Hormonal sex reversal of rainbow trout *(Oncorhynchus mykiss)* by ethynylestradiol-17α (EE_2)

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Abstract

Rainbow trout (*Oncorhynchus mykiss*) females grow larger and mature later than males, making all-female stocks economically advantageous. The objective of this research was to develop methods for the production of monosex populations of trout through the use of sex steroids. The synthetic estrogen ethynylestradiol-17α (EE_2) was administered in single period-immersion treatment of 400 µg/l for 1, 2, 4 and 8 h to groups of newly-hatched sac fries of rainbow trout and in a 30 day dietary treatment of 5, 10, 15 and 20 mg/kgf of newly swum up fries. 73.4 And 94.5% females were obtained with 1 and 2-h single-immersion of EE_2 respectively (38% female in control). However, higher doses progressively reduced the survival drastically. Sex ratios of dietary treatment of fry were 60, 57.4, 78 and 94% females respectively. Treatments also resulted in a slight increase of both weight and length. This increase was related to the method of hormonal exposure (dietary > immersion), but not dosage-dependent for utilized hormone. This demonstrates that the direct feminization of rainbow trout can be achieved and resulted in sex reversal ratio effectively but not decreased the growth, as observed in hormonal sex reversed females, and it would be a noticeable approach to the direct feminization of trout.

Keywords: Ethynylestradiol-17α, Rainbow trout, Immersion, Dietary

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Introduction

Some of the most important problems in fish farming that inevitably appear at the time of fish sexual maturation are decrease or complete cessation of growth, a decline in flesh quality, and an increase in mortality, especially in salmonid males (Devlin and Nagahama, 2002). Rainbow trout, *Oncorhynchus mykiss*, females grow faster, and their sexual maturation occurs later, than males (Piferrer, 2001), a characteristic also seen in other species of this family such as coho salmon, *Oncorhynchus kisutch* (Piferrer and Donaldson, 1994), and Chinook salmon, *Oncorhynchus tshawytcha* (Donaldson, 1986; Solar et al., 1987).

Production of all-female stocks can be achieved by direct or indirect feminization. Direct feminization involves the application of estrogens to sexually undifferentiated larvae and differentiating them to female, while the indirect method uses androgens to masculinize genotypic females, which are subsequently used in breeding programs to yield all-female populations (Hendry et al., 2003). In species, having females as the homogametic sex (XX genotype), sperm from masculinized females (i.e., XX-males) can be used to fertilize eggs from normal females to yield all-female second-generation offspring. However this indirect method is preferred because fish destined for market are not directly exposed to steroids (Tabata, 1991; Aida and Arai, 1998), but the indirect method, requires more than one generation for the production of all-female populations (Piferrer, 2001).

Several methods are currently available for fish hormonal treatment. Crim (1985) classified these methods as acute and chronic. The two methods currently feasible for utilization of sex hormones to large numbers of fish are using supplemented food as dietary treatment and utilization of static tanks or recirculating bath as immersion treatment, for chronic and acute administration of hormones, respectively (Piferrer, 2001).

The production of all-female stocks is now firmly designed for two types of direct and indirect production systems (Piferrer and Donaldson, 1992). Both of them involve application of producing and marketing of all-female stocks as it is practiced in the culture of Chinook salmon, *Oncorhynchus tshawytscha* (Donaldson, 1986; Solar et al., 1987) and rainbow trout, *O .mykiss* (Bye and Lincoln, 1986). The preferred method of producing monosex female population for either purpose is using the indirect method due to the use of monosex female spermatozoa to fertilize normal ova which were described elaborately in Piferrer (2001). Development of DNA sex-specific probes is a shortcut in the programs of all-female stocks production by the indirect method that make it possible to identify the genetic sex in some species. These probes, initially developed for Chinook salmon, have also been applied to coho, chum (*O. keta*) and pink salmon (*O. gorbuscha*) (Funk et al., 1973). However, the existing probes cannot identify the genetic sex of other salmonids such as the Atlantic salmon or the rainbow trout (Piferrer, 2001). Although, most studies on direct feminization of fish, particularly salmonid, have focused on using of the natural estrogen estradiol-l7ß (E_2) (Hunter et al.,

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1983; Piferrer, 1990; Piferrer and Donaldson 1992), but using a potent estradiol helps us to design a method of feminization with the lowest dosage and duration for producing a healthy marketing fish.

