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Gonadal Steroids Delay Spontaneous Flounder Metamorphosis and Inhibit T_3-induced Fin Ray Shortening *in vitro*

Evelyn Grace de Jesus, Tetsuya Hirano and Yasuo Inui[1]

Ocean Research Institute, University of Tokyo, 1-15-1 Minamidai Nakano, Tokyo 164, and [1]National Research Institute of Aquaculture, Nansei, Mie 516-01, Japan

ABSTRACT—Although it is now clear that the major hormones controlling metamorphosis of the Japanese Flounder, *Paralichthys olivaceus,* are the thyroid hormones, thyroxine and triiodothyronine, other hormones modulate their effects. The aim of the present study was to determine the effects of the sex steroids, estradiol and testosterone, on the resorption of the dorsal fin rays of flounder *in vitro,* and on spontaneously metamorphosing larvae. Neither estradiol nor testosterone (100 ng/ml) had any direct effect on fin ray shortening. However, they exerted inhibitory effects on thyroid hormone-induced fin ray resorption. Immersion of flounder larvae in 100 ng/ml solution of either estradiol or testosterone also resulted in a delay in metamorphic features such as fin ray shortening, eye migration and settling. Measurement of whole-body concentrations of estradiol and testosterone during metamorphosis revealed no significant changes, and levels remained low (below 1 ng/g) throughout the larval period. These results are discussed in relation to the possible interraction between thyroid hormones and sex steroids.

INTRODUCTION

The stimulatory effect of thyroid hormones on fish metamorphosis has recently been reported for the conger eel and the flounder [1–3]. Other hormones are also known to affect thyroid hormone actions. We have shown that cortisol enhances the stimulatory effects of both thyroxine (T_4) and triiodothyronine (T_3) on the resorption of the flounder fin rays *in vitro* [4]. Synergism between thyroid hormones and corticosteroids has been demonstrated in amphibians, whereas prolactin antagonizes thyroid hormone action and promotes growth of the tadpole and the retention of larval structures [5].

The reported effects of gonadal steroids on amphibian metamorphosis are contradictory. Kikuyama *et al.* [6] found no effect of progesterone, estradiol or testosterone on T_4- or T_3-induced shrinkage of the isolated tail fin disc of *Bufo bufo japonicus, in vitro.* A recent report by Gray and Janssens [7] showed that testosterone had only marginal effects on the shrinkage of the cultured tail fin of *Xenopus laevis,* whereas both estradiol and testosterone inhibited T_3-induced metamorphosis *in vivo.*

Information on the levels of sex steroids in metamorphosing amphibians is lacking. However, ovaries of tadpoles have the capacity to synthesize and release low levels of estradiol *in vitro* from an early stage, and levels increase toward the end of metamorphosis [8]. There is also a paucity of information on the levels of sex steroids during early development of fishes.

In this paper, we report an inhibition by testosterone and estradiol on T_3-induced fin ray shortening, *in vitro* as well as on spontaneous metamorphosis.

MATERIALS AND METHODS

In vitro experiment

Prometamorphic flounder larvae were obtained from a commercial source. The maintenance of the fish as well as the protocol for the *in vitro* experiments were as described previously [4]. Five

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fin rays were randomly distributed into sterile culture bottles containing 5 ml serumfree medium (SFM 101, Nissui, Tokyo) supplemented with T_3 (1 ng/ml, Wako, Tokyo), estradiol-17β (100 ng/ml, Wako) or testosterone (100 ng/ml, Wako), with combinations of T_3 and either estradiol or testosterone, or without supplement (control). Cultures were kept at 20°C for one week and the extent of fin ray resorption was monitored by measuring the length of the second fin ray daily. The results are presented as % of initial fin ray length.

In vitro experiment

Three hundred prometamorphic larvae of similar size were taken from the stock and randomly distributed into 3 groups. Each group was maintained in a 30-liter plasic tank corresponding to control, estradiol (100 ng/ml) and testosterone (100 ng/ml) treatment, respectively. Temperature was maintained between 18 and 21°C during the experiment. Twenty percent of the water was changed every day while keeping the concentrations of the hormones constant. The number of fish that settled and the number of dead fish were recorded daily. Twenty to thirty fish were sampled at the start of the experiment and at 1 and 2 weeks after treatment, and body length, fin ray length and the degree of eye migration were noted. Larvae from the stock were sampled at various stages of metamorphosis and frozen at −80°C until analysis of whole-body concentrations of estradiol and testosterone. Procedures for the extraction and radioimmunoassay of estradiol and testosterone were as described for chum salmon [9].

