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Influence of Testosterone on Precocious Sexual Development in Immature Rainbow Trout¹

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The influence of testosterone on plasma and pituitary levels of gonadotrophin (GTH) as well as on gonadal development was studied in immature rainbow trout. Among the animals receiving a testosterone-cocoa butter implant (200 µg) at the age of 5 months, gonadal puberty occurred 8 months later in half of the males (opposite to the controls which remained immature) and the beginning of oocyte maturation was observed in only one female. These animals were characterized by a higher pituitary GTH level. Owing to the multivariate statistical analyses made, it was possible to provide evidence for the presence of two populations with different reactions to the same steroid treatment. They also confirmed the existence of a positive testosterone feedback, in the male, leading to a precocious gonadal development. The pituitary GTH level obtained with 200 µg of testosterone seemed to be related to the age at first maturation. The secretion of an appropriate level of GTH resulting in the stimulation of gametogenesis required the availability of a relatively large pituitary GTH level and seemed to be possible because the animals were already in the pubertal period. The fact that the highest pituitary GTH level of the treated lot was found in the only female showing a beginning of sexual maturation suggests that testosterone may also act in females. © 1985 Academic Press, Inc.

In immature but already sexually differentiated teleosts, the effects of sexual steroids on gametogenesis vary according to species and experimental conditions (i.e., doses, mode of administration, treatment time and length).

Injection of estrogens seemed to stimulate growth of oocytes in the silver eel (Olivereau and Olivereau, 1979a) and gonial divisions and vitellogenesis in the carp (Pantic and Lovren, 1978), but had no effect in rainbow trout (Upadhyay, 1977; Billard *et al.*, 1982; Sower *et al.*, 1983) and led to cessation of vitellogenesis in pink salmon (Funk *et al.*, 1973).

Androgens stimulated spermatogenesis in *Poecilia latipinna* (Van den Hurk and Van de Kant, 1975) and in *Oryzias latipes* (Hamaguchi, 1979) as well as in salmonids such as *Oncorhynchus kisutch* (Fagerlund

and McBride, 1977), *Oncorhynchus tshawytscha* (Schreck and Fowler, 1982), and *Salmo gairdneri* (Fagerlund and McBride, 1977; Crim and Evans, 1982, 1983; Sower *et al.*, 1983). In *Salmo salar*, the percentage of mature males increased after treatment with testosterone (Crim and Peter, 1978; Dodd *et al.*, 1978). In hypophysectomized *Poecilia reticulata* (Pandey, 1969), *P. latipinna* (Van den Hurk and Van de Kant, 1975), and *O. latipes* (Hamaguchi, 1979), the mitotic divisions of the spermatogonial cells went on after androgen treatment. The multiplication of spermatogonial cells was not altered by methallibure in *P. latipinna* (Van den Hurk and Van de Kant, 1975) or cyproterone acetate in *O. latipes* (Hamaguchi, 1979). Large doses of methyltestosterone incorporated into the food, on the other hand, led to a degeneration of the testes (decrease in the number of spermatogonia, hypertrophy of the tissues) in *O.*

¹ Translated by K. Rerat.

Kisutck (McBride and Fagerlund, 1973, Fagerlund and McBride, 1977; Higgs *et al.*, 1977; Yu *et al.*, 1979), *O. gorbusha*, *O. keta* (Yamasaki, 1972), *S. gairdneri* (Billard *et al.*, 1982; Sower *et al.*, 1983), and *Carassius auratus* (Takahashi, 1972).

Ultrastructural and immunocytochemical studies of the pituitary gonadotrophic cells and assessment of pituitary and plasma levels of gonadotrophin (GTH) give information about the mode of action of steroids in immature animals: pituitary GTH increased after steroid treatment (Atlantic salmon: Crim and Peter, 1978; rainbow trout: Crim and Evans, 1979, 1980, 1982, 1983; Crim *et al.*, 1981; Gielen *et al.*, 1981; Gielen and Goos, 1983; eel: Dufour *et al.*, 1983a) and the pituitary gonadotrophic cells were stimulated (sockeye salmon: Van Overbreeke and McBride, 1971; rainbow trout: Gielen *et al.*, 1982; eel: Olivereau and Chambolle, 1978, 1979; Sokolowska *et al.*, 1978; Olivereau and Olivereau 1979a and b; carp: Pantic and Lovren, 1978). Like exogenous steroids, GTH or pituitary extracts led to an increase in the pituitary GTH level and a maturation of the gonadotrophic cells in male trout (Crim *et al.*, 1982; Gielen *et al.*, 1982) and female eel (Olivereau and Fontaine, 1966; Olivereau, 1967; Ueda and Takahashi, 1978; Dufour *et al.*, 1983b). The gonadal steroids were responsible for this increase of pituitary GTH in male trout (Crim *et al.*, 1982; Gielen *et al.*, 1982). According to Crim and Evans (1983), plasma GTH levels increased after testosterone treatment.