Ethynylestradiol-17α (EE_2) is a synthetic derivative of E_2 that has clinically important applications because of its high ovulation-inhibiting potency. Ethynylestradiol-17α has been used to regulate the menstrual bleeding patterns of millions of women (Piferrer and Donaldson 1992). In teleosts as a whole, the comparison of the estrogenic potency of EE_2 against other synthetic and natural estrogens has been carried out mainly in cichlids and cyprinodontids (Hunter et al., 1983), and the total numbers of references on this subject are few and none refer to salmonid and trout. Accordingly, the best comparisons of such relative potencies are dependent with review articles of Yamamoto and Piferrer (Yamamoto, 1969; Piferrer, 2001). Piferrer realized that, EE_2 was about 3 times more potent than E_2 and Yamamoto reported that EE_2 3.5 folds more potent than E_2 in medaka at the GD_{50} dosage level (dosage at which 50% of the genotypic males become phenotypic females) (Piferrer and Donaldson, 1992). Since steroids have been shown to affect fish growth and survival (Pandian and Sheela, 1995; Piferrer, 2001), the objective of this research was to determine the effects of EE_2 administration on development, growth and survival of Rainbow trout.

Materials and methods

Fish samples

The eggs of Rainbow trout (*Oncorhynchus mykiss*) fertilized on March 17th 2006 were prepared from a private farm (ZarQezel trout Farm. Ltd.) and they were transported to the farm laboratory as eyed eggs on April 9th 2006. Before hatching, eyed eggs were divided into ten groups containing 100 eggs each and were acclimated, over a period of 2 days (Piferrer and Donaldson, 1992). This time the laboratory water temperature was 10 $\pm 1^{\circ}C$. Each group of eggs was placed in a plexiglass chamber. four groups are prepared for single immersion, four groups served for dietary treatment and the two others were control groups. These chambers were placed in Heath tray incubators that comprised four chambers in each tray. By April 17th (31 DPF) 50% hatching had occurred and the remaining eyed eggs hatched out 2 days later.

Immerse treatments

Firstly Ethynylestradiol-17α (EE_2) (Iran Hormone Co., Ltd.,) was dissolved in ethanol at a concentration of 1 mg/ml. For estrogen treatments, 4 ml of ethanolic solution was added to 10 liters of water to obtain EE_2 at a final concentration of 400 µg/liter (Piferrer and Donaldson, 1992). Fish were treated with a single-immersion treatment around the time of sexual differentiation of their gonads (Piferrer and Donaldson, 1992). Four groups of newly hatched larvae were single-immersed for 1, 2, 4 and 8 hours in the bath described above 1 day after median hatch (50% of fish hatched out). Further details of this type of hormone administration are described elsewhere (Piferrer and Donaldson, 1989). Prior to swim-up, larvae were transported to the ZarQezel Fish Hatchery, they were kept in 70 liter fiberglass tanks supplied with well water,

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and reared using standard salmonid aquaculture procedure. Fish were fed with pellets with the appropriate size (Biomar Ltd.,) five times a day. The one remaining group served as control and was immersed only with ethanol.

Dietary treatments

Four of ten groups were transported to the hatchery, meanwhile 50% of larvae were swimming-up on April 29_{th} 2006 (42 DPF) and they were rare in a tray (80×18×10) up to yolk-sac absorption. They were then placed in 500-l fiberglass tanks.

Four dietary groups were fed a formulated diet supplemented with EE_2 at a concentration of 5, 10, 15 and 20 mg/kgf, respectively (Jhonstone et al., 1979). The duration of the treatment was 30 days (which started 3 days after median swim-up). In this stage fish were treated during the sex differentiation, as demonstrated by Piferrer (2001). The one remaining group served as control and was fed a diet supplemented only with ethanol.

To incorporate steroids into manufactured feed, a general method of spraying ethanol-dissolved steroids on to the food, was commonly used for salmonid sex reversal (Feist et al., 1996). However, this proved impractical because of the small size of the diet, and therefore a method of saturation was used (Carl Schreck, Oregon State University, personal communication, 1999).

