Aquat. Living Resour., 1989, 2, 225-230

Effect of methyltestosterone on sex differentiation and gonad morphogenesis in rainbow trout *Oncorhynchus mykiss*

Michelle Cousin-Gerber [1], Georges Burger [2], Claude Boisseau [1] and Bernard Chevassus [2]

[1] *Laboratoire de Biologie de la Reproduction, Université de Rennes-I, 35042 Rennes Cedex, France.*
[2] *Laboratoire de Génétique des Poissons, INRA, 78350 Jouy-en-Josas, France.*

Received April 17, 1989; accepted July 21, 1989.

Cousin-Gerber M., G. Burger, C. Boisseau, B. Chevassus. *Aquat. Living Resour.*, 1989, 2, 225-230.

Abstract

Oncorhynchus mykiss alevins originating from a monosex female population were treated with methyltestosterone incorporated in the diet (0.5 or 3 mg/kg) for 60 or 90 days from the beginning of first feeding. At sexual maturity the percentage masculinization achieved was high (89-98%) whatever the concentration or duration of treatment. However, the hormone concentration was the major factor in determining the frequency of functional males obtained. Of the 2-year-old neomales produced by the treatment with 0.5 mg/kg diet for 60 days 82% were able to emit their sperm under abdominal pressure compared to only 36% of those produced with 3 mg for 60 days. The increase in the duration of treatment also resulted in a decrease in the number of functional males from 82% (0.5 mg/60 days) to 72.6% (0.5 mg/90 days) and from 35.6% (3 mg/60 days) to 19.2% (3 mg/90 days). It is therefore recommended to use a low dose of hormone (0.5 mg/kg) for only 60 days to get a high proportion of functional males.

Keywords : Salmonidae, hormone, sex differentiation.

Effet de la méthyltestostérone sur la différenciation sexuelle et sur la morphogenèse des gonades chez la truite arc-en-ciel Oncorhynchus mykiss.

Résumé

Des alevins de *Oncorhynchus mykiss*, issus d'une population monosexe femelle, sont traités par la méthyltestostérone incorporée dans l'aliment (0,5 ou 3 mg/kg) pendant 60 ou 90 jours à partir de la prise de nourriture. Les animaux sont élevés jusqu'à leur maturité sexuelle. Les taux de masculinisation de la gonade sont systématiquement élevés (89 à 98%) quelle que soit la dose ou la durée du traitement. Par contre, le facteur dose a une action prédominante sur le pourcentage de mâles fonctionnels obtenus. 82% des néomâles âgés de 2 ans, produits par le traitement de 0,5 mg pendant 60 jours, peuvent émettre leur sperme sous une pression abdominale, comparativement à seulement 36% de ceux produits avec 3 mg pendant 60 jours. L'augmentation de la durée du traitement se traduit également par une diminution, moins marquée, du nombre de mâles fonctionnels qui passe de 82% (0,5 mg/60 jours) à 72,6% (0,5 mg/90 jours) et de 35,6% (3 mg/60 jours) à 19,2% (3 mg/90 jours). Il est donc recommandé d'utiliser une faible dose d'hormone (0,5 mg/kg) pendant seulement 60 jours pour obtenir une proportion élevée de mâles fonctionnels.

Mots-clés : Salmonidés, contrôle hormonal du sexe, différenciation sexuelle.

INTRODUCTION

The fundamental research of Yamamoto (1953) carried out on medaka (*Oryzias latipes*) has led to many studies on sex reversal in several fish species including the salmonids.

Besides their fundamental interest for the study of sex differentiation and genetic sex determinism these studies have been applied several times on the short and long term in aquaculture and especially to produce monosex populations (Chevassus *et al.*, 1979), sterile fish (Lincoln and Scott, 1983) or self-fertilizable hermaphrodites (Chevassus *et al.*, 1988).