The aim of the present work was to study the effects of testosterone on pituitary and plasma levels of GTH and on gonadal development in order to contribute to the understanding of the role of this steroid at the onset of puberty in rainbow trout.

MATERIALS AND METHODS

The rainbow trout used were 5 months old and weighed 5–10 g in July 1981. The stock from which they were taken was characterized by a sex ratio of

1:1 and by a percentage of first maturation at 2 years (during the winter of 1982–1983) of 54% for the males and 9% for the females (Chevassus, personal communication).

The study took place at the experimental fish farm of "Gournay sur Aronde" under natural photoperiod and temperature conditions. In July 1981, one lot of animals received an injection of cocoa butter alone (control, $n = 192$) and another one, cocoa butter combined with testosterone (experimental, $n = 166$) according to the method described by Billard and Peter (1977) and Crim and Evans (1979). Testosterone (17 β hydroxyandrostane-4-ene-3-one, Roussel), dissolved in molten cocoa butter, was injected intraperitoneally at the level of the pelvic fins at a dose of 200 μ g in a volume of 200 μ l. The presence of the solid implant within the perivisceral cavity was confirmed at the end of the experiment (95% positive results).

In March 1982, the effects of the treatment were analyzed and the criteria used were, on the one hand, the number of females having ovulated or the number of spermiating males in the whole population ($n = 358$) and, on the other hand, the body weight, gonadal weight, and histological stage, as well as the plasma and pituitary levels of GTH in the 55 sacrificed animals.

Gonadotropic Hormone Determination

Blood samples were collected in heparinized syringes at the level of the caudal vascularization. Immediately after their withdrawal, pituitaries were ground in 500 μ l saline. Samples of blood and pituitary homogenates were stored over crushed ice and centrifuged. The blood plasma and pituitary supernatants were removed and stored at -20°C until analyzed.

Pituitary and plasma levels of maturational glycoprotein GTH were determined by radioimmunoassay according to Breton *et al.* (1971) using an antibody produced against pure trout GTH (t-GTH; Breton *et al.*, 1976). The hormone used for labeling and establishment of standard curves was a purified salmon GTH hormone (Breton *et al.*, 1978).

Determination of the State of Gonad Development

The gonads fixed in Bouin–Holland's fluid were dehydrated and embedded into paraplast. The 5- μ m-thick sections were stained with Regaud's hematoxyline, orange G, aniline blue for the males and with Heindenhein's Azan for the females (Gabe, 1968).

Statistical Analysis

Classical statistical methods for a separate analysis of each variable were used in the first study involving results obtained in males and females (calculation of mean values, standard deviations, standard errors,

and coefficients of variation, comparison of groups by analysis of variance or by the Mann-Whitney (*U*-test). The data obtained with males were further studied using methods of multivariate analysis (Solari *et al.*, 1982; Lefebvre, 1983). These methods are based on the overall analysis of all measured variables and not on the separate study of each parameter as in classical statistics. The first method used was that of centered data. It is a descriptive method supplying a spatial graphical representation both of the animals and of the five measured biological variables (body weight, gonadal weight, state of gonads, plasma GTH, pituitary GTH). Owing to the more or less remote position of the individuals within the space, it was possible to form groups with the same characteristics. The position of the variables within the space may account for the difference between the groups of individuals.

The method of discriminating functions was used in addition to that of centered data when the difference between two groups could not be easily explained only by the position of the variables within the space. Those functions were linear functions of the biological variables by means of which new variables (*y*), the so-called discriminant variables, may be calculated.

The programs used for these multivariate analyses were developed by Lefebvre *et al.* (1981) and the data processed on the microcomputer Wang 2200P.

