Aquat. Living Resour., 1994, **7**, 127-131

Production of female triploid coho salmon (*Oncorhynchus kisutch*) by pressure shock and direct estrogen treatment

Fancesc Piferrer [1*], Tillmann J. Benfey [2] and Edward M. Donaldson [3]

[1*] Address of correspondence: *University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0947, USA.*
[2] *Department of Biology, University of New Brunswick, bag service number 45111, Fredericton, New Brunswick, E3B 6E1 Canada.*
[3] *Dept. of Fisheries and Oceans, 4160 Marine Drive, W. Vancouver B. C., V7V 1N6 Canada.*

Received August 10, 1993; accepted January 4, 1994.

Piferrer F., T. J. Benfey, E. M. Donaldson. *Aquat. Living Resour.*, 1994, **7**, 127-131.

Abstract

The aquaculture production of all-female triploid (sterile) populations is recognized as being of potential advantage for many species of fish where sexual maturation is not desired. All-female triploids have been produced by inducing triploidy in eggs that had been fertilized with monosex female sperm. However, monosex stocks to produce this type of sperm are currently available only for a limited number of economically important species. To circumvent this problem, an alternative method would be the direct feminization of tripoid embryos. In Pacific salmon, pressure shocks applied shortly after fertilization have consistently resulted in high yields of tripoids, and feminization by direct estrogen treatment has also been shown to be highly effective if applied shortly after hatching. In this study, coho salmon eggs were made triploid by a pressure shock of 633 kg/cm^2 for 4 min, applied 20 min after fertilization and incubation at 10 °C. Some of the resulting embryos were then feminized with estradiol-17β in single 2-hour immersion treatments at 400 μg/liter, administered 1 and 8 days after hatching. Six months later, when the fish were juveniles, analysis of the DNA content of erythrocytes by flow cytometry revealed a 100 % induction of triploidy, while histological examination of the gonads showed 82 % females, which were completely devoid of oocyte development and thus genetically sterile. These manipulations reduced survival during early stages of development but survival became stable later. Together, these results suggest that the production of sterile fish by direct feminization of induced triploids could be of particular advantage for those species in which all-female stocks are yet not available or difficult to obtain.

Keywords: Sterilization, triploidy, feminization, sex ratio, survival, coho salmon.

Production de femelles triploïdes de saumon coho (Oncorhynchus kisutch) *au moyen de chocs de pression et de traitement direct d'estrogène*

Résumé

La production aquacole de populations monosexes femelles et triploïdes (stériles) est reconnue comme étant un avantage potentiel pour de nombreuses espèces où la maturation sexuelle n'est pas désirée. Les triploïdes monosexes femelles étaient produites en induisant la triploïdie dans les oeufs qui avaient été fertilisés avec du sperme de femelles monosexes. Cependant, des stocks de monosexes pour produire ce type de sperme ne sont disponibles que pour un nombre limité d'espèces importantes économiquement. La féminisation d'embryons triploïdes permettrait de résoudre cette question. Chez le saumon du Pacifique, des chocs de pression appliqués peu après la fertilisation donnent des résultats satisfaisants pour des rendements importants de triploïdes; la féminisation par traitement aux estrogènes est également très efficace s'il est appliqué dès l'éclosion. Dans cette étude, les oeufs de saumon coho sont devenus triploïdes par un choc de pression de 633 kg/cm^2 pendant 4 min, appliqué 20 min après la fertilisation et l'incubation à 10 °C. Certains embryons ainsi obtenus ont ensuite été féminisés par traitement à l'estradiol-17β à raison d'une seule immersion dans une solution à 400 μg/litre, administré 1 et 8 jours après l'éclosion. Six mois

plus tard, chez les juvéniles, l'analyse de l'ADN des érythrocytes par cytométrie de flux révèle 100% de triploïde induite, tandis que l'examen cytologique des gonades montre 82% de femelles, mais sans développement ovocytaire et donc génétiquement stériles. Ces manipulations réduisent le taux de survie durant les premiers stages de développement qui devient stable plus tard.