For statistical analysis, the Duncan's Multiple Range test was used after checking that the variances of the experimental groups were not significantly different from each other by Bartlett's test and ANOVA.

RESULTS

The effects of estradiol and testosterone on T_3-induced fin ray shortening are shown in Figure 1. Estradiol (100 ng/ml) did not have any direct effect on the rate of fine ray shortening. T_3 (1 ng/ml) stimulated the resorption of the dorsal fine

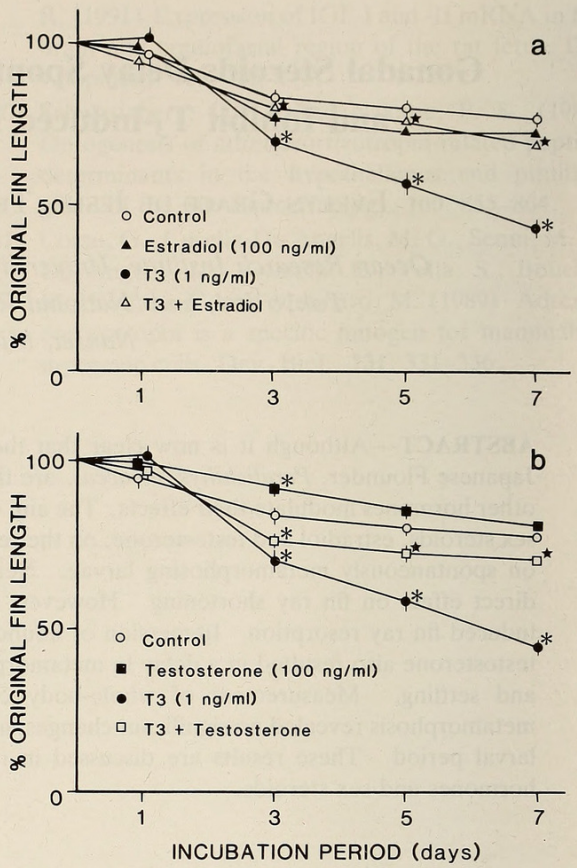

FIG. 1. Effects of estradiol (A) and testosterone (B) on T_3-induced fin ray resorption. Each point represents the mean of 8 measurements. Vertical bars indicate standard errors of the means. *,★ Significantly different ($P<0.01$) from the control and the T_3-treated group, respectively, at the corresponding incubation period.

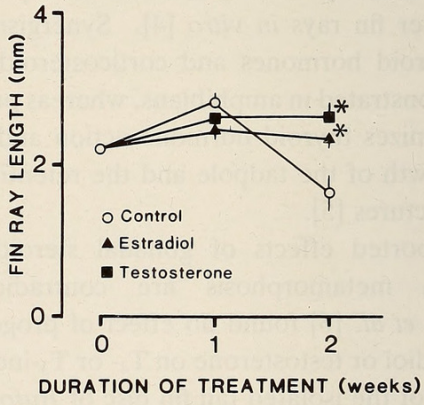

FIG. 2. Effects of immersion in 100 ng/ml estradiol or testosterone on the length of the second fin ray. Each point represent the means of 20–30 measurements. Vertical bars indicate the standard errors of the means. *, Significantly different ($P<0.01$) from the control.

rays, and a significant effect became apparent starting 3 days after culture. Treatment of the fine rays simultaneously with T_3 and estradiol did not cause significant shortening even after 7 days, indicating that estradiol blocked the effects of T_3. Testosterone likewise inhibited the effects of T_3. When testosterone (100 ng/ml) was added to the medium together with T_3, the stimulatory effect of T_3 was only observed on day 3.

Figures 2–4 show the changes in fin ray length, eye migration stage and settling rate of the flounder larvae from control, estradiol- and testosterone-treated groups. Immersion of the larvae in 100 ng/ml solution of either estradiol or testosterone resulted in a delay in the resorption of the dorsal fin rays. The effect was significant relative to the control after 2 weeks of treatment (Fig. 2). The hormonal treatment also caused a delay in the rate of eye migration (Fig. 3) and of settling (Fig. 4).