RESULTS

In March 1982 no female, among the 166 testosterone-treated animals, had ovulated, while 40 males (i.e., 50% of the males) produced sperm. No sexual maturation was observed in the 192 control animals.

Males

In the twenty-six males sacrificed in March 1982, the statistical analysis of each variable separately showed that there was a large heterogeneity within each of the two

lots (control and experimental), especially in the experimental (testosterone treated) one (Table 1). In the latter, this heterogeneity led to coefficients of variation exceeding 50% for all measured variables, except body weight (Table 1).

As the larger dispersion in the treated lot could result from the presence of several populations with different reactions towards testosterone, an analysis of the centered data was made in order to show the existence of these populations (Fig. 1). The position of each of the 26 males within the space of the first three canonical axes (which are linear functions of the biological variables) clearly shows that the treated animals can be divided into two groups: seven animals around the controls and nine completely discriminated in another area of the space. The histological examination of testes showed that these nine animals were all mature; a great number of spermatozoa were already present in the lobular lumen (Fig. 2b). Just before sacrifice, semen could be collected by abdominal pressure. These animals exhibited the heaviest gonads and the highest pituitary GTH levels (Table 2). On the other hand, the control and experimental animals grouped in another area of the space, were all immature. The germ cells of the testes were only spermatogonia (Fig. 2a). However, the testosterone-treated immature trout showed pituitary GTH levels between those of the controls and of the "testosterone-mature" animals (Table 2).

TABLE 1
IN THE MALES, INFLUENCE OF TESTOSTERONE ON BODY WEIGHT, GONADAL WEIGHT, PLASMA AND PITUITARY GTH

Treatment	Body weight (g)	Gonadal weight (g)	Plasma GTII (ng/ml)	Pituitary GTH (ng/pituitary)
Control (<i>n</i> = 9)	104.0 ± 9.3 (27)	0.066 ± 0.007 (34)	1.6 ± 1.1 (211)	3.2 ± 0.3 (24)
Testosterone (<i>n</i> = 17)	77.4 ± 5.5 (29)	0.712 ± 0.202 (117)	1.4 ± 0.2 (72)	51.9 ± 6.8 (54)

Note. Values are means ± SEM. Values in parentheses are coefficients of variation (%).

FIG. 1. Position of the five biological variables and distribution of the 9 control males and 17 testosterone-treated males in a three-dimensional space. This space is defined by the first three canonical axes obtained from linear combinations of the five biological variables. This graph shows that the testosterone-treated animals can be divided into two groups, i.e., one far from the controls, one close to the controls.

Among the testosterone-treated and mature animals, one individual with an exceptionally high gonadal weight (3.51 g) was different from the others and was located almost at the same place as the "gonadal weight variable." Likewise, a control animal could be distinguished from the others because of its high plasma GTH level (9.9 ng/ml; Fig. 1).

With the aim of determining the different variables involved in the separation of the

groups, the analysis was continued via the search for discriminant functions (Fig. 3). It was confirmed that pituitary GTH and gonadal weight intervened systematically in the separation of the three isolated groups by the method of centered data (controls, "testosterone-immature," "testosterone-mature") and this intervention was strong enough to isolate the "testosterone-mature" group from the controls. For separating the "testosterone-immature" group

FIG. 2.(a) Section of a testis from a control or "testosterone immature" animal. The germ cells are only spermatogonia (Sg) ($\times 280$). (b) Section of a testis from a "testosterone mature" animal. Spermatozoa (Sz) are present in the lobular lumen ($\times 280$). (c) Portion of an ovary with previtellogenic follicles (Pv) from a control or testosterone-treated animal ($\times 140$). (d) Portion of the ovary from the only testosterone-treated female showing follicles at a more advanced stage, i.e., formation of yolk vesicles (Yv) and oil droplets (Od) ($\times 140$).

