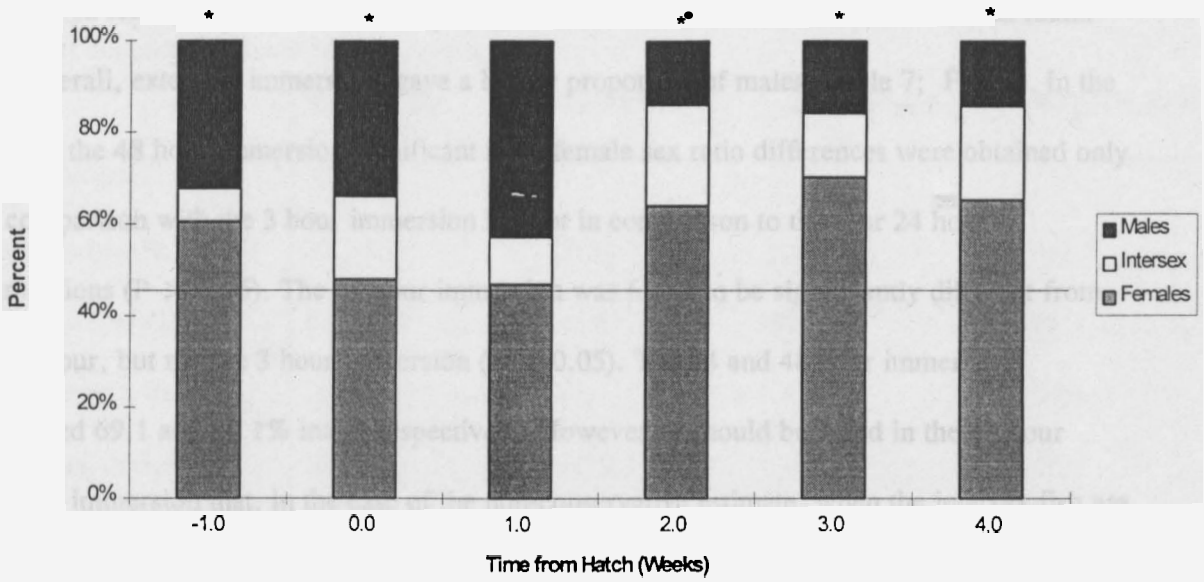

Figure 6: Sex ratios of fish treated through EE2 immersion at 1mg/l for 24 hours to compare efficacy of treatment timing. Expected female ratio noted by horizontal line at 45%. * denotes significantly different from expected ratio at P < 0.05.

Immersion Duration Effects:

In testing the effects of the duration of MDHT immersion on sex ratios it was found that overall, extended immersions gave a higher proportion of males (Table 7; Fig. 7). In the case of the 48 hour immersion significant male:female sex ratio differences were obtained only in comparison with the 3 hour immersion but not in comparison to the 6 or 24 hour immersions ($P > 0.05$). The 24 hour immersion was found to be significantly different from the 6 hour, but not the 3 hour immersion ($P > 0.05$). The 24 and 48 hour immersions produced 69.1 and 60.1% males respectively. However, it should be noted in the 48 hour MDHT immersion that, in the case of the non-conservative estimate, when the intersex fish are combined with the males the ratio increases to 73.8%, indicating that an even larger number of fish were affected by exposure to the steroid relative to the 24 hour MDHT immersion, and the 48 hour immersions produced sex ratios which were significantly different from the 24, 6 and 3 hour immersions. The 3 and 6 hour MDHT immersions produced sex ratios which were not significantly different from the expected ratios. The 12 hour immersion group was lost to hypoxia during the rearing stage and therefore samples were unavailable.

The duration treatments in the EE_2 groups showed an overall trend toward increased feminization with longer immersions, most notably when females and intersex fish are combined (Table 7a; Fig.8). The 48 hour immersion yielded a sex ratio significantly different from that of the 24, 12, 6 or 3 hour immersions whereas the 24 hour immersion was significantly different only from the 12 hour immersion ($P > 0.05$). When the intersex fish are combined with the female portion in the 48 hour immersion group 98.1% of the fish have had their gonadal development strongly influenced by exposure to the estrogen.

TABLE 7: Sex of fish in masculinization groups treated by duration of immersion in MDHT at 1mg/l concentration one week post-hatch.

	MALE(%)	FEMALE(%)	INTERSEX(%)	STERILE(%)
DURATION				
3 Hours (n=56)	30(53.6)	22(39.3)	3(5.3)	1(1.8)
6 Hours (n=51)	27(52.9)	16(31.3)	2(3.9)	6(11.8)
12 Hours(n=0)	0(0)	0(0)	0(0)	0(0)
24 Hours (n=55)	38(69.1)	16(29.1)	1(1.8)	0(0)
48 Hours (n=51)	31(60.1)	13(25.5)	7(13.7)	0(0)

TABLE 7a : Sex of fish in feminization groups treated by duration of immersion in EE_2 at 1 mg/l concentration one week post-hatch.

	FEMALES(%)	MALES(%)	INTERSEX(%)
Duration			
3 Hours (n=53)	27(51.0)	18(34.0)	8(15.1)
6 Hours(n=49)	29(59.2)	10(20.4)	10(20.4)
12 Hours(n=49)	23(46.9)	21(42.9)	5(10.2)
24 Hours(n=50)	28(56.0)	10(20.0)	12(24.0)
48 Hours(n=52)	31(59.6)	1(1.9)	20(38.5)