Treatments began on 1st of May 2006 (Day 0). Fish were fed by hand until satiation seven times a day at 7, 9, 11:30, 14, 16:30, 18 and 19 o'clock, throughout the experiment. Oxygen levels and temperature were measured regularly, and ranged from 9.5 to 12.3 mg/l and 10.3 to 13.1℃, respectively. Total lengths (±0.5 mm) and wet weights (±0.01 g) were assessed, during the experiment. Steroid treatments were finished after 30 days and fish were measured again. Then, the fish were fed on a steroid-free diet and similar measurements (e.g. length, weight and survival rate) were done at different times.

Sampling and histological procedure

Sampling took place on the 1st of October 2006, 5 months after the first feeding (Piferrer and Donaldson, 1992). During 5 months, gonads developed sufficiently, for histological observation and hormonal effects, since once the phenotype is well established it remains during the adult phase of the life cycle, as demonstrated by Johnstone et al. (1989), Chevassus et al. (1984) and Solar et al. (1984) in rainbow trout, and by Hunter and Donaldson (1983) in Pacific salmon (Piferrer and Donaldson, 1992). Fifty fish were randomly taken from each group and morphologic changes were examined due to the hormone treatment. Fish were killed with lethal doses of 2-phenoxyethanol and gonads were collected to determine sex ratios (Piferrer and Donaldson, 1992). To assess the effect of treatments on the sex reversal of the gonad, cross-sections were taken through the whole fish (Piferrer and Donaldson, 1992). Sections of fish were dissected and immediately placed in 10% neutral buffered formalin. After fixation for 48 h, pieces of gonadal tissues were prepared under a dissecting microscope and provided for histological analyses. (Hendry et al., 2003).

Histological sections were obtained using usual procedures (Presnell and Schreibman, 1997). Tissues were dehydrated in an ethanol series, depending on size (Hinton, 1990), cleared and

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infiltrated with toluene, and embedded with ParaPlast X-tra (m.p. 52 ℃) using a histomatic tissue processor (Fisher Scientific). Serial cross-sections were obtained on a microtome at a thickness of 6 µm, using disposable microtome blades, mounted on dH2O-covered ‘‘subbed’’ slides (Presnell and Schreibman, 1997) and dried on a slide warmer. After drying, sections were stained using Instant Haematoxylin, enhanced using ammonia water/acid alcohol (Presnell and Schreibman, 1997) and counterstained using eosin Y. Cover slips were mounted using Eukitt mounting medium (Hendry et al., 2003).

Statistical analyses

Analysis for alterations in the sex ratio was performed with the Chi-square test without Yate’s correction for continuity (Piferrer and Donaldson, 1992), although for conservative approach, the number of intersex fish was combined with that of males, but in any sample the intersex fish that were comprised of spermatogonia and contained oocytes were not considered. One-way analyses of variance (ANOVA) were performed on growth data.

Results

Analysis of data in each experimental group showed that Ethynylestradiol-17α affected the sex ratios significantly, since 1-h immersion of sac fries with concentration of 400 µg/l EE_2 resulted in 73.4% females, and redoubling the time of treatment to 2 hours, produced 94.5% females. Addition of immersion timing increased mortality up to 100% (e.g. in 8 hours). A 30 day period treatment with supplemented diet increased feminization of treated fish with 15 and 20 mg EE_2/kgf significantly. EE_2 treatments of 5, 10, 15 and 20 mg EE_2/kgf increased the sex ratio up to 60, 57.4, 78 and 94% female, respectively (Fig.1). Sex ratio in control groups was 57% male and 43% female. The histological analysis of the fish gonads from the control and the treated groups is presented in Fig. 2.

Estrogen-treated fish were bigger than control groups, although the growth increase rate was not significant in some groups compared to control. However, 1-h immersion treatment of sac fries with 400 µg/l didn't yield the most female (Fig. 2), but achieved the highest growth and 15 mg/kgf treated fish also gained the most growth in dietary treatment groups (Fig. 3). Survival rate of fish, which immersed in 1 and 2 hours didn’t affect significantly (Table 1), whereas 4 and 8 hours of EE_2 immersion seemed to be a lethal time for rainbow trout (Table 2).