Mots-clés : Stérilisation, triploïdie, féminisation, sexe-ratio, survie, saumon coho.

INTRODUCTION

From the perspective of fish farming, problems that inevitably appear when fish reach sexual maturation include a decrease or complete cessation of growth, a decline in flesh quality, and an increase in mortality, especially in salmonid males reared in seawater (Donaldson, 1986). These problems can be circumvented by the use of sterile fish, since such fish never mature and thus can be marketed at the producer's convenience (Donaldson and Benfey, 1987).

There are several potential methods for the sterilization of fish (Donaldson *et al.*, 1993), including the production of triploids (Chourrout, 1984). Induced triploidy in fish that are normally diploids causes genetic sterility due to the blockage of meiosis. However, triploid males still develop large gonads due to the great number of mitotic divisions of pre-meiotic cells in the testes. In fact, triploid males, despite being genetically sterile, still mature morphologically and endocrinologically (Benfey *et al.*, 1989) and produce aneuploid sperm (Benfey *et al.*, 1986). The solution, therefore, is to produce all-female triploids, which can be accomplished in two ways: the direct method consists of feminizing triploid embryos, whereas the indirect method involves inducing triploidy in eggs fertilized with milt produced by genetic females sex-reversed into phenotypic males (Chevassus *et al.*, 1984). However, the production and identification of genetic female-phenotypic male salmon requires either two generations of selection and progeny testing, which has only been achieved for a few economically important species, or the use of a Y-chromosome specific DNA probe (Devlin *et al.*, 1991). In the rainbow trout (*Oncorhynchus mykiss*), for example, sterile stocks have been produced by the indirect method, by using either heat shock (Lincoln and Scott, 1983; Bye and Lincoln, 1986) or pressure shock (Okada, 1985) to induce triploidy.

In Pacific salmon, induced triploidy was first achieved by Utter *et al.* (1983). Since then, several studies have followed on different aspects of the production and biology of triploids of the genus *Oncorhynchus* (reviewed by Benfey and Donaldson, 1988). Surprisingly, despite the economic potential of triploid Pacific salmon, there are no reports available on the production of sterile Pacific salmon by means of all-female triploid fish, by either the direct or by the indirect methods outlined above. In order to facilitate the examination of female triploid coho salmon as a production option, it has, in the absence of monosex female coho salmon sperm, been necessary to utilize direct feminization. This study, therefore, examines the feasibility of the production of sterile coho salmon by the feminization of triploid embryos.

MATERIALS AND METHODS

Gamete collection and triploidy induction

Coho salmon (*Oncorhynchus kisutch*) eggs and sperm from nine adults were obtained through the facilities of the Chilliwack River Hatchery (Chilliwak, B.C.) and transported to the West Vancouver Laboratory. After fertilization, the eggs were divided into two groups: one served as the diploid control and in the other triploidy was induced by subjecting eggs to a pressure shock of 633 kg/cm^2 for 4 min, applied 20 min after fertilization and incubation at 10°C. The equipment employed for applying the pressure shock has been described elsewhere (Benfey *et al.*, 1988). Fertilized eggs were placed in small plexiglass chambers and reared in vertically-stacked incubators supplied with flow-through well water at a constant temperature of 10±1°C.

Estrogen treatments

Triploids were further subdivided into two groups: one was the untreated triploid control and the other was treated with estrogen to obtain females. First, estradiol-17β (E_2) (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol at a concentration of 1 mg/ml. For estrogen treatments, 2 ml of the ethanolic solution were added to 5 liters of water to obtain E_2 at a final concentration of 400 μg/liter. Newly hatched alevins were immersed for 2 hours in the bath described above 1 day after median hatch (50% of fish hatched out), followed by a second immersion applied 8 days after median hatch. Further details of this type of hormone administration are described elsewhere (Piferrer and Donaldson, 1989). Prior to swim-up, alevins were transported to the Rosewall Creek Experimental Fish Hatchery (Fanny Bay, B. C.), where they were kept in 50 liter fiberglass tanks supplied with well water, and reared using standard salmonid aquaculture procedures. Fish were fed with pellets of the appropriate size (Whitecrest Ltd., Nanaimo, B. C.) four times a day.