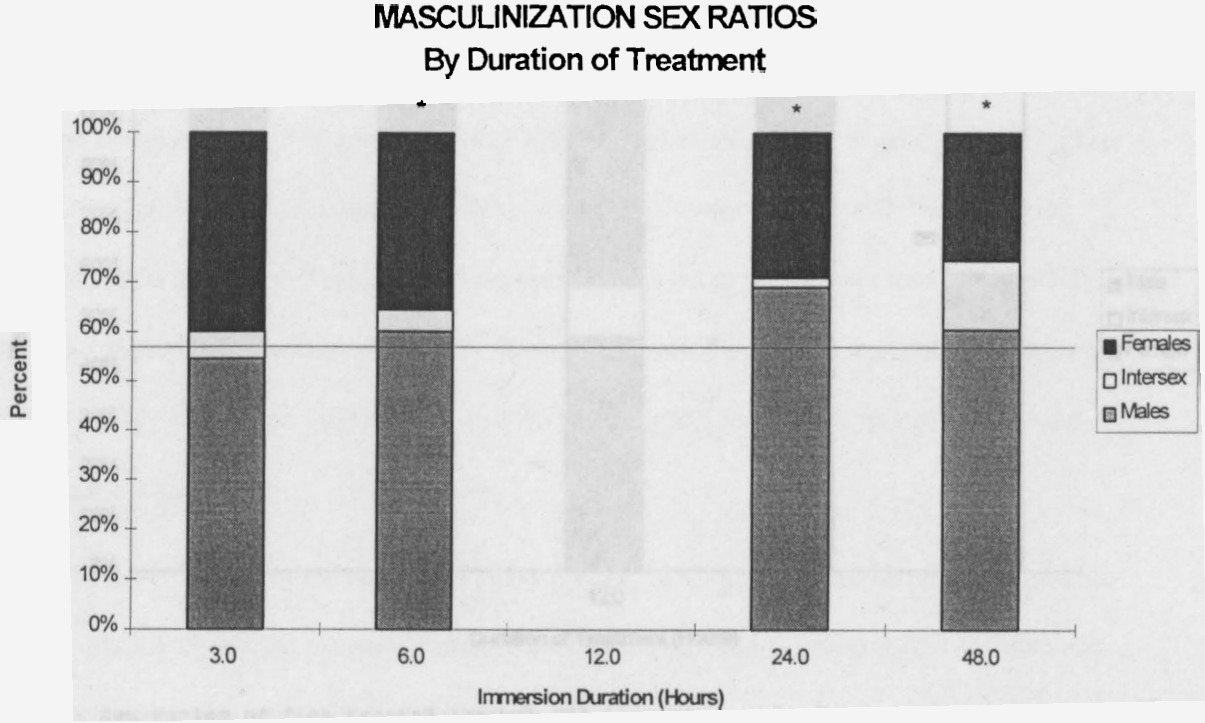

Figure 7: Sex ratios of fish treated through MDHT immersion at 1mg/l 1 week post-hatch to compare efficacy of treatment duration. Expected male ratio noted by horizontal line at 55%. * denotes significantly different from expected ratios at $P < 0.05$.

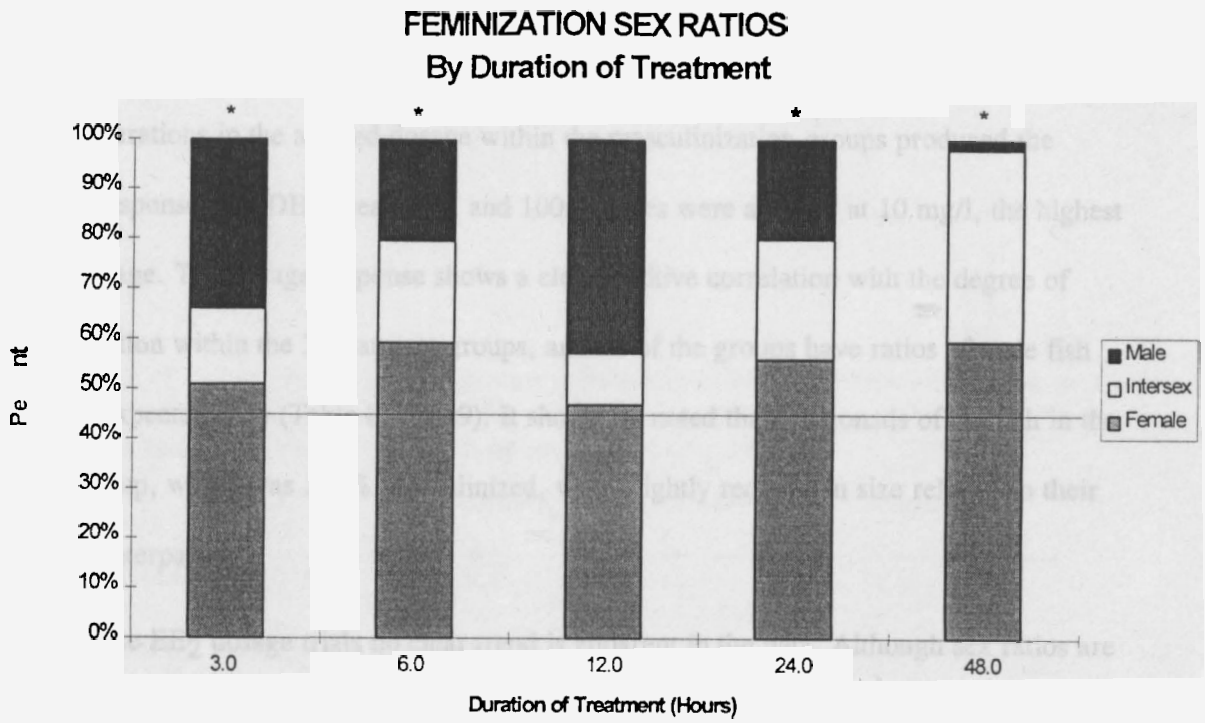

Figure 8: Sex ratios of fish treated through EE2 immersion at 1mg/l 1 week post-hatch to compare efficacy of treatment duration. Expected female ratio noted by horizontal line at 45%. * denotes significantly different from expected ratio at $p < 0.05$.

Immersion Dosage Effects:

Alterations in the applied dosage within the masculinization groups produced the strongest response to MDHT treatment, and 100% males were attained at 10 mg/l, the highest applied dosage. The dosage response shows a clear positive correlation with the degree of masculinization within the 3 treatment groups, and all of the groups have ratios of male fish above the expected ratio (Table 8; Fig. 9). It should be noted that the gonads of the fish in the 10 mg/l group, which was 100% masculinized, were slightly reduced in size relative to their control counterparts.

In the EE_2 dosage trials no clear trend is apparent in the data. Although sex ratios are elevated slightly above the expected ratios in the highest (2.0 mg/l) group this is not the case in either of the medium (1.0 mg/l) or low (0.5 mg/l) dosage treatment groups (Table 8a; Fig.10).

Significant differences from the expected sex ratios were obtained at the 95% confidence level in both the 0.5 mg/l (lowest) and the 2.0 mg/l (highest) dosages in these feminization procedures.

Table 8: Sex of fish in masculinization groups undergoing MDHT immersions for a 24 hour period one week post-hatch to determine the effects of immersion dosage on sex ratio. * Indicates sex ratios significantly different from expected ($P < 0.05$).