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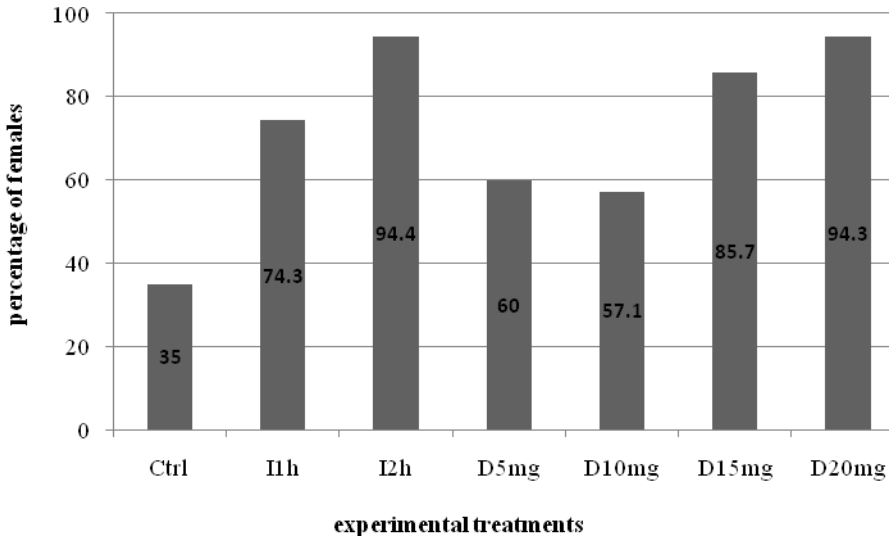


Figure1: The feminization rate (%) in immersion and dietary treatments
h = hour (s)
I = immersion
D = dietary

Table 1: Cumulative mortalities (%) of Rainbow trout up to 75 days after immersion with 400 µg/l Ethynylestradiol-17α

Day	Date	Ctrl	1h*	2h
4h	8h			
15	May 02 , 2006	33	47	59
450	735			
30	May 17 , 2006	40	50	64
890	995			
45	June 01 , 2006	45	54	70
1000	-			
60	June 15 , 2006	48	60	73
-	-			
75	June 30 , 2006	40	65	75
-	-			

(Ctrl = Control)
(* = duration of immersion in hour)

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Table 2: Cumulative mortalities (%) of Rainbow trout up to 75 days after the start of a 30 day dietary treatment with EE_2

Day	Date	Ctrl	5mg*	10mg	15mg	20mg
15	May 14 , 2006	30	25	33	43	48
30	May 29 , 2006	36	30	45	54	58
45	June 13 , 2006	40	38	49	60	64
60	June 28 , 2006	46	44	54	65	70
75	June 13 , 2006	51	47	59	67	73

(* = dosage of dietary treatment per kg feed)

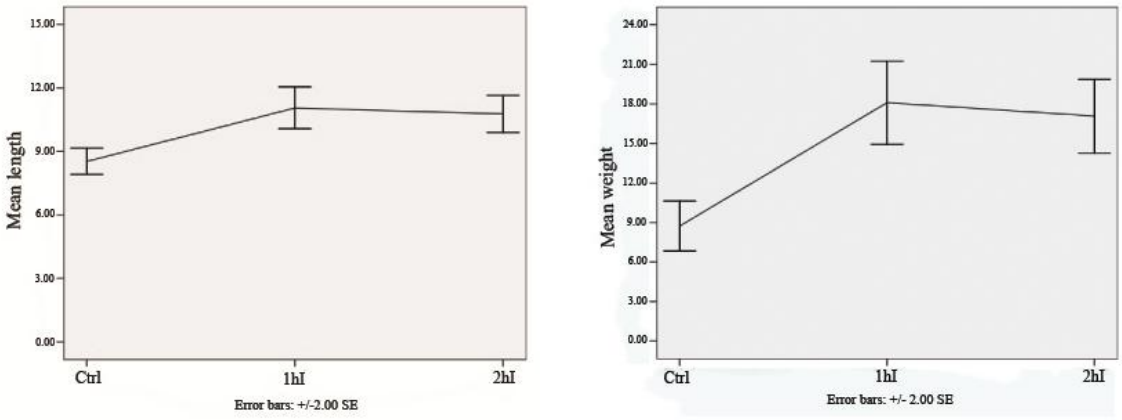


Figure 2: Growth rate in weight and length of rainbow trout treated with immersion of ethynylestradiol-17α