Among studies on masculinization induced by methyltestosterone, the first data on rainbow trout (*Salmo gairdneri*) were supplied by Jalabert *et al.* (1975), followed by several other studies that aimed

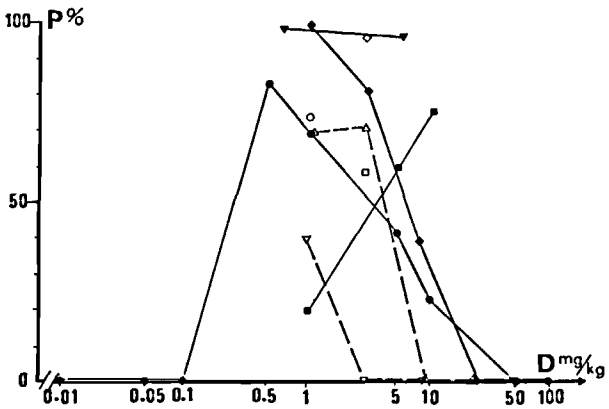

Figure 1. – Proportions (p%) of masculinization for different doses (D mg/kg) and durations of the methyltestosterone treatment (oral administration). In the case of bisexual populations, the proportion of masculinization is estimated by (2 × proportion of males − 1) %. From ● Okada *et al.*, 1981, 60 days; ■ Okada *et al.*, 1979, 58 days; Solar *et al.*, 1984, 60 days; △ Solar *et al.*, 1984, 90 days; ▽ Solar *et al.*, 1984, 120 days; ▼ Van den Hurk and Lambert, 1982, 58 days; Johnstone *et al.*, 1978 and 1979, 90 days; □ Johnstone *et al.*, 1978 and 1979, 30 days; ○ Yamazaki, 1976, 180 days.

at optimising the percentage of sex reversal (*fig.* 1). These studies determined the most effective dose (0.5 to 5 mg/kg diet) and treatment durations (50 to 60 days); higher doses or longer treatments lead to a significant increase in the proportion of sterile fish. However, the following two problems remained to be solved.

– Most of the studies were carried out on bisexual populations. The increase in the proportion of males due to the treatment may be the result either of a real effect on sex differentiation or of a possible difference in mortality between the two sexes. Thus, in the study of Johnstone *et al.* (1979), 98% were males, but one of the ten males tested showed to be a genetic female. The only study escaping this criticism is that of Okada *et al.* (1981 *in* Yamazaki, 1983) which showed a real sex reversal.

– Sex determination was generally carried out by histological examination of the gonads at an early stage. Several authors (Johnstone *et al.*, 1979; Bye and Lincoln, 1981) described frequent malformations of the genital ducts at a later stage (deferent ducts and urogenital papillae) likely to interfere with the emission of gonadal products, but no precise quantitative study of this phenomenon has been undertaken.

The purpose of our work was therefore to study the effect of masculinization treatments on genital duct morphogenesis and on the functional character of mature males obtained from monosex female populations.

MATERIAL AND METHODS

Rainbow trout (*Oncorhynchus mykiss*) alevins used in these experiments originated from a group of monosex females obtained by crossing two normal females (XX) with some ten neomales (also XX) originating from a previous experiment on sex reversal by methyltestosterone. Fertilization was carried out on December 3rd, 1984 according to usual fish farming procedures. Feeding of alevins started 66 days later, *i.e.* on February 8th, 1985.

Hormonal treatments

When active feeding behaviour appeared the alevins were fed 3 times daily *ad libitum*. Methyltestosterone (STELAROIDS) was added to the diet (TROUVIT) by ethanol impregnation of the pellets on the basis of 0.5 mg (groups "L", Light) or 3 mg (groups "H", High) of methyltestosterone per kilogram of feed. According to groups, the treatment was applied for a period of 60 or 90 days from the beginning of active feeding. The combination of these two factors led to the constitution of four experimental groups: L60, L90, H60 and H90.

At the same time 3 control groups were studied:

– one group of untreated monosex females, T1, placed downstream relative to groups L and H and thus receiving the catabolites of treated fish;

– one group of untreated monosex females, T2, placed upstream relative to treated groups bred in clean water;

– one bisexual group, T3, originating from a normal population. The experiments were conducted on the site of Gournay-sur-Aronde (the department of Oise, France). Taking into account the average water temperature (9.13°C during the first 60 days; 9.56°C during the 90 days of treatment), the degree day coefficients were 548°C days and 860°C days, respectively.

Examination of adult animals

Two series of anatomo-physiological examinations were carried out on treated animals and on the control group during the period of reproduction at the ages of 22 months (study α, 134 individuals) and 25 months (study β, 419 individuals), respectively.