	Males(%)	Females(%)	Intersex(%)	Sterile(%)
0.5mg/l* (n=53)	34(64.1)	17(32.1)	2(3.9)	0(0)
1.0mg/l* (n=55)	38(69.1)	16(29.1)	1(1.8)	0(0)
10.0mg/l*(n=27)	27(100)	0(0)	0(0)	0(0)

Table 8a: Sex of fish in feminization groups undergoing EE_2 immersions for 12 hour period one week post-hatch to determine effects of treatment dosages on sex ratios. *Indicates sex ratios significantly different from expected ($P < 0.05$)

	Females(%)	Males(%)	Intersex(%)
0.5mg/l* (n=52)	24(46.1)	12(23.1)	16(30.1)
1.0mg/l (n=49)	23(46.9)	21(42.9)	5(10.2)
2.0mg/l* (n=46)	25(54.3)	9(19.6)	12(26.1)

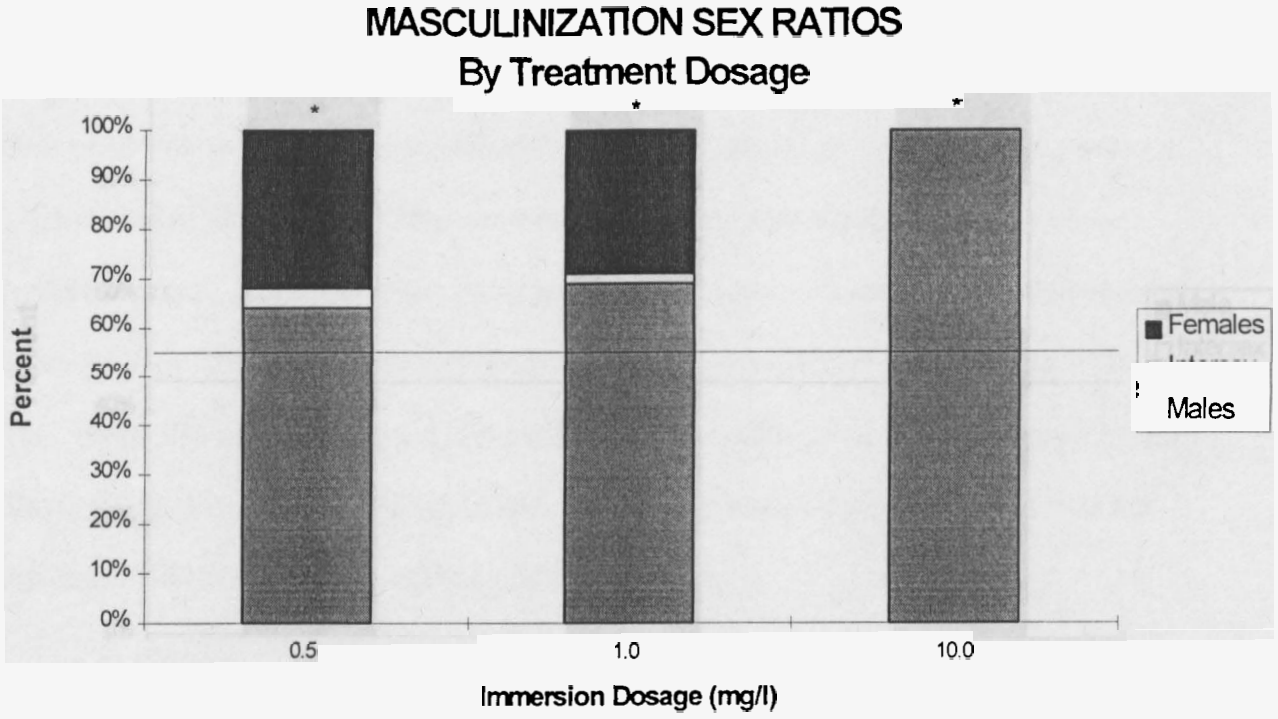

Figure 9: Sex Ratios of fish treated through MDHT immersion for 24 hours 1 week post-hatch to compare efficacy of treatment dosage. Expected male sex ratio noted by horizontal line at 55%. * denotes significantly different from expected ratio at P < 0.05.

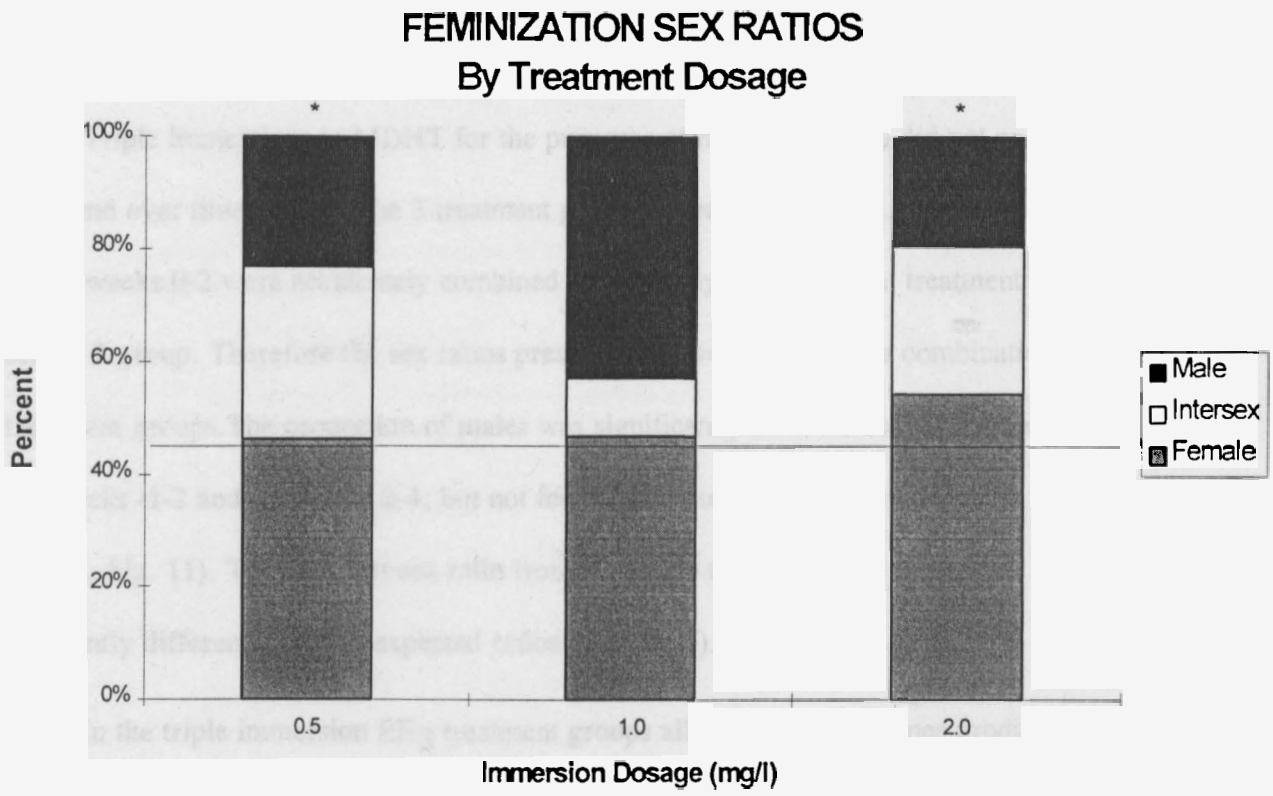

Figure 10: Sex ratios of fish treated through EE2 immersion for 12 hours at 1 week post-hatch to compare efficacy of treatment dosage. Expected female sex ratio noted by horizontal line at 45%. * denotes significantly different from expected ratio at $P < 0.05$.