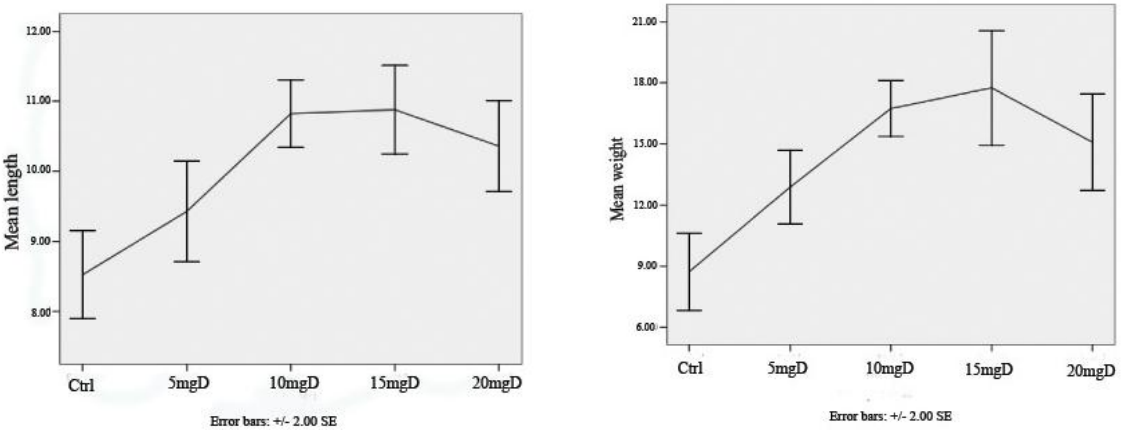


Figure 3: Growth rate in weight and length of rainbow trout treated with diet containing ethynylestradiol-17α

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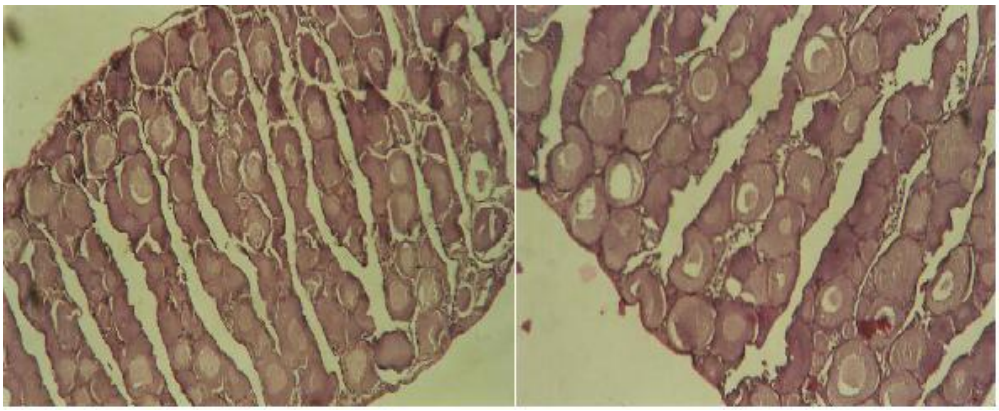


Figure 4: Effects of ethynylestradiol-17α treatment on the gonadal development of Rainbow trout after 200 days. (A) Treated with 2 hours immersion of 400 µg/l, (B) fed with 20mg/kg for a month