Sampling and histological procedures

Survival was determined in each group at 24 hours after fertilization, at the eyed-egg stage (22 days post-fertilization, DPF), at hatch (44 DPF), at first feeding (61 DPF) and at 200 DPF. At this time, a sample of about 50 fish was randomly taken for each group. Fish were killed in water containing a lethal dose of 2-phenoxyethanol (Syndel Ltd., Vancouver, B. C.), blood was obtained from the caudal vasculature for ploidy determination, and gonads were collected to determine sex ratios. Ploidy was determined by measuring the DNA content by flow cytometry of erythrocytes stained with propidium iodide (Sigma), using a Coulter EPICS V system (Coulter Electronics Inc., Hialeah, FL) at the Cancer Control Agency of British Columbia (Vancouver, B.C.). For gonadal histology, cross-sections were obtained from a point just behind the pelvic fins, fixed, dehydrated and embedded in paraplast using standard techniques for paraffin embedded tissue (Gabe, 1968). Gonadal sections of each fish were examined in duplicate at two different points along the cranial- caudal axis. Analysis to compare the survival of the triploids versus that of the diploids and for deviations in the sex ratio was performed with the Chi- square test (Sokal and Rohlf, 1981).

RESULTS

Survival was higher in diploids than in triploids and statistically significant ($p<0.001$) differences appeared by the eyed-egg stage at 22 DPF (*fig.* 1). However, there was no difference in survival between the estrogen treated and untreated triploid groups. Compared to that of diploids, the survival of triploids decreased especially during yolk absorption, between hatching and first feeding. Once these differences in survival had been established, any further decrease in survival of triploids was slight as compared to that of diploids.

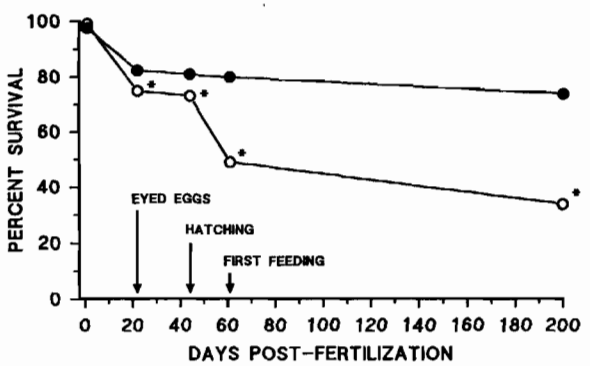

Figure 1. – Survival of diploid (•) and triploid (o) coho salmon at different stages of development, expressed in days post-fertilization. Estrogen treatment did not affect survival of triploids, therefore survival data for triploids includes both untreated and estrogen-treated fish. * denotes statistically significant differences ($p<0.001$) in survival of triploids when compared to that of diploid controls in a Chi-square test.

The determination of ploidy by flow cytometry and the proportion of sexes after histological examination appear in *table* 1. As expected, fish not treated with pressure shock after fertilization (controls) were all found to be diploids. In contrast, 100% triploidy was achieved with pressure shock treatment of fertilized eggs. Triploid fish had an external appearance indistinguishable from that of diploids except that some presented incomplete yolk absorption and died. Hormonal treatments did not produce any change in external appearance. During sampling at 200 days post-fertilization no abnormal fish were observed in any group.

Table 1. – Determination of ploidy and sex ratios of coho salmon after combined application of pressure shock to induce triploidy and direct estrogen treatment to induce feminization.

Group	Ploidy (N)	Sex-ratios			
		N	% M	% F	D
Diploids	100 % 2n (10)	52	40.4	59.6	NS
Triploids	100 % 3n (10)	51	54.9	45.1	NS
Feminized Triploids	100 % 3n (10)	61	18.0	82.0	$p<0.001$

Abbreviations: N = sample size, M = males, F = females, D = Significance of difference with respect to the 1:1 sex ratio, 2n = diploids, 3n = triploids, NS = not significant.