Triple Immersion Effects:

Triple immersions in MDHT for the purposes of masculinization did not produce a clear trend over time between the 3 treatment groups. Treatment groups representing weeks -1-1 and weeks 0-2 were accidentaly combined immediately after the final treatment of the weeks 0-2 group. Therefore the sex ratios presented for weeks -1-2 are a combination of these 2 treatment groups.The proportion of males was significantly elevated for the groups treated from weeks -1-2 and for weeks 2-4, but not for the intermediate group treated from weeks 1-3 (Table 9; Fig. 11). The resultant sex ratio from the group treated during weeks 1-3 was not significantly different from the expected ratios ($P > 0.05$).

In the triple immersion EE_2 treatment groups all 4 treatment regimes produced sex ratios which were significantly different from those expected ($P < 0.05$). The percentage of females found within the groups was postitively correlated with the timing of the treatments, with the later treatments giving an increased ratio of females.The highest proportion of females was found in the group treated from weeks 2-4. (Table 9a; Fig. 12).

TABLE 9: Sex of fish treated by triple immersion in MDHT at 1.0 mg/l concentration for 12 hours per treatment over a 3 week period.*Indicates sex ratios significantly different from expected ($P < 0.05$).

	Males(%)	Females(%)	Intersex(%)	Sterile(%)
Treatment Weeks				
-1 - 2 (n=55)*	39(71.0)	11(20.0)	5(9.1)	0(0)
1 - 3 (n=58)	34(58.6)	22(37.9)	1(1.7)	1(1.7)
2 - 4 (n=57)*	40(70.2)	15(26.3)	2(3.5)	0(0)

TABLE 9a: Sex of fish treated through triple immersions in EE_2 at 1.0mg/l concentration for 12 hours per treatment over a 3 week period.* Indicates sex ratios significantly different from expected ($P < 0.05$).

	Females(%)	Males(%)	Intersex(%)
Weeks of Treatment			
-1 - 1 (n=58)*	20(34.5)	25(43.1)	13(22.4)
0 - 2 (n=51)*	25(49.0)	11(21.6)	15(29.4)
1 - 3 (n=62)*	32(51.6)	21(33.9)	9(14.5)
2 - 4 (n=45)*	26(57.8)	8(17.8)	11(24.4)

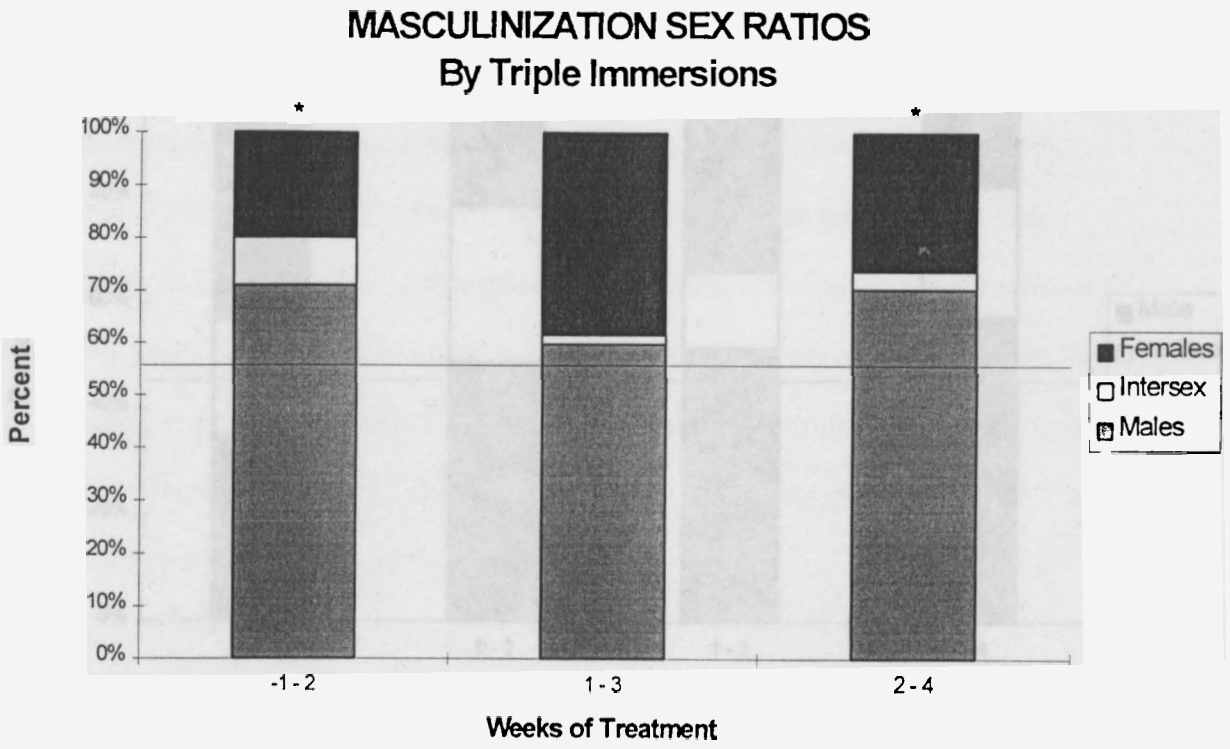

Figure 11: Sex ratios of fish treated through triple MDHT immersions at 1.0mg/l concentration for 12 hours over a 3 week period to compare efficacy of treatment. Expected male sex ratio denoted by horizontal line at 55%. * denotes significantly different from expected ratio at P < 0.05. (NOTE: Weeks -1 - 1 & 0 - 2 combined).