There was no significant change in the whole-body concentrations of estradiol and testosterone during metamorphosis; they remained low throughout the larval stages (Fig. 5).

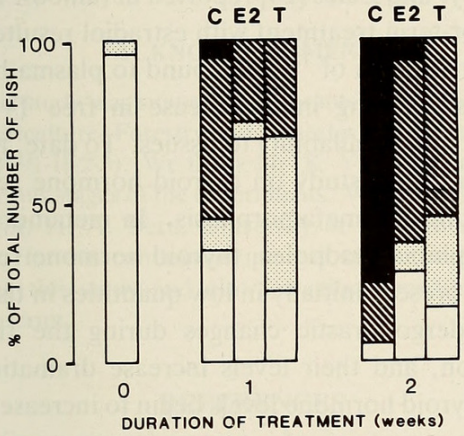

FIG. 3. Effects of estradiol (E2) and testosterone (T) on the rate of eye migration in flounder larvae. C: control (without hormone). Each bar represents the results of estimation of eye stage (based on Miwa and Inui, 1987 [7]) from 20–30 fish. Clear bars, eyes are situtated on both sides of the body; dotted bars, the right eye is moving although both eyes are still seen one over the other; hatched bar, eyes are seen separately; solid bar, the right eye has crossed the midsagittal line and is situated on the left side of the body.

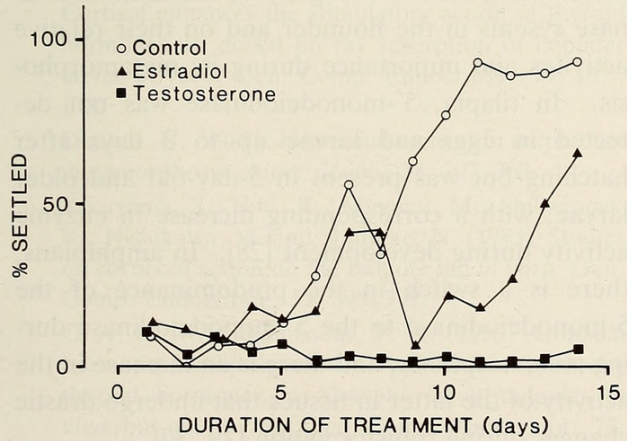

FIG. 4. Effects of estradiol and testosterone on the settling behavior of flounder larvae, expressed as % of fish exhibiting benthic behavior against the total number of fish during each sampling day.

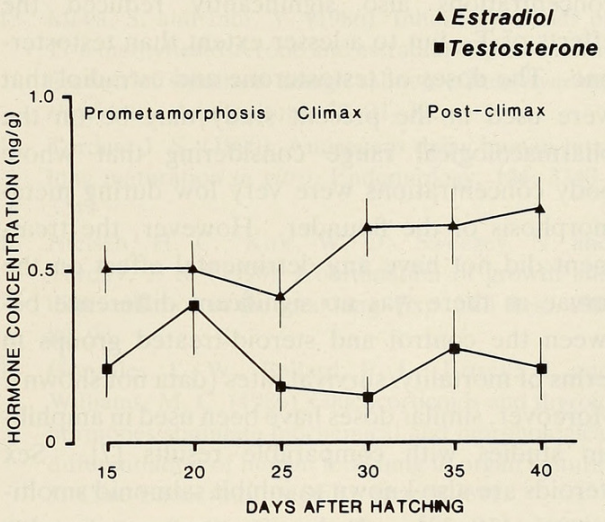

FIG. 5. Changes in whole body concentrations of estradiol and testosterone during metamorphosis of the flounder. Vertical bars indicate the standard errors of the means (n=5).

DISCUSSION

The results of the present *in vitro* experiments confirm previous reports that thyroid hormones induce fin ray shortening in flounder larvae. Fin rays treated with 1 ng/ml T_3 accelerated shortening significantly compared with the control group from days 3–7 [4]. Estradiol and testosterone did not have direct effects on fin ray shortening. However, both steroids blocked the action of T_3 on the resorption of dorsal fin rays when both T_3 and either of the steroids were present in the culture medium. These findings are contrary to reports on amphibians by Kikuyama *et al.* [6] and

Gray and Janssens [7] who found no effect of sex steroids on thyroid hormone-induced tail fin shrinkage of tadpoles *in vitro*.