Discussion

The results of the present study showed that using ethynylestradiol- 17 α has a positive effect on sex reversal of rainbow trout. The synthetic estrogen 17α-ethynyloestradiol (EE_2) has been used in a variety of species and is among the most potent feminizing agents tested so far (Piferrer, 2001). Feminizing estrogens potencies vary among species and depend on the stage of gonadal development. Rosenstein and Hulata (1994) mentioned Diethylstilbestrol (DES) more potent than EE_2 in sex reversing of *Oreochromis aureus,* but Gilling et al. (1996) found the opposite in feminization report of *Oreochromis niloticus.* However, considering a sufficient number of trials and species, EE_2 is, on average, about three times more potent than E_2 in feminizing fish (Piferrer and Donaldson, 1989). Farmers prefer shorter treatment duration at relatively higher doses (if proven that they do not result in undesired deleterious side effects), rather than comparatively longer treatments at lower doses, since this allows reduction of the work of preparing treated diets or immersion baths (Piferrer, 2001).This research has shown us the importance of treatment timing. Sexually undifferentiated gonads in fish are much more sensitive than differentiated ones, and hormonal treatment is more effective when the gonads are undifferentiated yet (Piferrer, 2001). In the process of gonadal development, which period of time when the still sexually undifferentiated gonads are more responsive to the effects of exogenous steroids, known as the labile period (Piferrer, 2001). Piferrer (2001) has demonstrated the localization of labile period for several species, and described that the labile period of Rainbow trout is in the stages of swimming up, first feeding and yolk- sac absorption. This assay showed highly significant differences in the proportion of females with respect to the expected 1:1 sex ratio (Piferrer and Donaldson, 1992). The results showed that Ethynylestradiol-17 α has extreme potential for sex differentiation induction in rainbow trout, since a single 2-h immersion treatment with a concentration of 400 µg/liter EE_2 resulted in 94.5% females and a 30 day dietary treatment period with a concentration of 20 mg/kgf resulted in 94% females, furthermore growth has increased in some groups.

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However, longer immersion treatment durations resulted in a progressive decrease in the survival. This phenomenon may be due to a toxic effect of this hormone directly on the gonads or to a more generalized effect on the pituitary gonadal axis (Yamazaki, 1983). In this regard, however, Hopkins et al. (1979), showed that EE_2 was more potent than E_2 in induction female differentiation in tilapia *(Oreochromis aureus)*, when fish were treated with high dosages (25 to 200 mg/kg of diet) of both steroids for 5 and 8 weeks (Piferrer and Donaldson, 1992).

However growth rate of estrogen-treated fish is generally affective (Papoulias et al., 2000), and estrogen normally results in reduction of growth (Nagahama, 2000), but in this research, exposure of rainbow trout to EE_2 increased the growth rate. Enhancement of growth in feminized fish may be due to changes in growth hormone, insulin secretion and thyroxine hormones (Rao and Rao, 1983). Some studies have reported yielded growth after treatment with estrogens. Piferrer and Donaldson (1992) reported that, 2 hours immersion of Chinook salmon (*Oncorhynchus tshawytscha*) with EE_2 resulted in a slight increase of both weight and length (Piferrer and Donaldson, 1992). Similarly, Cowey et al. (1973) found that diethylstilbestrol stimulated the weight increase of plaice, (*Pleuronectes platessa*), and Malison et al. (1996) found that E_2 stimulated weight gain and food consumption but did not affect food conversion efficiency in the yellow perch (*Perca flavescens*). These authors also recognized sex-related dimorphic growth. In this regard, the enhanced growth of females in these species may be a consequence of the endogenous estrogens. Reduced growth has been reported in the Pacific salmon following treatment with E_2 (nakamura et al., 1998).

However, consumption of feminized trout treated with EE_2 (as a synthetic steroid) directly, is questionable for consumers, there is no risk, because the marketable fish consumes months or years after hormonal treatment. To put things into perspective, the intake of EE_2 for women who take the contraceptive pill is in the range of 30–50 µg a day (Anonymous, 1992). In order to ingest the same amount of EE_2 one should eat tens or hundreds of kilograms of feminized fish every day (Piferrer, 2001). It seems the main reason of high mortality in steroid-treated fish (especially estrogens) is related to the toxicological characteristics of estrogens and occurrence of disruption in hormonal axis (Folmar et al., 2000; kime, 1998). Although this research showed both methods (immersion and dietary treatment) are effective for inducing feminization of trout in particular concentrations of EE_2, analysations of side effects, growth and survival rate of treatments suggested that a dietary method of hormonal exposure with dosage of 20 mg/kgf of EE_2 is preferred, since monosex milt is not available for all of the hatcheries. The present method, due to its simplicity and effectiveness offers potential for the efficient direct feminization of trout.

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