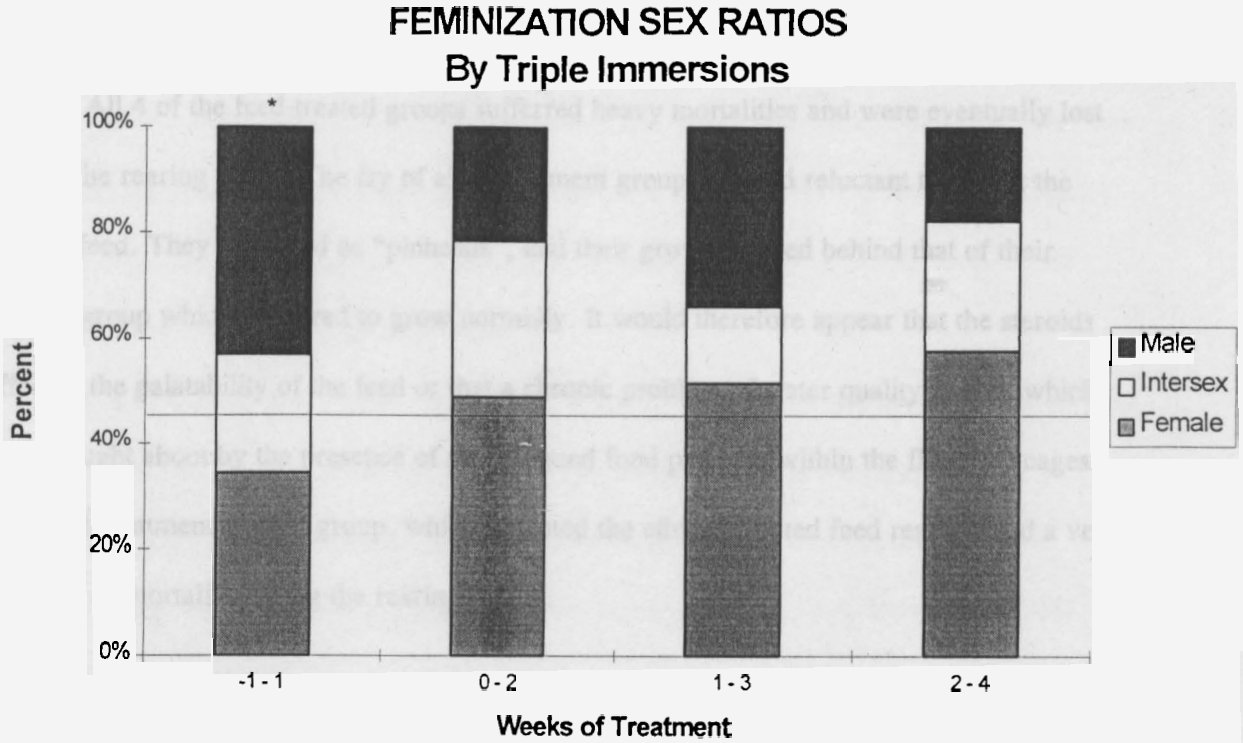

Figure 12: Sex ratios of fish treated through triple EE2 immersions at 1.0 mg/l concentration for 6 hours over a 3 week period to compare efficacy of treatment. Expected female sex ratio noted by horizontal line at 45%. * denotes significantly different from expected ratio at $P < 0.05$.

Feed Treatments:

All 4 of the feed-treated groups sufferred heavy mortalities and were eventually lost during the rearing phase. The fry of all 4 treatment groups seemed reluctant to accept the treated feed. They remained as "pinheads", and their growth lagged behind that of their control group which appeared to grow normally. It would therefore appear that the steroids affected the palatability of the feed or that a chronic problem of water quality existed which was brought about by the presence of unconsumed food particles within the floating "cages". The feed-treatment control group, which accepted the ethanol treated feed readily, had a very low rate of mortality during the rearing period.

DISCUSSION

SEX RATIO ANALYSIS:

The sex ratios resulting from the immersion treatments with either MDHT or EE_2 have shown that significant control of gonad differentiation is attainable for the Arctic char for the purposes of feminization, and that 100% rates of masculinization are possible, even under conditions of low ambient water temperature.

It is of interest to note that the methods of steroid application which yielded the highest rates of either feminization or masculinization differed. Maximal rates of feminization occurred in response to an extended duration of steroid exposure, while optimal rates of masculinization were obtained in conjunction with an exceptionally high dosage of MDHT.

TREATMENT TIMING:

The series of experiments examining the effects of treatment timing on rates of masculinization and feminization following steroid immersion have yielded important information regarding the labile period of the Arctic char to the influences of sex steroids on gonadal development. The highest degrees of masculinization were obtained in response to a single immersion treatment administered during the week following hatch. This timing for optimum masculinization in the Arctic char coincides precisely with that of coho salmon, which were found to be maximally sensitive to a single steroid treatment administered from 6 to 13 days post-hatch (Piferrer & Donaldson, 1989). In the case of Arctic char, the treatments carried out one week post-hatch were completed at 471 ATUs post-fertilization whereas in the coho salmon one week post-hatch represented ATUs. This difference in the most effective timing of treatment may be attributable to a variance in the physiological rates of gonad development between the two species, possibly indicating that the char undergo gonad differentiation earlier than coho salmon. Because no study of gonadal development in the

Arctic char has been carried out to date it is presently difficult to test the validity of this hypothesis. The response of masculinization ratios to timing of treatment found here for the Arctic char show a nearly identical trend to that found for the coho salmon by Piferrer and Donaldson (1989). In both studies the rates of masculinization in response to androgen immersion exhibited increases over the week before, and of hatch, attaining a maximum at one week post-hatch. Over the course of the 2 following weeks, the rates of masculinization successively decreased, returning to a level not significantly different from that which would be expected for their control counterparts. The similarity of the results obtained in this study when compared to Piferrer and Donaldson's (1989) study, provides compelling evidence that the labile period of the Arctic char for the purposes of masculinization through androgen immersion has been delineated.

The results of the experimental series aimed at identifying the labile period of the arctic char for the purposes of feminization through a single EE_2 immersion do not present a clear picture. The maximum ratios of feminization occurred in response to treatments completed 3 weeks post-hatch. As would be expected, during the weeks before and after the maximum, slightly lower rates of feminization existed, respectively indicating a decreased sensitivity to the estrogen treatments. The results obtained here are different from those reported by Pifferer & Donaldson (1989), who found that for coho salmon the maximal rate of feminization occurred when the eggs were estrogen immersed one week pre-hatch. However, in this experiment, utilizing the Arctic char, the rate of feminization is also elevated to a level significantly different from the expected ratio during the week before hatch. It is difficult to reconcile these results in light of the existing model for predicting the labile period of steroid sensitivity in regard to control of gonad differentiation. The model states that physiological

differentiation of the gonad occurs concurrently with a peak of endogenous estrogen (Feist et al., 1990). When steroid application coincides with physiological differentiation of the gonad, the optimum rate of gonad differentiation control will be achieved. In this instance, where two peaks appear to be present in the labile period of the Arctic char to the effects of EE_2 immersion, a more complex scenario would appear to exist. Pifferer and Donaldson (1994) found that eyed eggs retained steroids for an extended period following immersion treatments relative to alevins or fry. They also found that upon hatching, 16% of the total steroid content of the egg was lost in conjunction with the egg capsule. This effect may explain why the char treated during the week before hatch showed an elevated rate of feminization. The presence of the yolk sac and egg capsule would allow a greater portion of the steroid to be retained following completion of immersion treatment, producing an elevated exposure to the steroid, and therefore a possible corresponding increase in the rate of feminization. Results obtained in the duration study carried out here for the Arctic char have shown that optimum rates of feminization occur in response to extended durations of steroid exposure. The unhatched eggs would be exposed to the steroids for a longer period than those treated during later weeks as alevins, as the lipid portion of the yolk in addition to the eggshell capsule would act as a steroid reservoir. If, as is suspected, the cold water temperatures slow rates of steroid uptake and clearance, the presence of both yolk and eggshell would play a critical role in determining total steroid exposure. The fish treated during the week of hatch would initially retain a similar amount of steroid as those treated one week pre-hatch, but would lose a significant portion of their steroid content upon shedding the egg capsule. This effect would translate to an overall lower degree of exposure for the eggs treated during the week of hatch relative to those treated one week pre-hatch, and may explain the differences in rates of feminization which occurred between these two groups. The fish treated one week post-hatch would not be exposed to the