Animals were electrically killed, weighed and opened ventrally for examination of the genital duct. When gonads had developed into testicles and deferent ducts seemed to be complete, the permeability of the genital papillae was tested by pressing the ducts manually. The tests carried out led us to distribute the animals into three categories:

1. functional males, in which a moderate pressure produced a flow of milt (or seminal liquid) and which exhibited at least one deferent duct associated with a permeable papilla;

2. non-functional males in which even a strong pressure on the ducts did not produce any emission of genital products or in which deferent ducts did not reach the papilla;

3. Females, Immature, Sterile, Hermaphrodites (FISH), a category grouping non-inverted animals (females), immature males, sterile individuals or hermaphrodites (ovarian nodules associated with more or less developed testes).

Moreover, during the first series of tests (α) a distinction was made among functional males, of those having only one duct leading to the papilla. This fourth category was designated by the term semi-functional.

Statistical analysis

The homogeneity of the results between the two slaughterings was estimated using the G-test and the χ^2 test. The latter was also used to compare the relative range of the different categories of animals according to experimental groups; this study was supplemented by tests on confidence intervals of percentages. For groups of less than 100 individuals, confidence intervals were obtained from specialized tables concerning small numbers.

RESULTS

Homogeneity of the two series of tests and study of control groups

Distribution of the various categories in the two series of tests is given in *table* 1.

The overall distribution of animals between the three main functional categories did not vary significantly between the two series of killings, α and β (χ^2: 1.93 (2 *dl*)). A more detailed analysis taking into account the distribution of functional categories in each of the four experimental groups carried out with the G-test did not show more heterogeneity between the two series of killings (*table* 3). Unless otherwise stated the following results concern regrouped data from these two series.

In control (T3) animals (*table* 2), at an age of 22 months a normal sex-ratio was revealed and only females were in group T2. The control "upstream" group T1, exhibited two non-functional males among

Table 2. — Number of the different categories in the control groups. T1, monosex upstreams control; T2, monosex downstreams control; T3, bisexual control; *, males with abnormal genital ducts.

Groups	T1	T2	T3
Females	36	27	16
Males	2*	0	15
TOTAL	38	27	31

Table 1. — Number and percentage of the different categories of animals in the four experimental treatments. α, examination at 22 months of age; β, examination at 25 months of age; Σ, regrouping of the two series.

Treatment	L 60			L 90			H 60			H 90		
	α	β	Σ	α	β	Σ	α	β	Σ	α	β	Σ
Mature Males						Non-functional						
	3	23	26	5	20	25	18	52	70	22	74	96
	8.3	17.5	15.6	14.7	22.2	20.1	58	51.5	53	66.7	76.3	73.8
						Functional						
	31	106	137	26	64	90	9	38	47	8	17	25
	86.1	81	82	76.5	71.1	72.6	29	37.5	35.6	24.2	17.5	19.2
Others (females, immatures, steriles, hermaphrodites)	2	2	4	3	6	9	4	11	15	3	6	9
	5.5	1.5	2.4	8.8	6.7	7.3	13	11	11.4	9.1	6.2	7
TOTAL	36	131	167	34	90	124	31	101	132	33	97	130
Percentage of sex reversals	–	–	97.6	–	–	92.7	–	–	88.6	–	–	93
G tests (α,β)	0.74 (NS)			1.57 (NS)			3.4 (NS)			0.93 (NS)		

the 36 females which was probably the result of a misclassification. However, it cannot be excluded that some spontaneous neomales exist in monosex female populations (unpublished data).

Percentages of sex reversals

Masculinization percentages, including functional and non-functional males, varied little between the different experimental groups and ranged from 88.6 (H60) to 97.6% (L60); the only significant difference ($p < 0.01$) being between these two groups (*table* 1).

Percentages of functional males

In contrast to the relative stability of sex reversal percentages, the porportions of functional males (giving sperm by abdominal pressure) widely varied according to experimental groups (*fig.* 2) as also shown by the χ^2 analysis of the overall distribution of animals (*table* 3, line *a*). This variability could also be studied according to the two controlled factors in the experiments: steroid concentration and treatment length.

Influence of steroid concentration

The study of functional male percentages showed a distinct difference between groups treated at a low dosis (82 and 72.6%, respectively for L60 and L90) and groups treated at a high *dosis* (35.6 and 19.2% for H60 and H90) (*fig.* 2). The χ^2 analysis confirmed the high significance ($p < 0.001$) of these differences whether between L60 and H60 or L90 and H90 (*table* 3, lines *b* and *c*).