The results of the *in vivo* experiment also indicated that treatment with either estradiol or testosterone (100 ng/ml) retarded the course of natural metamorphosis. Testosterone consistently showed greater effects than estradiol especially on the settling of the larvae. Similar observations were reported by Gray and Janssens [7] in *Xenopus* when they treated the tadpoles with T_3 and either estradiol or testosterone. Testosterone (3.4 μM or about 1 μg/ml) completely blocked the effects of T_3 on head length, width and body weight and considerably on gut length. Estradiol, at same concentrations also significantly reduced the effects of T_3, but to a lesser extent than testosterone. The doses of testosterone and estradiol that were used in the present study may be on the pharmacological range considering that whole body concentrations were very low during metamorphosis of the flounder. However, the treatment did not have any detrimental effect on the larvae as there was no significant difference between the control and steroid-treated groups in terms of mortality/survival rates (data not shown). Moreover, similar doses have been used in amphibian studies with comparable results [7]. Sex steroids are also known to inhibit salmonid smoltification [10–13]. Androgen treatment is also reported to retard the development or maturation of mouse and human fetal lung [14, 15], whereas corticosteroids and thyroid hormones have opposite effects [16].

There was no significant change in the levels of either estradiol or testoerone, and they remained low in comparison with thyroid hormone and cortisol levels throughout the metamorphic period of the flounder. Whole-body concentrations of cortisol and T_4 were around 4 ng/g and 1 ng/g, respectively, during the early stages of metamorphosis, increased to peak levels of 11 and 12 ng/g, respectively, during climax and declined by approximately 50% in the juveniles [17]. Likewise, there was no change in the levels of estradiol and 11-ketotestosterone during smoltification in coho salmon when both plasma T_4 and cortisol levels were remarkably elevated [18]. Similar hormonal patterns were seen during the emergence and downstream migration of chum salmon [9].

Inhibition of thyroid hormone action by sex steroids may occur at the level of the target tissues. Although corticosteroids enhance thyroid hormone action by increasing the nuclear binding capacities for T_3 in bullfrog tadpoles [19], Gray and Janssens [7] found that testosterone had no such effect in *Xenopus* tadpoles. On the other hand, Cyr and Eales [20] reported in rainbow trout that shor-term treatment with estradiol resulted in a higher fraction of ^{125}I-T_3 bound to plasma binding sites resulting in a decrease in free T_3 and possibly in T_3 availability to tissues. To date, there is no published study on thyroid hormone receptors during fish metamorphosis. In metamorphosing amphibian tadpoles, thyroid hormone receptors are present initially in low quantities in tissues that undergo drastic changes during the transformation, and their levels increase dramatically when thyroid hormone levels begin to increase [21, 22]. The occurrence of receptors for sex steroids in the flounder fin rays is not known.

Sex steroids may also act on T_4-T_3 conversion and thus affect thyroid hormone kinetics. There are several reports that treatment of rainbow trout with estradiol depresses T_3 levels by depressing the 5′-monodeiodinase system which converts T_4 to T_3 [23–25]. Reports of the effects of testosterone are contradictory: inhibitory in rainbow trout [23, 26] and stimulatory in Arctic charr [27]. There is no information on the ontogeny of the monodeiodinase sysems in the flounder and on their relative activites and importance during its metamorphosis. In tilapia, 5′-monodeiodinase was not detected in eggs and larvae up to 3 days after hatching but was present in 5-day-old and older larvae, with a corresponding increase in enzyme activity during development [28]. In amphibians, there is a switch in the predominance of the 5-monodeiodinase to the 5′-monodeiodinase during metamorphosis, and there is an increase in the activity of the latter in tissues that undergo drastic changes during transformation [29, 30].

Sex steroids may also act at the level of the pituitary-thyroid axis. Sex steroids blunt the response of the thyroid to TSH stimulation in the

rainbow trout [23] and in the eel [31]. Sex steroids, especially estradiol, may also act through prolactin. Estradiol potentiates TRH-induced prolactin secretion in tilapia pituitaries [32]. Increased prolactin in circulation may in turn antagonize thyroid hormone action.

Studies of thyroid hormone receptors and of monodeiodinase systems are needed to make the picture of flounder metamorphosis and its endocrine control more complete.

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