Control diploids and triploids that had not been hormonally treated had a sex ratio not significantly different from the expected 1:1 in a Chi-square test (*table* 1). Treatment of triploids with E_2 significantly ($p<0.001$) increased the proportion of females to 82%. At the age examined, male triploids had testes of normal appearance both in size and shape and were packed with spermatogonia. In contrast, female triploids presented ovaries of reduced size compared to their corresponding control diploids. Furthermore, the germ cells observed were oogonia blocked in their first meiotic division. Thus no oocytes were observed in any of the triploid females analyzed, neither from the untreated triploids nor the estrogen- treated triploids.

DISCUSSION

In this study we show the feasibility of combining the induction of triploidy with direct feminization as a means to produce sterile coho salmon. These procedures provided 100% triploidy and 82% females, and therefore the yield of steriles was 82% in the surviving fish. The sex ratio of triploids not treated with E_2 was not significantly different from 1:1, denoting that XXX and XXY individuals are equally viable, but in this study the survival of triploids

in general was lower than expected, according to our previous experience in coho salmon. In practice, the 100% tripoidy achieved is of importance, even if some mortality occurs since, from the economic standpoint, mortality early in the production cycle is less important than later mortality (Sutterlin *et al.*, 1987). With the 1 liter capacity device used in this experiment, 250,000 coho salmon eggs can be treated daily (Benfey *et al.*, 1988), and with larger motorized pressure shockers still greater numbers of triploids can be produced. Further, pressure shocks have provided more consistent results, with a higher triploid yield, than heat shocks, which are characterized by having a narrower window of effectiveness and of exhibiting higher interindividual variability (Johnstone, 1993).

The approach used here permits the production of female triploid fish in a single generation, but requires the application of estrogens to fish that will be marketed for human consumption. However, sex steroids are cleared rapidly from fish tissues (Fagerlund and Dye, 1979). Furthermore, since this study was completed, we have optimized the direct feminization technique such that only one short immersion treatment of newly hatched alevins in a water bath containing estrogen is necessary. Thus, 100% females were obtained after a single 8-hour immersion treatment with E_2 at 400 μg/liter, or after a single 2-hour immersion treatment with the synthetic estrogen ethynylestradiol-17α at 400 μg/liter (Piferrer and Donaldson, 1992). In diploids of the same species, a single 2-hour immersion treatment with E_2 at 400 μg/liter resulted in 84% females (Piferrer and Donaldson, 1989). Our results with triploids, where two immersion treatments in E_2 resulted in 82% females, suggest that triploids are not particularly different from diploids regarding their sensitivity to exogenous steroids. Nevertheless, differences encountered between the feminization response obtained with diploids and triploids could reflect differences in development rates (Allendorf and Leary, 1984; Leary *et al.*, 1985; Happe *et al.*, 1988), presumably also affecting the rate of formation of the gonadal primordium. Feminization with only one or two immersion treatments requires precise timing of treatment. Therefore, it would be worthwhile to test whether patterns of sensitivity to exogenous steroids differ between diploid and triploid fish of the same species.

In conclusion, we have shown the feasibility of producing all-female triploids by the direct method, *i.e.*, by the feminization of 1:1 male:female triploids. As discussed above, it is possible to induce 100% triploidy and feminization in coho salmon with acceptable survival, thus providing yields of sterile fish similar to those obtained with the indirect method. However, the combination of genetic and endocrine manipulations used in this study would be of particular advantage in many species for which no all-female stocks are available and therefore the indirect method of producing all-female triploids cannot be employed.

Acknowledgements

Thanks are due to the staff of Rosewall Creek Experimental Fish Hatchery and to Andy Lamb at the West Vancouver Laboratory for rearing the fish; to Ian Baker and Igor Solar for their assistance during sampling, and to Gary de Jong for doing the flow cytometry.

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