Table 3. – Comparison of the proportions of functional males in the different groups.

Comparison	Degrees of freedom	χ^2 value	Significance
a. Global	6	159.92	$p < 0.01$
b. L 60-H 60	2	67.4	$p < 0.01$
c. L 90-H 90	2	78.3	$p < 0.01$
d. L 60-L 90	2	5.4	NS
e. H 60-H 90	2	12.3	$p < 0.01$

Moreover, in groups L60 and L90 there were significantly ($p < 0.01$) more functional than non-functional males and this situation was reversed in groups H60 and H90. On the other hand, there were no significant differences between the various groups as far as the FISH group was concerned (*fig.* 2).

Influence of treatment duration

Figure 2 shows that for the same steroid concentration the proportion of functional males decreased with increasing treatment duration. This decrease was 9.4% between L60 and L90 and 16.4% between H60 and H90, where only the latter difference was statistically significant (*table* 4, lines *d* and *e*). The proportions of non-functional males varied almost conversely taking into account the low variability of the FISH rate.

Table 4. – Proportion of semi-functional males (only one functional deferent duct) among the functional males (giving sperm by abdominal pressure) in the α sample.

Groups	L 60	L 90	H 60	H 90
Number	36	34	31	33
Proportion of functional males	86.1	76.5	29	24.2
Proportion of semi-functional males	12.9	32.1	33.3	62.5

Anatomo-functional characteristics of the animals

Table 4 gives detailed results of the first series of tests (study α) in which a distinction was made between fully functional males (two permeable ducts) and semi-functional males (only one permeable duct). In spite of the low number of semi-functional males, our observations seem to indicate that among functional males in its widest sense (all males giving sperm by abdominal pressure) the proportion of semi-functional males increased with the steroid concentration as well as with the length of the treatment. This trend like that of non-functional males seemed to reflect an increase in dysgenesis as a function of one or the other factors.

The main types of genital duct abnormalities in non-functional and semi-functional males are indicated in *figure* 3. They consisted either in the absence of the deferent duct (B, C, D) or in its interruption at some level between the testis and the urogenital papilla (E, F). When the deferent duct was absent, a conjunctive rudiment sprinkled with more or less widespread oedematous vesicles, could be observed.

DISCUSSION AND CONCLUSION

Effect of the treatment on sex reversal

The present study shows that methyltestosterone administered at doses of 0.5 to 3 mg/kg diet during a period of 60 or 90 days after first-feeding induces high sex reversal rates (89 to 97%) in a monosex female population. These results agree with those of the literature as far as low doses are concerned: for a period of 60 days, Okada *et al.* (1981, *in* Yamazaki 1983) obtained 84% males at a *dosis* of 0.5 mg/kg; Hurk and Lambert (1982) obtained 99% males at 0.6 mg/kg. Solar *et al.* (1984) found 100%.

On the other hand, treatments of a longer duration or at higher doses, which remained effective in our study, gave less coherent results: Johnstone *et al.*

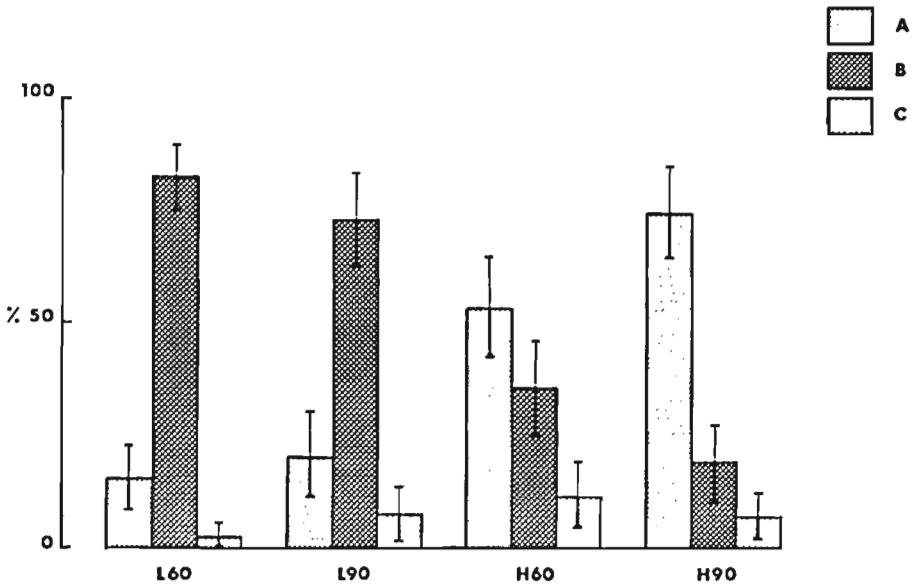

Figure 2. — Proportion of non-functional (A) and functional (B) mature males, resulting from the different treatments, C=non-mature males (females, immatures, steriles, hermaphrodites).