portion of steroid contained within the eggshell and at this later developmental stage would contain even less yolk than the fish treated in the two previous weeks. Therefore, although the fish treated in the week before and of hatch are not treated at the optimal point in the labile period they are probably exposed to the estrogen for a longer period of time. This extended exposure may have a compensatory effect ultimately promoting an increase in the rate of feminization.

TREATMENT DURATION:

Duration of treatment effects were less pronounced in the masculinization series relative to those of the feminization groups. In the assessment of these results it is important to note that the treatments were carried out one week post-hatch which has been shown above as optimum treatment timing for the purposes of masculinization, but two weeks earlier than optimum for feminization.

In the masculinization series data, there appears to be a correlation betweeen the duration of treatment and the degreee of resulting masculinization, with increased rates of masculinization occurring in conjunction with longer durations. Piferrer and Donaldson (1994) noted that the time required to reach saturation levels of steroid concentration at all developmental stages was shorter for testosterone than for the estrogen estradiol-17 β. Assuming corresponding effects with MDHT and EE_2, the fact that there is no increase in the rate of masculinization in the 48 hour immersion relative to that of the 24 hour immersion may be explained by the fact that MDHT saturation has occurred for this concentration of steroid, or alternatively that a minimum necessary dosage level has not been attained. Baker et al. (1988) have shown using chinook salmon, that while both dosage and duration affect rates of

masculinization, dosage appears to be the more critical factor for achieving a 100% level of masculinization. Utilizing MDHT, a strong positive correlation between dosage and rates of masculinization has been established employing immersion durations as short as 2 hours (Piferrer et al. 1993; Piferrer & Donaldson, 1991). In the Arctic char, an increase in immersion duration beyond 24 hours does not appear to produce a corresponding increase in the degree of masculinization at moderate dosage levels. It has also been established that EE_2 is exceptionally effective at relatively low dosage for the purposes of sex control in salmonids (Piferrer & Donaldson, 1992). Exposure to high dosages of the estrogen estradiol and the compound diethylstilbestrol through oral adminstration has proven to be toxic in Atlantic salmon (Sower et al., 1984). As will be discussed further on, high rates of masculinization were achieved utilizing a 24 hour immersion in conjunction with elevated dosage, whereas the most effective route toward feminization involves an extended immersion at a moderate dosage level.

The results from the feminization duration series are difficult to reconcile in concordance with the currently accepted model of sex control. There is clearly a trend shown in the data toward increasing rates of feminization with extended immersion duration. Also, a higher degree of partial feminizations occurred, resulting in the production of an elevated proportion of intersex individuals in the longer immersions. As mentioned earlier, the duration series treatments were carried out two weeks previous to the time at which immersion treatments proved optimum for feminization. The rates of feminization from the treatment timing trials, discussed earlier, have shown a large increase beginning with the group treated 2 weeks post-hatch and peaking during the third week after median hatch. One of the known effects of low ambient water temperatures on fish is to slow metabolic rates as indicated by decreased rates of oxygen consumption per unit body weight (Brett, 1973). In this classic

study, it was shown that a compensatory effect existed in the sockeye salmon (Oncorhynchus nerka) which caused an increase in the rate of respiration which seems to compensate for the metabolic slowing brought about by decreased ambient temperatures. However, another study by Holeton (1973) indicated that this metabolic compensatory mechanism was not observed in a subspecies of Arctic char from the Rankin Inlet area of the NWT, the same geographical region as Nauyuk Lake, where the char in this study originated. If this slowing of respiratory metabolism is similarly indicative of the effects of cold water on steroid metabolism it would cause both the uptake and clearance rates of the estrogen from the body of the alevins to be impeded. In the cases of the 24 and 48 hour immersions, the body content of the steroid would rise to a correspondingly higher level than that witnessed in cases of the shorter immersions. If clearance rates were slowed significantly by the low ambient water temperatures, a larger proportion of the steroid would be retained within the body of the fish as its physiological development progressed toward the most sensitive point in the labile period. This effect would be expected to produce an elevated rate of feminization, in excess of that which could be attributed to the effects of extended immersion durations alone. The steroid content would have fallen to a somewhat lower level as the fish entered the second and then third weeks post-hatch, an effect which could produce an increased tendency toward partial feminizations in conjunction with extended EE_2 immersions due to underdosing. Therefore, although the fish do exhibit increased feminization in response to increased duration of estrogen immersions, the factors which cause this effect may be compounded by body steroid contents remaining high, due to low water temperatures, as they develop to the point in the labile period where they are most susceptible to feminization procedures. The higher rates of feminization witnessed in the 6 hour immersion may be a statistical anomaly as it is difficult to understand why both longer

and shorter durations would produce lower rates of feminization, unless the extended exposures caused a decrease in the number of estrogen receptors within the target cells. The effects of extended immersion durations as a critical factor in achieving high rates of feminization are clearly illustrated in a study by Nakamura (1984) wherein it was shown that at very low doses of estradiol-17β, in the range of 0.00025 - 0.005 mg/l, administered at 15 °C for 17 and 66 days in masu salmon (Oncorhynchus masou) and chum salmon (Oncorhynchus keta) respectively, total feminization was attainable. In these experiments it was found that extended treatment durations appeared to have a more significant effect than dosage level with regard to affecting rates of feminization.

TREATMENT DOSAGE:

The effects of treatment dosage for the purposes of masculinization clearly indicate that at a relatively high dosage level (10.0 mg/l) administered for 24 hours, 100% masculinization is attainable at temperatures in the range of 2-3 °C. The 10.0 mg/l dosage level, which represents the current upper known safe limit of MDHT dosage, produced fish with slightly reduced gonads and spermatogonia which were smaller than those of their control counterparts. Another problem encountered at this dosage was attributed to the effects of the 0.1% ethanol concentration on the alevins as a result of the relatively large aliquot of stock solution added to the immersion bath. At the conclusion of the treatment the alevins appeared quite lethargic and did not respond to light or movement when returned to the egg trays. However, they appeared to have recovered fully from the effects of the treatments after 24 hours in fresh water, and did not suffer any identifiable long term effects. The reduced size of the spermatogonia may be indicative of an overdosing of MDHT, and that exposure to this dosage level causes the gonads to approach sterility, an effect known to occur in conjunction

with very high levels of androgen. It is therefore likely that reducing the dosage somewhat, perhaps into the range of 7.5 mg/l for a 24 hour immersion, may produce equivalent rates of masculinization without incurring smaller gonads/spermatagonia and possibly sterility.