TABLE 2
MEAN VALUES OF THE VARIABLES MEASURED IN CONTROL AND TESTOSTERONE-TREATED IMMATURE
AND MATURE MALES

Treatment	Body weight (g)	Gonadal weight (g)	Plasma GTH (ng/ml)	Pituitary GTH (ng/pituitary)
Control (n = 9)	104.0 ± 9.3 (27)	0.066 ± 0.007 (34)	1.6 ± 1.1 (211)	3.2 ± 0.3 (24)
Testosterone "Immature" (n = 7)	78.8 ± 8.9 (30)	0.084 ± 0.025 (77)	0.8 ± 0.3 (104)	22.2 ± 4.9 (58)
"Mature" (n = 10)	76.4 ± 7.3 (30)	1.151 ± 0.267 (73)	1.8 ± 0.3 (52)	72.6 ± 3.5 (15)

Note. The subdivision of the "testosterone" lot results from the multivariate analysis. Values are means ± SEM. Values in parentheses are coefficients of variation (%).

from the controls, the body weight played a part in addition to the former two variables. Plasma GTH only intervened for a better discrimination between testosterone-treated mature and immature animals.

FIG. 3. Frequency histogram of the discriminant variable y (the discriminant variable y for each male is obtained by replacing the value of each biological variable in the discriminant function Y); n = number of males.

Females

Control as well as testosterone-treated females (except one) were immature, in the state of previtellogenesis (Fig. 2c). With regard to body weight and plasma GTH (Table 3), the univariate statistical analysis did not show any difference between control and treated lots. Plasma GTH was rather variable in the two lots (from 0 to 5.5 ng/ml, coefficient of variation > 90%).

Pituitary GTH levels in the treated females were significantly higher than those of the controls ($P < 0.05$). In the testosterone lot, the coefficient of variation was 87%. This high variability revealed the large heterogeneity of the pituitary responses to the steroid: 65% of the treated females (11/17) showed the same range of pituitary levels as the controls; the six other treated females exhibited higher pituitary GTH levels (from 18 to 49.3 ng/pituitary) than the controls. Beginning of vitellogenesis could only be observed in the female with the highest GTH level (49.3 ng/pituitary; Fig. 2d).

Comparison between Male and Female Pituitary GTH Levels (Tables 2,3)

The pituitary levels of GTH were significantly higher in control females than in control males ($P < 0.005$). However, after

TABLE 3
IN THE FEMALES, INFLUENCE OF TESTOSTERONE ON BODY WEIGHT, GONADAL WEIGHT, PLASMA
AND PITUITARY GTH

Treatment	Body weight (g)	Gonadal weight (g)	Plasma GTH (ng/ml)	Pituitary GTH (ng/pituitary)
Control (n = 11)	96.6 ± 7.3 (26)	0.141 ± 0.100 (42)	0.8 ± 0.5 (224)	6.3 ± 0.9 (48)
Testosterone (n = 18)	79.3 ± 7.7 (41)	0.146 ± 0.040 (23)	1.1 ± 0.2 (92)	16.3 ± 3.5 (87)

Note. Values are means ± SEM. Values in parentheses are coefficients of variation (%).

steroid treatment, the pituitary GTH level of the mature males was higher than that of the females ($P < 0.005$). On the other hand, the levels of the immature treated males and those of the treated females were the same.

DISCUSSION

After administration of a single implant of testosterone at the age of 5 months, gonadal puberty occurred 8 months later in half of the males (i.e., 1 or 2 years before the normal date of first reproduction). These animals were characterized by a higher level of pituitary GTH. Use of discriminant functions showed that the plasma level of GTH also intervened in the separation of these mature males from the immature ones. A high pituitary GTH level in relation to the onset of gonadal maturation was observed in only one female.

According to our results, the hypothesis of a direct stimulation of the testes by exogenous testosterone should be rejected. Indeed, only part of the individuals exhibited spermatogenesis while the testes of all animals had already reached a sufficient degree of differentiation for production of gametes and secretion of sexual steroids, as affected by gonadotrophin (Magri *et al.*, unpublished data). The mechanism of indirect action of testosterone could be the following: accumulation of GTH in the pituitary gland, release of GTH into the blood circulation, and, at last, stimulation of gametogenesis.