(1978) found 98% at 3 mg for 90 days; Solar *et al.* (1984) indicated 91 and 86% at the same *dosis* for 60 and 90 days; Okada *et al.* (1981) found only 42% at 5 mg for 60 days. The latter study, as well as ours, used a monosex female population and indicated that doses higher than 3 mg might lead to a lower efficiency. Hurk and Lambert (1982) certainly obtained 98% males with a treatment of 6 mg/kg for 58 days, but they mixed the steroid with low-fat feed and stressed that a better stability of the steroid can be obtained with this method. Moreover, all studies (except Okada *et al.*, 1979) concluded that efficiency was lower when the *dosis* reached 9 or 10 mg/kg.

Effect of the treatment on the functional morphogenesis of deferent ducts

Our results clearly demonstrated a marked influence of the *dosis* and the duration of the treatment on the functional character of the deferent ducts. This influence was characterized by a gradual decrease in the number of functional males from 82 to 19% and by an increase (from 13 to 63%) in the frequency of the same animals exhibiting one non-functional deferent duct. These results confirm the problems arising from doses that are too high and sometimes masked by their high apparent efficiency on the

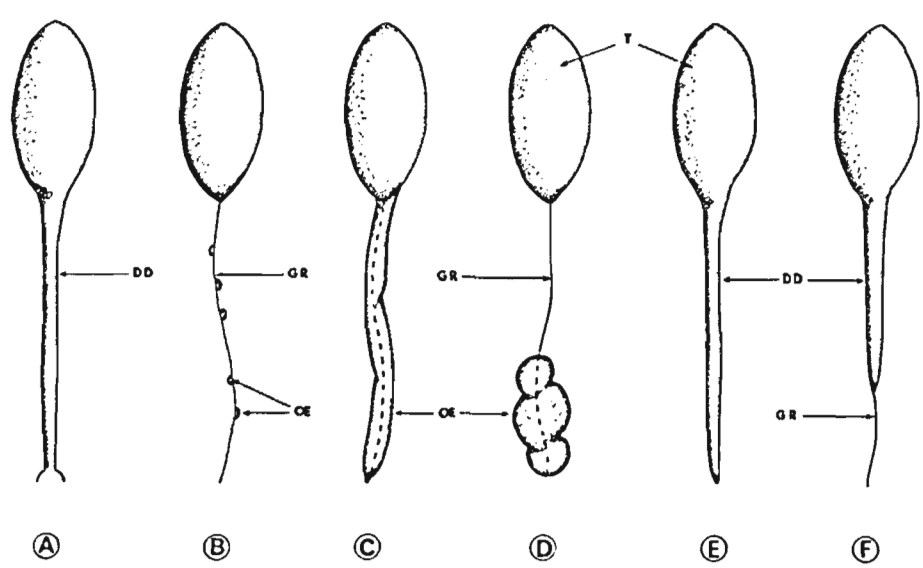

Figure 3. — Main type of genital duct abnormalities in the treated groups. A: normal deferent duct (D.D.). B, C, D: absence of the deferent duct. A conjunctive rudiment (GR) sprinkled with punctual (B) or continuous (C, D) oedematous vesicles can be observed. E, F: the deferent duct is interrupted before the genital papilla.

masculinization rate. Thus, in a treatment of 90 days at 3 mg/kg Johnstone *et al.* (1979) observed 98% males by early histological test of the gonad. However, among 37 individuals of the male type only 6 proved to be functional at the maturity stage. The 16% frequency was close to the 19% we observed. However, in our case, the use of an all-female population allowed us to confirm that it was a question of effect of the treatment on the process of sex reversal and not a possible effect of dysgenesis on animals of the male genotype.