The results obtained here clearly indicate that a high dosage of MDHT is the critical factor necessary to elicit 100% masculinization in the Arctic char at temperatures in the 2-3 °C range. The question which arises from the results obtained in this investigation is; why does a high dosage of the steroid produce a maximum rate of masculinization, while an extended immersion at a lower dosage does not? It would appear that there is either a species or temperature related difference which requires a significantly higher dosage to attain complete masculinization in the Arctic char relative to the requirements of Pacific salmon. In comparison, MDHT immersion dosages of 0.4 mg/l administered for only 2 hours elicited a 98% male population in the chinook salmon at 10 °C, and a dose of 2.0 mg/l administered under identical conditions produced a 100% male population (Piferrer et al. 1993). Comparing the relatively low dosages and short durations needed to masculinize chinook salmon at 10 °C it would appear that the lower rate of masculinization in the 48 hour immersion group occurred because of inadequate steroid exposure. Inter-species differences cannot be completely ruled out however, as in the coho salmon, the relatively high MDHT immersion dosage of 6.4 mg/l administered for 2 hours at 10.0°C produced only 90% males (Piferrer & Donaldson, 1991). It is therefore not possible at this time to determine whether it is an inter-species difference or an effect of reduced metabolic rate, due to low ambient water temperature, which demands the high dosage of androgen necessary to attain complete masculinization in the Arctic char. Further experimentation with masculinization immersion treatments over a range of temperatures would provide answers to these questions.

The need for a high dose of MDHT in the presence of low ambient water temperatures to attain complete masculinization, should also be examined in light of Piferrer and Donaldson's research (1994) wherein it was shown that in response to immersion treatments, testosterone was incorporated at a higher rate than estradiol-17 β, and attained saturation much earlier. Assuming that the results obtained utilizing testosterone can be extrapolated to MDHT effects, some insight may be gained regarding the rate of masculinization which occurred in response to the 3 dosage levels. With a slowed metabolism, the rate of steroid turnover at the receptor cells will be decreased. A high dosage level would ultimately provide a pool of steroid which would accumulate in the lipid components of both the yolk and the fish's body. Upon termination of treatment those individuals treated at the highest dosages would have a larger reservoir of retained androgen which would be available to them for an extended period of time, relative to those treated at a lower dosage. Therefore, those treated at high dosages would undergo a significantly greater exposure. This effect would compensate for the slowed rates of steroid uptake and clearance. Depending on the degree to which androgen uptake and clearance are slowed by low water temperatures, those fish treated over a 48 hour period at a 1.0 mg/l dosage may not experience the same degree of exposure as those treated for 24 hours at 10.0 mg/l This would explain the differences in rates of masculinization between the two groups. Alternatively, an effect peculiar to the species cannot yet be ruled out. The Arctic char may be less sensitive to the effects of gonadal steroids relative to other species and therefore a greater exposure would be required. There may be a "threshold" minimum dosage level necessary to attain complete masculinization in the Arctic char. As exceeding a minimum dosage level has been shown to be the critical factor in achieving high rates of masculinization in Pacific salmonids, a minimum dosage level may also exist for the Arctic char which is higher than required for other species of salmonids. If this species difference exists, meeting

the minimum dosage level would be necessary and may be independent of ambient water temperature effects. Further experimentation is needed to reveal which factors are critical for this species.

The results obtained during the feminization dosage trials are difficult to assess for several reasons. The most important of these being that the immersion procedures were completed two weeks prior to the point in the labile period optimal for the purposes of feminization. It is apparent from the data that underdosing of the alevins occurred as indicated by the large number of intersex fish which resulted. When the fish were treated at a dosage of 1.0 mg/l one week post-hatch the number of females in the sample was not significantly different from the expected ratio, yet the same dosage applied 3 weeks post-hatch produced a population containing over 70% females. Although the 2.0 mg/l dosage produced a slightly higher percentage of females than the 1.0 mg/l dosage it is difficult to determine to what degree this difference would have been enhanced had the treatments been carried out at the time of maximum hormone sensitivity. It is also significant that the lowest dosage produced a number of intersex individuals which was larger than those of the two higher dosages. This may be indicative of the phenomenon discussed earlier which showed that duration of estrogen immersion is more critical than dosage for the purposes of achieving high degrees of feminization. The higher rate of feminization attained in conjunction with the higher dosage may be due to a residual effect of higher estrogen concentrations migrating into the body from the yolk after cessation of treatment, thereby effectively increasing the duration of steroid exposure. The most important point to note here is that the 48 hour immersion at medium dosage produced a larger number of both females and intersex individuals than the 24 hour immersion at twice the dosage. This provides important information supporting the theory that

duration is more important than dosage in feminization procedures, and also that duration and dosage do not have equivalent effects in terms of total effective "exposure" to the steroid.

The term "dosage" as it has been used in these procedures would be more accurately described as total "exposure" of the fish to a given concentration of hormone, as the actual proportion of steroid reaching the gonadal tissues is unknown. It should be kept in mind when analyzing the results obtained here, that a more direct application of steroid, e.g. by injection, may significantly increase the effective dosage and resulting sex ratios.

TRIPLE IMMERSIONS:

The decision to include triple immersion treatments in these procedures was made in an attempt to test for the presence of any inductive effect which might occur in response to the initial immersion, increasing the efficacy of masculinization/feminization with the subsequent treatments. When the results obtained in this study are examined overall, there is no apparent evidence which would indicate the presence of an inductive effect in response to multiple immersions.

The masculinization rates obtained in the fish treated over the course of weeks -1 to 2 showed a masculinization response to treatment very similar to those fish given the same dosage in a single 24 hour immersion one week post-hatch. This would be expected as one week post-hatch, the optimum point in androgen sensitivity, occurs near the mid-point of these treatments. These particular data should be examined with caution however, because the masculinization groups treated over weeks -1 to 1 and weeks 0 to 2 were accidentaly combined in the incubation stacks at the conclusion of the final treatment. If a large difference existed between the masculinization rates of the two treatment groups this would be masked in the combined sample, producing an intermediate ratio which is unrepresentative of either treatment regime. It can be safely concluded however, that at least one, and possibly both of

these treatment series produced a ratio of males significantly greater than the expected ratio. There is no apparent reason why the fish treated over the course of weeks 1 to 3 would yield a lower male ratio than those treated over weeks 2 to 4. As the initial immersion in this treatment series occurred during the week of maximum sensitivity, and there is strong evidence that dosage is more critical than immersion duration for the induction of masculinization, a larger number of males would have been expected in this group. The group treated during weeks 2 to 4, a time period when androgen sensitivity was on the wane, showed an elevated masculinization ratio. It can only be concluded that one or both of these groups likely represents a skewed, or otherwise unrepresentative sample, and therefore do not clearly indicate the effects of the MDHT immersions.