Accumulation of GTH in the Pituitary Gland

According to data from the literature, the same dose of steroid leads to large individual variations in the level of pituitary GTH (c.g., Crim *et al.*, 1981; Dufour *et al.*, 1983a). Our results, obtained with 200 µg of testosterone or with 20 µg (Magri *et al.*, unpublished data), reveal the existence of two populations with different pituitary GTH responses induced by the steroid. In the females, one group gave the same pituitary response as the controls and in another group, the response was higher. The males could also be distributed into two groups: one with a higher pituitary GTH level and another group with a much higher level than that of the controls. This heterogeneity, which is marked for the two sexes, might reveal the existence of genetic differences in relation to the age at puberty. In the stock of fish used, 54% of the males and 9% of the females were sexually mature at the age of 2 years, the others at 3 or 4 years (Chevassus, personal communication). The population of individuals with the same response as the controls (♀) or exceeding it (♂) corresponded to late-maturing animals (3 or 4 years), and those with a superior (♀) or highly superior (♂) response corresponded to early-maturing animals (2 years). According to our results, there was a large difference between "early-maturing" males and females in the pituitary sensitivity towards the steroid; the

pituitary GTH level of the males exceeded that of the females and this was all the more marked as the level was higher in March in female controls.

These considerations can be compared with the results obtained in the platyfish, where the more or less late-maturation processes are controlled by a gene P (Kallman and Schreibman, 1973; Kallman *et al.*, 1973; Schreibman and Kallman, 1978). The sequence of luteinizing hormone-releasing hormone (LH-RH) appearance in the brain (Schreibman *et al.*, 1982a and b; Halpern-Sebold and Schreibman, 1983), the development of the gonadotrophic area in the pituitary (Schreibman and Kallman, 1978), and the response of gonadotrophic and germinal cells to exogenous LH-RH (Bao and Kallman, 1982) follow a more or less precocious temporal scheme depending on the genotype of the animal.

Thus, the magnitude of the pituitary GTH response, as affected by testosterone, seems to depend on the age at puberty, but it also depends on the steroid dose used: pituitary gonadotrophin increased with increasing doses of testosterone (from 0.2 to 20 μ g) (Crim and Evans, 1979). The dose-response effect exists at higher doses since the pituitary GTH level reached 8 months after administration of 200 μ g of testosterone was about four times higher than that obtained with 20 μ g after 1 month (Magri *et al.*, unpublished data). When very high doses of testosterone were administered (3 or 7 mg), the pituitary GTH increased very rapidly and remained high during the 3–4 months of study performed by Crim and Evans (1982, 1983). With the dose of 200 μ g testosterone, our experimental design did not supply any information about the variation in the pituitary GTH level according to time. It was therefore impossible to determine whether the pituitary level reached a maximum during the first months and then decreased, as it did 1 month after administration of 20 μ g

testosterone (Magri *et al.*, unpublished data).

Release of GTH Accumulated in the Pituitary Gland

The statistical method of multivariate analysis shows the role of plasma GTH in the separation of mature from immature males. Thus, in some of these individuals, GTH was released from the pituitary and this release was confirmed by the onset and achievement of spermatogenesis. It was in these animals that the highest pituitary GTH levels were recorded. The results of Crim and Evans (1983) obtained with 7 mg testosterone clearly show the existence of a positive testosterone feedback on the gonadotrophic function since very high plasma levels of GTH (10–20 ng/ml) were reached from very large contents of pituitary GTH. All these results show that the existence or detection of gonadotrophin secretion in the circulation require the availability of a sufficiently large mobilizable pituitary load induced by an adequate steroid stimulation.

The mechanism of indirect action of testosterone envisaged for males could be applied to females, but would perhaps require larger steroid doses and treatment lengths. Indeed, the beginning of vitellogenesis was observed in a female whose pituitary GTH level exceeded that of the other individuals remaining in the state of previtellogenesis.

In conclusion, owing to an appropriate pituitary GTH load, it was possible to obtain a secretion of GTH in the circulation capable of inducing a precocious spermatogenesis. However, this secretion seemed only to be possible because the animal was already in the pubertal period. Indeed, the period during which the experiment took place was considered as a period of initiation of the gonadotrophic function (Magri *et al.*, unpublished data). This suggests that during that period, the hypothalamo-pituitary system becomes progressively able to

respond to high doses of testosterone by an adequate secretion of GTH in the circulation. In that case, the positive action of testosterone on the gonadotrophic function and on the gonadal development constitutes, in the young, one of the endocrine adjustments of the pubertal period (for instance, via the elevation of the pituitary GTH level during puberty observed in precocious Atlantic salmon, (Crim and Evans, 1978; Stuart-Kregor *et al.*, 1981)) but not the cause of puberty.

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