It is interesting to note that the reproductive anatomy of female salmonids is characterized by the absence of oviducts. It seems, however, difficult to explain the abnormalities from this observation; Hunter and Donaldson (1983) who carried out masculinizations in Chinook salmon (*Oncorhynchus tshawytscha*) did not report functional abnormalities of deferent ducts. On the other hand, the abnormalities of the genital duct in general and the deferent ducts in particular described in trout have been reported in other species treated with methyltestosterone (*Poecilia reticulata*, Takahashi, 1975).

The practical interest of functional males

The possibility of modulating the proportion of functional males, preserving at the same time a high sex reversal rate, makes it possible to consider two extreme applications of this method:

The use of neomales in the production of monosex female populations in fish farming will be of practical interest in order to obtain a high proportion of functional males, in particular when a selected line is involved in which males are repeatedly used due to their possible high cost.

Conversely, in order to maintain the line of neomales it might be preferable to induce a high proportion of non-functional males. Being characterized by an abnormal gonad morphology revealing their character as genetic females, they cannot be mistaken for possible contaminants and will be preferentially used.

Acknowledgements

This study was supported by an IFREMER grant (GIS Nord Vilaine 1984). We thank Marianna Perrier-Olesen for the English translation of the manuscript.

REFERENCES

Bye V. J., R. Lincoln, 1981. Get rid of the males and let the females prosper. *Fish Farmer*, **4**, 1-3.

Chevassus B., D. Chourrout, B. Jalabert, 1979. Le contrôle de la reproduction chez les poissons. I. Les populations « monosexes ». *Bull. Fr. Pisc.*, **274**, 32-46.

Chevassus B., A. Devaux, D. Chourrout, B. Jalabert, 1988. Production of YY Rainbow Trout males by self-fertilization of induced hermaphrodites. *J. Heredity*, **79**, 89-92.

Hunter G. A., E. M. Donaldson, J. Stoss, I. Baker, 1983. Production of monosex female groups of Chinook Salmon (*Onchorhynchus tshawytscha*) by the fertilization of normal ova with sperm from sex-reversed females. *Aquaculture*, **33**, 355-364.

Hurk (Van den) R., J. G. D. Lambert, 1982. Temperature and steroid effects on gonadal sex differentiation in Rainbow Trout. Int. Symp. Reprod. Physiol. of Fish, Wageningen, 69-72.

Jalabert B., R. Billard, B. Chevassus, 1975. Preliminary experiments on sex control in trout production of sterile fishes and simultaneous self-fertilizable hermaphrodites. *Ann. Biol. anim. Bioch. Biophys.*, **15**, 19-28.

Johnstone R., T. H. Simpson, A. F. Youngson, 1978. Sex reversal in Salmonid culture. *Aquaculture*, **13**, 115-134.

Johnstone R., T. H. Simpson, A. F. Youngson, C. Whitehead, 1979. Sex reversal in Salmonid culture. Part II. The progeny of sex-reversed Rainbow Trout. *Aquaculture*, **18**, 13-19.

Lincoln R. F., A. P. Scott, 1983. Production of all female triploid Rainbow Trout. *Aquaculture*, **30**, 375-380.

Okada H., H. Matumoto, F. Yamazaki, 1979. Functional masculinization of genetic females in Rainbow Trout. *Bull. Jap. Soc. Sc. Fish.*, **45**, 413-419.

Okada H., H. Matumoto, Y. Murakami, 1981. Ratio of induced males from genetic females at various dietary concentrations of methyltestosterone. Ann. Meet. Jap. Soc. Sci. Fish., Abstract, p. 33 [in Japanese].

Solar I. I., M. E. Donaldson, G. A. Hunter, 1984. Optimization of treatment regimes for controled sex differentiation and sterilization in wild Rainbow Trout (*Salmo gairdneri*) by oral administration of 17 α-methyltestosterone. *Aquaculture*, **42**, 129-139.

Takahashi H., 1975. Functional masculinization of female Guppies, *Poecilia reticulata*, influenced by methyltestosterone before birth. *Bull. Jap. Soc. Sc. Fish.*, **41**, 499-506.

Yamamoto T. O., 1953. Artificialy induced sex reversal in genotypic males of the Medaka (*Oryzias latipes*). *J. Exp. Zool.*, **123**, 571-594.

Yamazaki F., 1976. Application of hormones in Fish culture. *J. Fish Res. Board Can.*, **33**, 948-958.

Yamazaki F., 1983. Sex control and manipulation in fish. *Aquaculture*, **33**, 329-354.