While the results obtained in the feminization triple immersion series did not produce the higher ratios of females, as had been expected at the outset of the experiments, the trend toward increasing rates of feminization in the later treatments lends added support for the data obtained in the weekly treatment series. The highest rate of feminization occurred in the group treated over weeks 2 to 4 which encompasses precisely the period previously identified as optimal for estrogen treatment. The ratios obtained in the triple immersion series are slightly lower than in the corresponding weekly treatments. However, the individual immersion durations, which appear to be the critical factor in feminization procedures, were only half of those in the weekly treatment series. The results obtained in the feminization multiple immersion series provide support for the evidence shown earlier that optimum feminization in the Arctic char occurs in response to extended immersions at a moderate dosage level when treatment is completed 3 weeks post-hatch. In other multiple immersion feminization procedures utilizing salmonids, the results have indicated that multiple immersions do not

produce sex ratios which exceed those of single immersions of equal duration of estrogen exposure. In a study by Hunter et al. (1986) chinook salmon were given double estradiol-17β immersions of 2 hours each, at a dosage of 0.4 mg/l at 2 and 9 days post-hatch, which resulted in an 82% female population. In a subsequent study utilizing the same species, steroid and dosage, a single 4 hour immersion yielded approximately 85% females (Piferrer & Donaldson, 1992).

In this investigation the total exposure to the steroids for groups of the triple immersion series was 1.5 times that of the weekly immersion series. This increased exposure did not increase the rates of either masculinization or feminization over those of the weekly single immersion treatments. These results concur with the results of the other treatment regimes which have shown that for masculinization, dosage level is the critical factor(Piferrer & Donaldson, 1991; Piferrer et al. 1993), whereas for feminization procedures immersion duration appears to be more critical for high success rates (Nakamura, 1984; Hunter et al.,1986). It is interesting to note that the feminization group treated over the course of weeks 0 to 2 and weeks 1 to 3, which underwent total EE_2 exposures of 18 hours at 1.0mg/l produced female ratios intermediate to those given single immersions at equivalent dosages for 12 and 24 hours. The fact that intermittent estrogen exposure produced results which correlate with those of a continuous exposure raises the question of whether the estrogen exposure period in feminization treatments needs to be continuous if high rates of feminization are desired.

FEED TREATMENTS:

A combination of technical factors make it difficult to pinpoint the reasons behind the mortalities which arose in the feed only and immersion-feed treatment groups. None of the four groups which received steroid-treated feed fed readily, and remained as "pinheads". This was not the case in the immersion only groups which received untreated feed and rose to the surface feeding readily whenever feed was introduced. It is believed that the mortalities in the feed treatment groups arose from problems of hypoxia which was due in part to flow restriction brought about and/or aggravated by the presence of unconsumed food particles in the water. The feed treatment groups were maintained in separate holding tanks from both the immersion- treated and control groups as well as the groups treated with the other sex steroid. For this reason problems within the water supply alone cannot be ruled out as factors contributing to the hypoxias; however, it seems unlikely that water supplies in the hatchery would contain chronically low levels of dissolved oxygen and not be noticed, or affect fish in other tanks. The reasons behind the unpalatibility of the feed are not known. It is difficult to accept the idea that feed treated with two separate steroids would be equally unacceptable to the fish, but again this possibility cannot be eliminated. In other feed experiments of this type the use of ethanol has not proven to cause unpalatability of the feed (Guerrero, 1975; Goudie et al. 1983; Varadaraj, 1989). There are currently no reports in the literature of the feeding of either MDHT or EE_2 for the purposes of sex control. Because of this unusual set of circumstances these feed treatment experiments should be repeated before any conclusions are drawn regarding the effects of feeding of these two steroids.

CONCLUSIONS

Analysis of the data obtained in these procedures allows a number of significant conclusions to be drawn regarding the development of reliable sex control protocols through immersion therapy for the Arctic char at ambient water temperatures in the range of 2-3 °C.

The sex ratios of the control groups have shown that ethanol immersions at the concentrations used to dissolve the steroids used in these procedures did not significantly alter the expected sex ratios. The amalgamated sample of all control fish used in these procedures (n = 417) shows an expected male:female sex ratio of 55:45 for the Nauyuk strain of Arctic char.

Optimum treatment timing for masculinization and feminization procedures is not the same. The largest proportion of masculinizations occur when alevins are immersed one week post-hatch (466°C-days) whereas the highest rates of feminizations are attained when immersions are completed 3 weeks post-hatch (508 °C-days).

There is some evidence from the procedures carried out here that the duration of immersion treatments may play a more critical role in attaining high rates of feminization relative to masculinization.These procedures showed a higher degree of positive correlation between the duration of EE_2 immersion and the numbers of fish with ovarian characteristics, than occurred in the masculinization series. It is believed that this occurs because of lower rates of estrogen uptake and time to saturation, which may be caused by either cold water temperatures or a species effect.

The results of the masculinization procedures carried out here have clearly illustrated that 100% masculinization of the Arctic char is attainable, when an immersion dosage of MDHT in the range of 10.0 mg/l is employed for a 24 hour duration. In contrast to the results obtained in the feminization trials, it was the dosage of MDHT which proved to be the critical

factor in achieving an all-male population. The hypothesis is put forward here that this effect occurs because there is an accumulation of steroid in the yolk and lipid portion of the body which increases the total exposure to the steroid. There may also be a minimum dosage requirement to achieve a high rate of masculinization.

Triple immersions do not appear to be an exceptionally efficient method of steroid application for the purposes of either masculinization or feminization. The sex ratios produced as a result of triple immersions were, for the most part, within the range which would be expected for a single immersion carried out at the same time during physiological development, dosage and duration. There does not appear to be any inductive effect from multiple immersions. It is possible that in the case of feminization procedures, total exposure to the steroid does not have to be continuous, but may be carried out over the course of several shorter immersions spanning the labile period, with similar sex ratios resulting.

It appears likely that ambient water temperature has an effect on the outcome of sex control procedures. The dosages and durations of the immersions carried out in these procedures equal or exceed those which have been reported in the literature as having elicited 100% masulinizaton and feminization in Pacific salmon. While an interspecies difference in steroidal effects cannot be ruled out it seems the less plausible of the two explanations.

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